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

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Short running title: Diet and gut microbiome of Grauer's gorillas

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Abstract

Conservation efforts tend to focus on populations that are genetically differentiated without paying attention to their ecological differentiation. However, isolated populations may be ecologically unique, an important aspect for the design of appropriate conservation measures for endangered species. Here we investigate the interplay between diet and gut microbiome in several geographically isolated and genetically differentiated populations of the critically endangered Grauer's gorilla. We find that dietary and gut microbial profiles are population-specific, likely due to geographic isolation and environmental differences. In addition, social groups within each population also differed in diet and, to a lesser extent, in gut microbial composition and diversity. Individuals at low elevation consumed a larger variety of plant taxa than those at high elevation, consistent with the notion that dietary choice is constrained by food availability that changes with elevation. Despite no detectable correlation between the diet and gut microbiome in richness or evenness, dietary and gut microbial composition covaried significantly. As we did not find evidence for an effect of genetic relatedness on the composition of the gut microbiome of Grauer's gorillas, this pattern is likely a result of long-term social, ecological, and geographic factors acting on both diet and microbiome. These results reveal that isolated and genetically distinct populations of Grauer's gorillas are also ecologically distinct, highlighting the need to dedicate separate conservation efforts for each population.

Introduction

Biodiversity world-wide is declining rapidly due to habitat loss, overexploitation, and climate change (Ceballos et al., 2020; Haddad et al., 2015; IUCN, 2021; Ripple et al., 2019). The aim of conservation is to prevent this decline and to preserve unique species and populations (DeWoody et al., 2021; Teixeira & Huber, 2021). Across a species' range, variation in environmental conditions combined with restricted gene flow can lead to population differentiation, due to both neutral (drift) and adaptive (selection) processes. From a conservation perspective, such differentiated populations are important, as they contain unique traits that could be lost from the species' pool of variation if the individual populations were to disappear (Cadotte et al., 2011). Therefore, effective conservation requires investigating the ecology and genetics of populations. This necessitates a holistic approach.

Non-invasive faecal sampling is a powerful tool for genetic studies of elusive animals (Beja-Pereira et al., 2009; Schwartz et al., 2007). In addition to DNA from the individual itself, faecal DNA also contains clues to its ecology, including diet and gut microbiome (Valentini et al., 2009). Food consumed reflects individual feeding behaviour and health, and can be informative about foraging strategies, ranging patterns, and social and cultural behaviour (Kamil et al., 2012). Further, diet can reflect local adaptation, if populations specialise due to local availability of different foods or different foraging behaviours and cultures (Alberdi et al., 2020).

Tightly linked to diet, the gut microbiome impacts long-term health and behaviour of its host, by aiding digestion, modulating dietary choice, and stimulating the immune system (Agranyoni et al., 2021; Colston & Jackson, 2016; Davidson et al., 2020; Ley et al., 2008; Moran et al., 2019). Isolated populations in different habitats have been shown to possess unique gut microbiomes, which is likely a result of ecological and genetic differences (Bueno de Mesquita et al., 2021; Couch et al., 2020; Fietz et al., 2018). The gut microbiome may even facilitate local adaptation and drive genomic evolution (Alberdi et al., 2016; Rudman et al., 2019). It is therefore important to consider gut microbiome when evaluating conservation needs and values (Trevelline et al., 2019; West et al., 2019).

Here, we jointly investigate dietary and gut microbial diversity and composition in isolated and genetically differentiated populations of Grauer's gorillas (*Gorilla beringei graueri*) using DNA metabarcoding. This gorilla subspecies is endemic to the eastern Democratic Republic of Congo (DRC)

73 and is classified as critically endangered due to recent drastic population decline (IUCN, 2016; Plumptre
74 et al., 2016, 2021). Our study populations occupy the low and high altitudinal extremes of the species'
75 range (600-700m, 800m and 2100-2400m), with limited connectivity and gene flow between
76 populations (Baas et al., 2018; Plumptre et al., 2021). We hypothesized that ecological differences
77 would cause diet and gut microbiome to differ between these isolated populations, co-varying with host
78 genetic differentiation. In addition, we investigated whether the taxonomic composition of the gut
79 microbiome is influenced by the dietary profile and examined microbiome stability in individuals
80 through time.

Materials and 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 approaching or disturbing wild animals.

Sample collection

Faecal samples (n=220) were opportunistically collected from night nests of Grauer's gorillas in eastern DRC between 2015 and 2018 at three sites: Kahuzi-Biega National Park (2.32˚S, 28.72˚E; KBNP; 2500 m above sea level [asl]), Nkuba Conservation Area in Walikale territory, North Kivu (1.38˚S, 27.47˚E; NCA; 600 m asl), and Maiko National Park (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 immediately upon 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. We included biological replicates for several individuals for genotyping and gut microbiome analyses.

DNA extraction

Faecal 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)

109 in a dedicated primate faecal extraction laboratory. We implemented the following modifications to the
110 manufacturer's protocol: faecal samples were incubated under shaking (500 RPM) in the C1 solution
111 overnight at 23°C. They were then transferred into a heating block and incubated at 65°C for 10 minutes,
112 followed by bead beating on a vortex at maximum speed for 1 hour. Incubation in C2 and C3 solution
113 was on ice. We incubated the samples in C6 solution at room temperature for 5 minutes before elution.
114
115 *Gorilla genotyping, individual identification, relatedness and population differentiation analyses*
116
117 We genotyped all 220 samples at 12 microsatellite loci (vWF, D1s550, D4s1627, D5s1457,
118 D5s1470, D6s474, D6s1056, D7s817, D8s1106, D10s1432, D14s306, and D16s2624) following the
119 two-step multiplex protocol (Arandjelovic et al., 2009) and sexed them with the amelogenin assay
120 (Bradley et al., 2001). PCR products were run on an agarose gel to confirm amplification success and
121 absence of contamination in blanks. Up to four loci were pooled, based on fluorophores and product
122 sizes, and run on the ABI GeneAnalyzer (ThermoFisher Scientific). We scored genotypes manually in
123 GeneMapper v5.0 (Chatterji & Pachter, 2006) and used Cervus v3.0.7 (Kalinowski et al., 2007) to
124 identify individuals. Samples were considered from the same individual if their genotypes had no
125 mismatches and matched at five or more loci, with the probability of identity assuming full-sibling
126 relationship (PIDsib) less than 0.05. We manually generated consensus individual genotypes from
127 matching samples, taking into account their time and place of sample collection, and evidence about
128 the presence of other individuals from the same group.
129 We tested for deviations from Hardy-Weinberg equilibrium, heterozygote deficiency, and
130 linkage disequilibrium at each locus in GenePop v4.7.5 (Raymond & Rousset, 1995; Rousset, 2008).
131 Genetic population structure was assessed using STRUCTURE v2.3.4 (Porras-Hurtado et al., 2013)
132 with 20 independent runs for K = 1-11 (corresponding to the eleven social groups), an 100,000-iteration
133 burn-in, and data collection for 1,000,000 runs, assuming population admixture and correlated allele
134 frequencies (Falush et al., 2003). Results from different runs of K were merged in CLUMPP (Jakobsson
135 & Rosenberg, 2007; Kopelman et al., 2015), and analysed and visualized in 'pophelper' in R (Francis,
136 2017; R Core Team, 2021). The most likely value of K was determined using ΔK (Evanno et al., 2005).

137 We used the 'adegenet' R package for Principle Component Analysis (PCA) based on individual
138 genotypes (Jombart, 2008). Population differentiation statistics F_{ST} and F'_{ST} (Meirmans & Hedrick,
139 2011) were calculated in GenoDive v3.04 (Meirmans, 2020), and significance assessed with 9999
140 permutations. We compared genetic relatedness between populations and social groups using an
141 AMOVA in the R package 'poppr' (Kamvar et al., 2014) and calculated pairwise relatedness (*r*)
142 between all individuals within KBNP and NCA separately in ML-Relate (Kalinowski et al., 2006).
143
144 *Characterization of gorilla diet*
145
146 We characterized the diet of 92 unique individuals identified by genotyping (see Results), from
147 nine social groups and two lone silverbacks (**Table S1, S2**). Where possible, we selected a single nest
148 site per group, to maximize the number of unique individuals. When multiple samples per individual
149 were available, we chose the sample that performed best in genotyping as indication of quality.
150 We amplified the P6 loop of the *trn*L chloroplast intron (Taberlet et al., 2007), a locus that has
151 been successfully used for dietary metabarcoding in primates, and for which a large database of tropical
152 plants is available (Mallott, Garber, et al., 2018). We used the standard *trn*L g and h primers (**Table**
153 **S3**), tagged with 96 eight-base-pair (bp) barcodes. Each barcode differed from all others at a minimum
154 of three positions. DNA amplifications were carried out in 20 µl reactions containing 2 µl faecal DNA
155 extract, 1 U Platinum II *Taq* Hot-Start DNA polymerase, 1x Platinum II Buffer, 0.2 mM each dNTP, 2
156 mM $MgCl_2$, and 1 µM each primer. Each DNA sample was amplified twice. The duplicates were placed
157 randomly on different PCR plates to avoid potential batch effects and biases due to cross-contamination
158 of sample and/or barcoded primer (**Table S1**). We included one PCR negative and two to three empty
159 wells per plate, to check for contamination during PCR (Taberlet et al., 2018). In addition, we included
160 five DNA extraction blanks. PCR conditions consisted of 2 minutes denaturation at 94˚C followed by
161 35 cycles of 94˚C for 30 seconds, 51˚C for 30 seconds, and 68˚C for 15 seconds, without final extension.
162 PCR products were checked on a 2% agarose gel to confirm amplification without contamination.
163 The barcoded PCR products were pooled column-wise (16 µl for each sample, duplicates in
164 separate pools), mixed with 640 µl PB Buffer, and purified using MinElute columns (Qiagen, The

165 Netherlands), eluting in 50 µl EB buffer. Double-indexed next-generation sequencing libraries (Kircher
166 et al., 2012) were prepared as detailed (Brealey et al., 2020; Rohland et al., 2015) but using not-barcoded
167 incomplete adapters after blunt-end repair. Two library preparation blanks were carried through all
168 steps. Each pool was quantified using qPCR with PreHyb primers (**Table S3**; Rohland et al., 2015) and
169 amplification settings as in Brealey et al. (2020).

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

184 Sequence processing and analysis was done in OBITools v1.2.13 (Boyer et al., 2016). Paired
185 reads with quality scores >40 and overlap >10 bp were retained and merged. Sample of origin for each
186 read was established through its index and barcode, requiring an exact sequence match. Sequences were
187 clustered into molecular operational taxonomic units (MOTUs), each representing a unique plant taxon
188 (Valentini et al., 2009). A large number of MOTUs had fewer than 10 sequences across all samples and
189 were removed as recommended (*e.g.*, Shehzad et al., 2012). We also removed sequences that differed
190 by exactly one nucleotide from a more abundant sequence and had a total count less than 5% of the
191 more abundant sequence, following Boyer et al. (2016).

192 Finally, taxonomic assignment used a custom-made reference database (below). Based on a
193 frequency plot of identity to the reference database (**Figure S1**) and similar *trn*L-based studies of
194 tropical primate diet (*e.g.,* Quéméré et al., 2013), we removed sequences below an identity threshold
195 of 0.90. Below this, sequences were regarded as likely chimeric, enriched in sequencing or PCR
196 errors. No singletons were present after this filtering step.
197
198 *Compiling plant* trn*L reference database*
199
200 We built a local DNA barcoding reference library by downloading all 324,502 available
201 sequences from NCBI GenBank using the search query: "(trnL[All Fields] OR complete genome[All
202 Fields]) AND (plants[filter] AND (chloroplast[filter] OR plastid[filter]))" (last accessed 2 December
203 2021). In OBITools v1.2.13, the sequence list was annotated with taxonomy information downloaded
204 from NCBI (ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz, last accessed 3 December 2021). To
205 complete the database of *trn*L genes, we followed established protocol (Boyer et al., 2016), using the
206 same *trn*L g-h primers as in the wet laboratory to extract *trn*L variants *in silico* in the program ecoPCR
207 v2.1 (Ficetola et al., 2010). We kept sequences that were between 10 to 230 base pairs long with at most
208 three primer mismatches total (Taberlet et al., 2018). The final database contained 21,308 *trn*L *in silico*
209 amplicons, in 608 families and 5,662 genera.
210 To evaluate the resolution of our reference database with respect to local plant diversity, we
211 compared plant taxa present in our database to a list of plants known to occur in the Kahuzi and Itebero
212 regions of KBNP (Yumoto et al., 1994). To enable this comparison, we updated the taxonomic
213 classification of the KBNP plant list (Yumoto et al., 1994) by searching for species names in the Global
214 Biodiversity Information Facility (GBIF). The updated list contained 328 taxa, in 81 unique families
215 and 234 genera. Of these, all families and 77.4% of genera were present in our *trn*L database.
216
217 *Characterization of gorilla gut microbiome*
218

219 We determined gut microbial composition of 86 samples belonging to 68 individuals from
220 KBNP and NCA populations (**Table S2**). (Samples from MNP were not yet available at the time of
221 microbiome characterization.) The dataset included multiple samples from 15 individuals (2-3 each),
222 collected 0-257 days apart (mean = 27 days, median = 0), which we used to evaluate intra- versus inter-
223 individual variation in microbiome composition. We also carried nine random extraction blanks through
224 the entire data generation process, on par with samples.

225 The V4 region of the *16S rRNA* gene was amplified with primers 515F/806R (**Table S3**) for
226 each sample in duplicate. The PCR reaction contained 2 µl of extracted DNA, 5 µM each of the forward
227 and reverse primer, 1x Phusion High-Fidelity Buffer, 0.02 units Phusion HF DNA polymerase (2U/µl),
228 0.012 mg DMSO and 0.05 µM (each) dNTPs, with the volume made up to 20 µl with Ultrapure H2O.
229 Thermal cycling conditions were as follows: 30 seconds at 98°C, 25 cycles of 98°C for 10 seconds,
230 52°C for 20 seconds and 72°C for 20 seconds, and 10 minutes at 72°C. PCR cycles were limited to 25
231 to minimize the risk of unspecified products and chimeras. Duplicate reactions were pooled and cleaned
232 with AmPure beads (Qiagen).

233 Next-generation sequencing libraries were prepared from PCR products following the double-
234 barcoding, double-indexing strategy (Kircher et al., 2012; Meyer & Kircher, 2010; Rohland et al., 2015;
235 van der Valk et al., 2017). As a result, each sample had a unique combination of two barcodes and two
236 indices, which enabled bioinformatic filtering of potential chimeric molecules and misassigned reads
237 resulting from index hopping (van der Valk et al., 2017, 2020). For indexing, we determined the suitable
238 number of PCR cycles (8-11) based on qPCR of barcoded libraries, as above. Indexed libraries were
239 quantified by qPCR and pooled in equimolar amounts for sequencing on a single MiSeq lane, using
240 version 2 chemistry and 250 bp paired end sequencing at the Uppsala Science for Life Laboratory
241 sequencing facility.

242 Sequencing reads were demultiplexed and adapters removed using a Python script (Brealey et
243 al., 2021). We followed established protocol to estimate microbial amplicon sequence variants (ASVs)
244 using DADA2 (Callahan et al., 2016), rather than clustering sequences, which avoids biases due to
245 arbitrary similarity thresholds (Edgar, 2018). Forward and reverse reads were truncated to 200 and 150
246 bp, respectively, at which point read quality scores dropped below 35. We merged paired-end reads,

247 requiring an overlap of at least 12 bp, and removed sequences outside the 250-256 bp range and those
248 with any barcode mismatch, as recommended (Callahan et al., 2016).
249 Taxonomy was assigned using the SILVA 132 reference database, released in December 2017
250 (Quast et al., 2012). Species-level assignment required a strict 100% match (Edgar, 2018). We removed
251 singletons and ASVs labelled 'Unassigned', 'Eukaryota', "mitochondria", or "chloroplast". We
252 retained Archaea, although archaeal amplification from the V4 region of the *16S rRNA* is limited
253 (Raymann et al., 2017), because within-dataset comparisons are nonetheless informative. We built a
254 bacterial phylogenetic tree by aligning sequences to the Greengenes 13_5 mega-phylogeny (203,452
255 99% OTUs; DeSantis et al., 2006) in SEPP using default parameters (Mirarab et al., 2012).
256
257 *Statistical analyses of* trn*L and* 16S *datasets*
258
259 To examine dietary and microbiome diversity, we analysed the *trn*L and *16S rRNA*
260 metabarcoding datasets, after first filtering out rare sequence variants below 0.5% relative abundance
261 in at least one sample, as suggested (Deagle et al., 2019). We evaluated sampling effort and sequencing
262 depth accumulation curves in the R packages 'vegan' (Oksanen et al., 2020) and 'ranacapa' (Kandlikar
263 et al., 2018), respectively. We checked whether the predicted number of taxa (asymptote of the
264 sequencing accumulation curve) minus actual number of taxa (richness) related to any of the considered
265 biological variables or sequencing depth (read count) using a generalised linear model (GLM) with
266 quasi-Poisson error distribution in the R package 'lme4' (Bates et al., 2015).
267 We calculated two alpha diversity metrics for each dataset: richness, or the number of taxa, and
268 Shannon's diversity index, or evenness (Chao et al., 2014). As recommended by McMurdie & Holmes
269 (2013), we did not rarefy to minimum sequencing depth. To test the effects of population, social group,
270 altitude, sex, and age class on diversity metrics, we fitted a GLM with quasi-Poisson (for richness) or
271 gamma (for evenness) error distribution with log link, followed by Tukey honestly significant
272 difference (HSD) *post-hoc* comparisons between levels of categorical variables that were overall
273 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 centred 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 modelled 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). We assessed the temporal stability of individual microbiomes using a pairwise Wilcoxon test between Aitchison dissimilarities of replicate and unique individuals. The influence of genetic distance (*1* - genetic relatedness) was modelled separately within each population using Mantel and partial Mantel tests (controlling for social group identity).

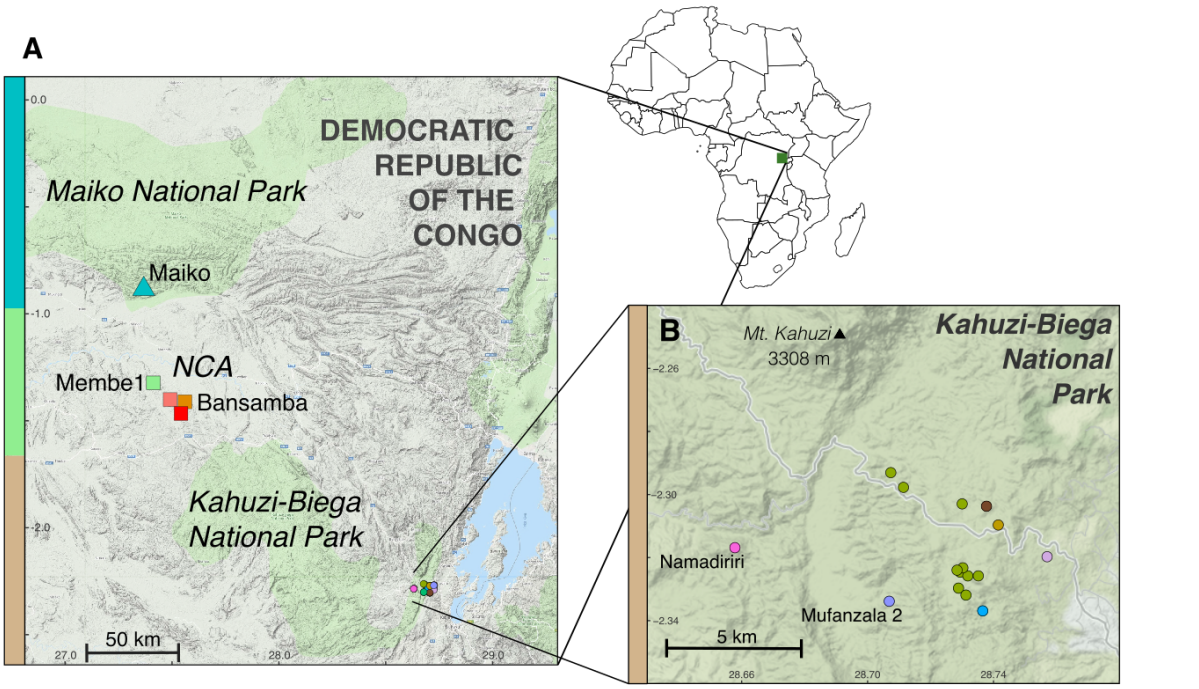
We estimated the covariance between diet and microbiome using a co-inertia analysis between the two matrices in the package 'omicade4' and calculated the RV coefficient (Escoufier, 1973; Robert & Escoufier, 1976) and its significance using a Monte Carlo test with 999 permutations (Meng et al., 2014). 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.

301 Differences in beta diversity can be due to differential abundance of a few key organisms, or
302 subtle differences across an entire community. To identify dietary and microbial taxa that may have
303 driven compositional differences, we used the R package 'ALDEx2' and focused on significant
304 differences (Wilcoxon rank sum test with correction for false discovery rate (FDR) $p < 0.05$) with effect
305 sizes >1, as recommended (Gloor et al., 2017).
306

Results

Study populations of Grauer's gorillas are genetically differentiated

We identified 92 unique individuals in the three study populations by microsatellite genotyping: 59 in KBNP, 28 in NCA, and 5 in MNP (**Figure 1A, Table S2**). Individuals belonged to six different social groups in KBNP, two in NCA, and one in MNP. We sampled four to 17 individuals from each social group. In addition, there were two solitary adult male gorillas (lone silverbacks) in KBNP.



Social group		N (f,m)	Year	Month	Altitude (m)	$N_{genotypes}$
Bonane	Kahuzi-Biega NP	1 (0,1)	2016	June	2328	1
Chimanuka		14 (4,9)	2016	April-June	2300	44
Mankoto		12 (1,11)	2016	July	NA	13
Mpungwe		8 (6,2)	2016	June	2200	21
Mufanzala 2		4 (3,1)	2016	July	2443	5
Mugaruka		1 (0,1)	2016	June	2123	2
Namadiriri		13 (8,5)	2016	June	2174	23
Nouvelle Famille		6 (5,1)	2016	July	2282	16
Bansamba	Nkuba-Biruwe	11 (9,2)	2015	June	619	46
Bansamba		3 (1,2)	2016	April	630	
Bansamba		3 (0,3)	2018	July	675	
Membe 1		11 (5,6)	2017	August	650	33
Maiko	Maiko	5 (3,2)	2016	November	835	6

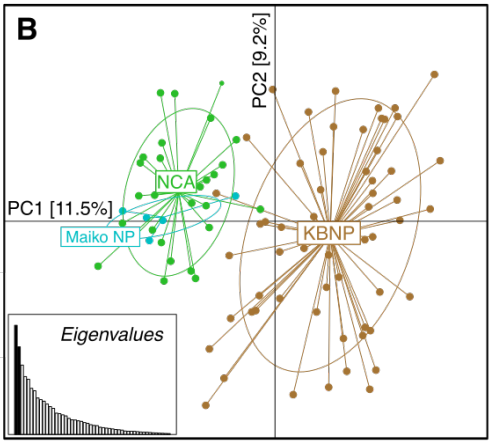


Figure 1. Geographic location and genetic differentiation of Grauer's gorilla populations. (**A**) Broad geographic location of Grauer's gorilla populations in Maiko National Park (MNP; designated with cyan on the left-hand side of the map), Nkuba Conservation Area (NCA; green) and Kahuzi-Biega National Park (KBNP; brown). Multiple social groups were collected in NCA and KBNP. Each social group is given its own symbol colour, whereas repeated sampling of the same social group is shown by multiple identical or, for the group sampled in multiple years, similarly coloured symbols. The inset (**B**) zooms in on KBNP. Circle colours designate

different social groups, as shown in the table (C). Note that multiple circles are present for the Chimanuka group, consistent with repeated, opportunistic sampling of identified individuals. Geographic coordinates were not available for Mankoto group. (**C**) Overview of sample size for dietary characterization (N=number of unique individuals), sex composition (f=number of females, m=number of males), collection year and month, altitude and the total number of successfully genotyped samples from each group. Gut microbiome data is not available for groups shaded in grey. Group Bansamba (NCA) was sampled repeatedly, but only few samples were included in dietary analyses in later years (3 in 2016 and 3 in 2018). (D) PCA of genetic distances among individuals, coloured by population. KBNP is clearly separated from NCA and MNP.

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

Analysis of the three populations using STRUCTURE (Porras-Hurtado et al., 2013) revealed two distinct genetic groups (optimal K=2 according to ΔK; Evanno et al., 2005; **Figure S2**). The clusters differentiated gorillas in high-altitude KBNP from those in low-altitude NCA and MNP (**Figure S3**). Only three KBNP individuals (from two different social groups) showed a higher genetic similarity to the low altitude populations. This population structure was consistent with the PCA, which divides gorilla genotypes in KBNP from NCA and MNP (**Figure 1B**). All three populations were significantly differentiated from one another ($p < 0.001$), with largest differences between MNP and KBNP (F'_{ST} = 0.45; **Table S4A**), which are furthest apart geographically (215km). Individuals within social groups were more closely related than individuals in different groups in the same population (AMOVA ϕ = 0.12, $p < 0.001$; **Table S4B**), consistent with gorilla social structure (Harcourt & Stewart, 2013).

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

Metagenomic studies rarely report treatment of negative control samples, although stringent quantification of contamination is of utmost importance for any molecular study. To quantify contamination in the diet (*trn*L) dataset, we included and analysed five DNA extraction blanks, two

355 PCR negative controls, five unused barcode combinations, all sequenced in duplicates, and two library
356 preparation negative controls (**Table S2**; **Table S5**). There were 286 *trn*L reads belonging to 19
357 different taxa in the negative controls in total. With two exceptions, the detected taxa showed very low
358 abundance in the blanks (1-3 reads) but were highly abundant in the samples (average > 200,000 reads).
359 This pattern is consistent with low-level cross-contamination from high quantity into low quantity
360 samples typical for large-scale sequencing studies (Eisenhofer et al., 2019). The two exceptions with
361 high abundance in blanks (77% and 15% of all blank reads, respectively) had low abundance in the
362 samples (<0.01%) and were in the families Fagaceae (chestnut) and Rutaceae (citrus). We thus
363 concluded that they may derive from reagent contamination. Both taxa were automatically removed
364 from the dataset during abundance-based filtering, as they were below the specified 0.5% cut-off. Zero
365 reads were identified with unused barcode combinations, suggesting that cross-contamination of
366 barcodes during PCR and library preparation was negligible (Taberlet et al., 2018).
367 In the microbiome (*16S rRNA*) dataset, five out of nine DNA extraction blanks (**Table S2**)
368 contained zero reads. The remaining four blanks had six to 72 reads each, in ten different *16S* ASVs
369 (**Table S6**). These ten taxa were among the most abundant in the samples and thus, as above, likely
370 represent cases of low-level cross-contamination. We found no indication of ASVs exclusive to the
371 blanks, and hence all ASVs were retained in the dataset.
372
373 ***Diet of Grauer's gorillas***
374
375 We characterised the diet of 92 Grauer's gorilla individuals using the chloroplast *trn*L *P6 loop*
376 locus. After data filtering, we retained 536,160 *trn*L sequencing reads, 45% of raw reads, belonging to
377 120 unique taxa (**Table S7A**). PCR duplicates were more similar to each other than to other samples in
378 alpha and beta diversity ($p < 0.001$, **Figure S4**), and hence their sequencing data were pooled. Sample
379 accumulation curves suggested that additional samples would uncover only few novel *trn*L variants
380 (**Figure S5A**), except in MNP, where only five individuals were sampled. Although deeper sequencing
381 could have potentially uncovered novel taxa (**Figure S5B**), sequencing depth did not have a strong
382 effect on *trn*L richness (three additional taxa per 47,000 reads, $p = 0.03$). Furthermore, the discrepancy

per sample in observed minus predicted richness did not corelate with sequencing depth (p = 0.3, nor with any of the tested variables (population p = 0.9; social group p = 0.06; sex p = 0.6; age class p = 0.8; altitude p = 0.4). Taken together, this suggests that our dataset is suitable to carry out comparisons across study populations.

Of the 120 detected taxa, 115 could be identified to at least the order level (in 29 different orders), 110 to family (in 49 families), and 44 to genus (in 35 genera) (**Table S8**). Based on previous studies of Grauer's gorilla diet in KBNP, NCA, and Mt. Tshiaberimu (Kambale, 2018; van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 1994, 2005; Yumoto et al., 1994) and plant distribution in the region (Spira et al., 2018), we manually confirmed that all taxa assignments were possible. This revealed 21 taxa that have not been previously reported to be consumed by gorillas but occur in the study region (**Table S8**).

Each Grauer's gorilla faecal sample contained 36 – 80 *trn*L taxa (mean 58.52 ± 10.83) (**Figure 2A**). The most abundant taxon across the dataset was *Urera* sp. (MOTU1, 25.6% of all reads), followed by Apocynaceae sp. (MOTU2, 14.8%), Apocynoideae sp. (MOTU5, 6.7%), Urticaceae sp. (MOTU6, 4.6%), and *Maesa lanceolata* (MOTU9, 4.3%) (**Table S8**). The five most abundant plant families were Urticaceae (31.9%), Apocynaceae (23.0%), Moraceae (7.1%), Myristicaceae (5.0%), and Primulaceae (4.4%).

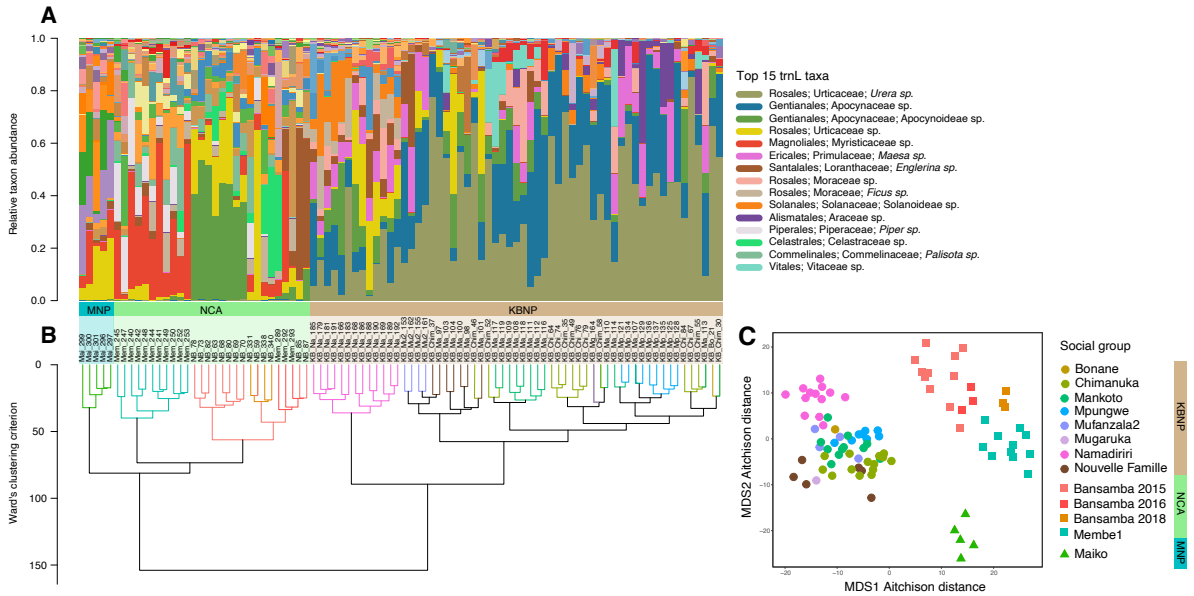


Figure 2. (**A**) Plants consumed by Grauer's gorillas in KBNP, NCA, and MNP, with the 15 most abundant taxa across all samples indicated in the legend on the right. Populations are designated with coloured bars below

(Maiko NP cyan, Nkuba Conservation Area green, Kahuzi-Biega NP brown). (**B**) Hierarchical cluster dendrogram of Ward's sum of squares based on minimum variance of squared dissimilarities (Murtagh & Legendre, 2014) of centred-log-ratio (CLR) transformed taxon abundance. Relative taxon abundance clustered by population (aligned to the bars below (A)) and by social group, shown as coloured tips in the dendrogram (see legend to right of (C)). (**C**) Non-metric multidimensional scaling (NMDS) ordination of *trn*L CLR-transformed Aitchison distances, representing dietary composition dissimilarity across KBNP, NCA, and MNP. Groupings by population (shape) and social group (colours) were significant (PERMANOVA $p < 0.001$, $R^2 = 0.28, 0.22$, respectively).

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

Dietary richness and evenness differed significantly by population and social group identity ($p < 0.001$, **Table S9**). Both richness and evenness were higher in low altitude populations (NCA and MNP) than in high-altitude KBNP (mean richness: 66.8 taxa in MNP, 65.6 in NCA vs. 54.5 in KBNP, evenness: 10.0 in MNP, 8.4 in NCA, vs. 5.4 in KBNP, $p < 0.001$; **Figure S6**). Within the KBNP population, the diets of social groups Namadiriri and Nouvelle Famille were richer than other groups (>62 vs. <53 plant taxa), and the diets of groups Namadiriri and Mankoto were more even ($p < 0.01$; **Table S9**; **Figure S6**). One of these groups, Namadiriri, was sampled at lower elevation than any other social group in KBNP. In this population, one standard deviation of altitudinal increase (84 m) from the mean (2250m asl) corresponded in a 6% decrease in richness and 25% decrease in dietary evenness ($p < 0.001$; **Figure S7**). In contrast, neither individual's sex nor age class (age class available for N=70) had an effect on dietary richness or evenness ($p > 0.3$).

Hierarchical clustering of dietary composition first separated high altitude (KBNP) from low altitude (NCA and MNP) locations (**Figure 2B**). Within populations, individuals clustered together by social group. NMDS ordination showed a similar pattern (**Figure 2C**). After accounting for slight differences in sequencing depth, dietary composition was significantly influenced by population ($p < 0.001$), which explained 27.9% of the variance, and social group ($p < 0.001$), which explained an additional 21.6%, but not sex ($p = 0.7$) nor age class ($p = 0.2$; **Table 1**). All between-group *post-hoc* comparisons were significant ($p < 0.05$), suggesting that the group Chimanuka was not an outlier, though it was sampled from April-May 2016 rather than June-July 2016 like other KBNP groups.

435 **Table 1.** PERMANOVA model of factors influencing dietary composition

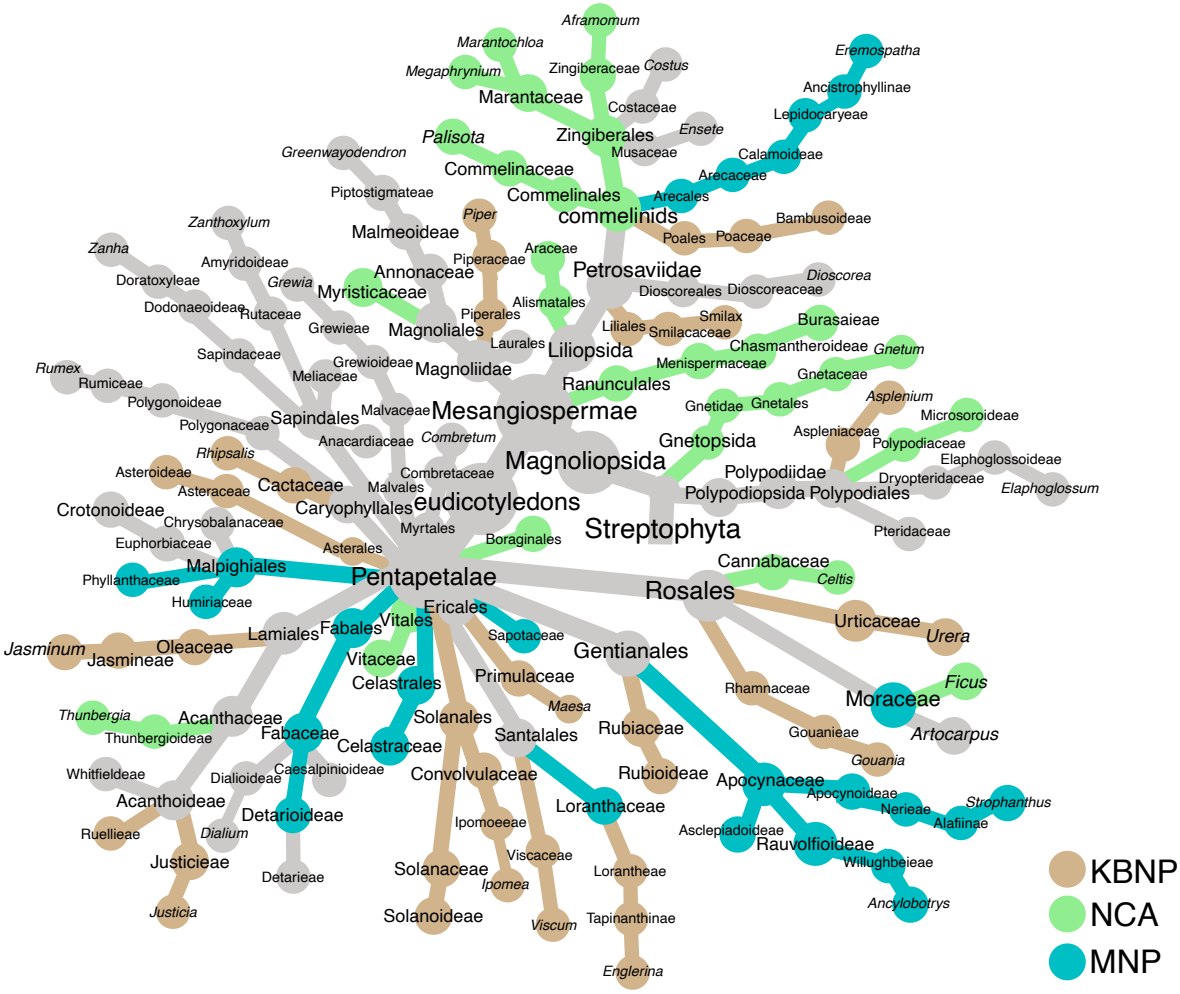
Variable	Df	R^2	F	*p*		*Post-hoc* tests	
						Significant pairwise comparisons	$p_{Bonferroni}$
Read Count	1	0.01	1.73	0.05		–	
Population	2	0.279	17.80	<0.001		NCA – KBNP	< 0.001
						KBNP – MNP	< 0.001
						MNP – NCA	< 0.001
Social Group	6	0.216	4.56	<0.001	KBNP	Chimanuka – Nouvelle Famille	0.02
						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 modelled separately using a reduced dataset, since only 70/92
437 samples had age estimates. In this model the other predictor variables had similar estimates, so for all other variables the model for the
438 complete dataset (n=92) is presented.
439

440 We identified differentially abundant taxa by population and social group using ALDEx2
441 (**Figure 3**, **Table S10**). Eight taxa were enriched in KBNP relative to MNP and NCA (Wilcoxon test
442 with FDR < 0.05) and were considered important contributors to population dietary uniqueness (effect
443 size > 1). These were dominated by the family Urticaceae, especially the genus *Urera*, and also included
444 *Piper sp.* (Piperaceae), *Maesa lanceolata* (Primulaceae), and taxa in Apocynaceae and Solanaceae. In
445 NCA, seven taxa including *Megaphrynium, Marantochloa, Aframomum, Palisota,* and Myristicaceae
446 spp., were enriched relative to the other populations. We found 14 enriched taxa in MNP, notably
447 Apocynaceae spp. Out of the 21 previously undescribed food items, 13 were more common in low-
448 altitude populations in comparison to the high-altitude population. These taxa included *Gnetum* sp.,
449 more abundant in NCA relative to KBNP and MNP (MOTU34, effect = 0.93) and Humiriaceae sp.,
450 enriched in both NCA and MNP relative to KBNP (MOTU48, effect = 0.79) (**Table S8, S9**). Within

451 populations, each social group consumed twelve differentiating taxa on average (range = 0 – 30, median

452 = 8).



453

454 **Figure 3.** Grauer's gorilla diet across three study populations. Differentially abundant taxa are coloured according
455 to the population in which they are significantly more abundant (ALDEx2 Wilcoxon test $p < 0.05$). For taxa that
456 differ between two or more population pairs, the colour corresponds to the population where the effect size is
457 greatest. Gray taxa do not differ significantly in abundance between populations. Branch lengths do not reflect
458 phylogenetic distance. This diagram was generated with the 'metacoder' package in R (Foster et al., 2017).

459 ***Gut microbiome of Grauer's gorillas in Kahuzi-Biega National Park and Nkuba Conservation Area***

460

461 We sequenced the *V4* region of *16S rRNA* to characterize the microbiome of 88 samples from
462 a subset of individuals for which we had dietary data (n=70, **Figure 1A**; **Table S2**). Two samples had
463 low read count (5 and 348, compared to other samples: mean=43,611 ± 11,357) and were thus excluded.
464 Our dataset contained multiple individuals (n=15) that were sampled repeatedly (2-3 times each),
465 allowing us to assess individual gut microbiome stability over time. Microbiome composition was more
466 similar in samples from the same individual collected zero to 257 days apart (mean compositional
467 dissimilarity = 35.0) than between different individuals from the same social group sampled zero to 231
468 days apart (Wilcoxon Test $p < 0.001$). Therefore, for further analysis we focused on the 68 unique
469 individuals and picked the same faecal samples used for dietary analyses. The final dataset contained
470 2,965,516 reads in 421 unique microbial taxa (**Table S11**).

471 The sample accumulation analyses suggested that additional sampling of faeces from more
472 individuals could uncover novel gut commensals at the population level (**Figure S8A**). However, per
473 sample sequencing depth was sufficient to obtain a good representation of host microbiome diversity
474 (**Figure S8B**). We detected 16 different phyla and 48 different families of microorganisms in the gut
475 microbiome of Grauer's gorillas. All taxa were identified at least to the family level; 309 taxa were
476 refined further to genus and 17 to species (**Table S11**). None were closely related to dominant soil
477 microorganisms (Delgado-Baquerizo et al., 2018). There were seven Archaea in our dataset, from the
478 Methanomethylophilaceae and Methanobacteriaceae families. Each faecal sample contained on average
479 200.29 ± 19.6 unique taxa (min = 160, max = 237), each with average abundance 0.2% ± 0.4%. The
480 most abundant taxa were *Christensenellaceae* R-7 group sp. (3.07% per sample), *Flexilinea* sp.
481 (2.97%), and *Faecalibacterium* sp. (2.46%). Eleven taxa were present in every gorilla faecal sample,
482 constituting a "core microbiome" of Grauer's gorillas (**Table 2**).

483 At a broader taxonomic scale, the average Grauer's gorilla gut microbiome was dominated by
484 the phyla Firmicutes (64.7%), Bacteroidetes (21.0%), Spirochaetes (3.8%), Chloroflexi (3.0%),
485 Proteobacteria (2.9%), and Actinobacteria (1.9%) (**Figure S9**). The most abundant families were
486 Ruminococcaceae (25.1%), Lachnospiraceae (14.8%), and Rikenellaceae (13.1%) (**Figure 4A**).

487

488 **Table 2.** Grauer's gorilla core microbiome, taxa present in 100% of samples.

Core Microbiome	Abundance†	Taxonomy to genus
ASV1	2.97%	Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae; *Flexilinea*
ASV3	2.41%	Bacteria; Firmicutes; Clostridia; Clostridiales; Family XIII; *AD3011 group*
ASV6	2.13%	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; *UCG-004*
ASV22	0.94%	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; *Prevotella 7*
ASV21	0.87%	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *UCG-005*
ASV31	0.85%	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Oribacterium*
ASV30	0.82%	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; *Sutterella*
ASV33	0.74%	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Ruminiclostridium 9*
ASV59	0.41%	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *UCG-002*
ASV70	0.35%	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Eggerthellaceae; *Senegalimassilia*
ASV152	0.15%	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; NA

489 †Abundance per sample, averaged over the entire dataset (N=68).

490

491 *Diversity and composition of the gut microbiome in Grauer's gorillas correlates with population and*

492 *social group identity*

493

494 Opposite to observations for diet, gut microbiome richness and evenness were higher in the

495 high-elevation population (richness: mean KBNP = 202.2 taxa vs. NCA = 190.2; $p = 0.05$; evenness:

496 83.7 vs. 74.2, $p = 0.01$), though neither diversity metric was related to altitude within KBNP ($p = 0.07$,

497 0.9; **Table S12**; **Figure S10**). Microbial richness did not differ by sex ($p = 0.2$), but females had higher

498 microbial evenness than males (85.7 vs. 78.5, $p = 0.002$). There were no differences by age class ($p =$

499 0.3, 0.1). In KBNP, as with diet, the gorilla group Namadiriri had the highest microbial richness and

500 evenness (**Table S12**). Neither gut microbial richness nor evenness were directly related to dietary

501 richness or evenness ($p > 0.2$), but dietary composition, scaled to one-dimension by NMDS, did

502 correlate with richness ($p = 0.002$; **Table S12**), a result we investigated further in the following section.

503 The composition of individual gut microbiome differed between the two populations (KBNP

504 and NCA) (**Figure 4B-D**). Population explained 10.5% of the total variance, and social group in KBNP

505 explained an additional 17.8% ($p < 0.001$; **Table 3**). Intergroup differences were not only due to

506 Chimanuka group (**Table 3**). Overall, gut microbiome dissimilarity was largest between individuals of

507 different populations, followed by individuals from different social groups, and individuals sharing a

508 social group were least dissimilar (**Figure S11**). We modelled altitude separately, as it was distinct for
509 each population and social group (**Figure 1**). It explained 12.7% of the variance across populations
510 (N=56, $p < 0.001$) but only 3.8% in KBNP (N=45, $p = 0.01$). Host genetics did not explain variance in
511 gut microbiome composition in KBNP when social group was considered as a factor ($\rho = 0.015$, p =
512 0.3). Despite low sample size, for the one social group in NCA (N=11), we also found no correlation
513 between gut microbiome and genetic distance ($\rho = -0.080$, p = 0.7). Microbiome composition did not
514 differ by sex or age class ($p > 0.05$, **Table 3**). Results using phylogeny-informed (phILR) distances
515 were qualitatively similar (**Table S13**).

516 We identified 111 taxa that differed significantly in abundance between NCA and KBNP ($p <$
517 0.05), of which 42 had effect size greater than one (**Table S14**). At the family-level, gorilla gut
518 microbiomes in KBNP had a higher abundance of Muribaculaceae and Erysipelotrichaceae, while gut
519 microbiomes in NCA had more Spirochaetaceae and Christensenellaceae. At a finer phylogenetic level,
520 populations differed in abundance of specific ASVs belonging to common, shared families like
521 Rikenellaceae, Lachnospiraceae, and Ruminococcaceae.

522

523 **Table 3.** 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	0.004
					Chimanuka – Mankoto	0.01
					Chimanuka – Mpungwe	0.007
					Chimanuka – Namadiriri	0.004
					Nouvelle Famille – Mankoto	0.02
					Nouvelle Famille – Namadiriri	0.01
					Mankoto – Mpungwe	0.007
					Mankoto – Namadiriri	0.004
Sex	1	0.014	1.18	0.2	–	
Age class†	2	0.031	1.22	0.2	–	

524 †Age class (Infants (N=3), Juveniles/subadults (N=21), Adults (N=38)) was modelled separately using a reduced dataset, since only 62/68
525 samples had age estimates. In this model the other predictor variables had similar estimates, so for all other variables the model for the
526 complete dataset (n=68) is presented.

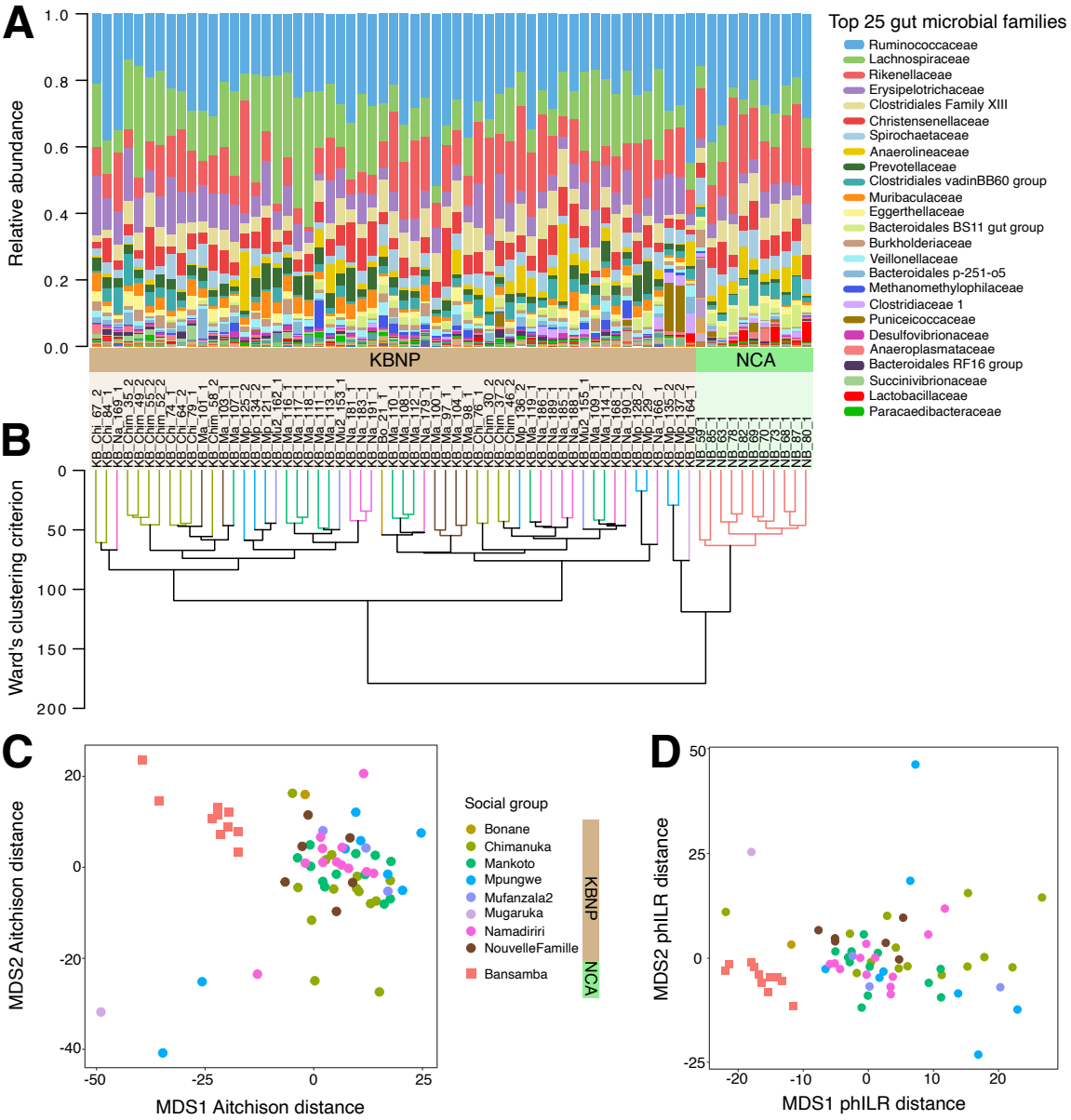


Figure 4. Gut microbiome composition at the family level (**A**) and differentiation based on compositional abundances of microbial ASVs (**B-D**). Gut microbiome clusters by population in CLR Aitchison distances dendrogram based on Ward's clustering criterion (Murtagh and Legendre 2014) (B), CLR Aitchison distances using NMDS (PERMANOVA $R^2 = 0.14$, $p = 0.001$) (C), and phILR Euclidean distances using NMDS ($R^2 = 0.15$, $p = 0.001$) (D).

533 ***Dietary and microbial profiles are correlated with each other***

534

535 Although neither dietary richness nor evenness directly correlated with gut microbial richness
536 or evenness ($\rho < 0.2$, $p = 0.2$; **Figure 5A**), compositional differences in dietary and gut microbial
537 profiles showed significant co-inertia (RV = 0.557, $p < 0.001$; **Figure 5B**) and were correlated ($\rho =$
538 0.32, $p < 0.001$; **Figure 5C**). Microbiome composition is known to change with diet in individuals and
539 also differs between populations with different dietary preferences (*e.g.,* Muegge et al., 2011;
540 Youngblut et al., 2019). To investigate whether dietary differences across our study populations could
541 explain differences in gut microbial composition or, alternatively, if external factors influenced diet and
542 microbiome independently, we compared the effects of diet composition and geographical and social
543 variables on microbiome composition using an MRM model (**Table 4**). Population and social group
544 were the only predictor variables significantly correlated with microbiome composition, while neither
545 dietary composition nor geography had an effect after accounting for social group and population of
546 origin.

547

548 **Table 4.** MRM model comparing the effects of geography, diet, and sociodemographic factors on Grauer's gorilla
549 gut microbiome composition†

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

550 † Microbiome and diet composition in Aitchison distances.

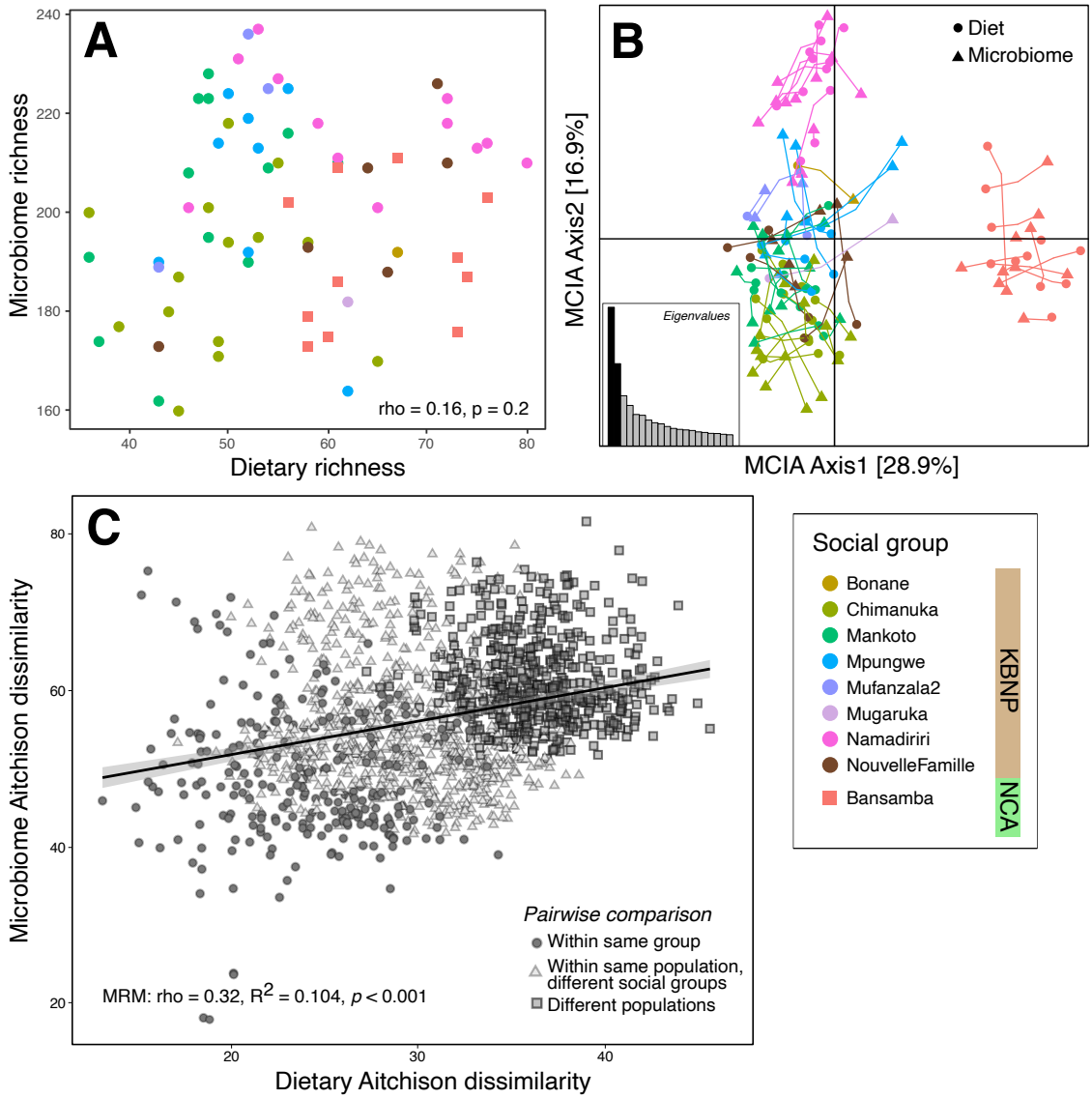


Figure 5. Relationship between diet and gut microbiome. (**A**) Microbiome and dietary richness, assessed as per-sample total sequence count, are not correlated (p = 0.2). (**B**) High multiple co-inertia (MCIA) between microbiome and diet composition in CLR-transformed space with Aitchison distance (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.

558 **Discussion**

559

560 We jointly characterized genetic and dietary profiles of three isolated populations of Grauer's
561 gorillas, one of which has never been studied before (Maiko NP). For two of these populations (Nkuba
562 Conservation Area and Kahuzi-Biega NP), we also characterized the gut microbiome. These
563 complementary datasets enabled direct evaluation of the role of social factors, host relatedness, and diet
564 on gut microbial diversity and composition, thus allowing us to speculate on the importance of
565 ecological differentiation for geographically isolated populations. Whether such a relationship exists
566 and how it is determined is a central topic in ecology (Amato et al., 2019; Bolnick et al., 2014; Bueno
567 de Mesquita et al., 2021; Gomez et al., 2016; Hicks et al., 2018; Muegge et al., 2011). We find that diet
568 and microbiome profiles are correlated and differ between the study populations, likely as a result of
569 habitat differences and social factors. Habitat- or behavioural-driven differentiation in dietary and gut
570 microbial composition, concurrent with genetic differentiation, might be an indicator of processes
571 relevant to local adaptation (Alberdi et al., 2016, 2020; Fietz et al., 2018; Henry et al., 2019; Kolodny
572 et al., 2020; Moeller & Sanders, 2020), which is of particular relevance to the design of effective
573 conservation policy for these critically endangered apes.

574

575 *Molecular analyses complement field observations of Grauer's gorilla diet*

576

577 In addition to traditional methods based on direct observations and feeding traces, molecular
578 approaches provide further opportunities to detect rare dietary items, revealing a more complete picture
579 of cryptic and rare species' nutritional ecology (Ait Baamrane et al., 2012; Bradley et al., 2007;
580 Goldberg et al., 2020; Hibert et al., 2013; Mallott, Garber, et al., 2018; Nawaz et al., 2019; Osman et
581 al., 2020; Quéméré et al., 2013; Valentini et al., 2009). The approach is scalable to many individuals,
582 is not biased towards habituated or approachable individuals, and provides data which can be compared
583 across species (Kartzinel et al., 2019). Importantly, analysis of faecal samples minimizes disturbance
584 and avoids influencing animal behaviour, aligning data collection with conservation goals (Jewell,
585 2013).

586 Nevertheless, as every method, molecular dietary analyses are not perfect. For instance, while
587 Grauer's gorillas predominantly feed on vegetative plants, they occasionally consume insects and fungi
588 (van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 1991). Yet by choosing a chloroplast gene, *trn*L,
589 we restricted dietary characterization in this study to plants only. There are also additional technical
590 limitations. By relying on a reference database for identification of plant taxa, metabarcoding
591 approaches are necessarily limited by the content of these databases (Taberlet et al., 2018). Similarly,
592 chosen thresholds for sequence identity and relative abundance could impact results. We used a
593 sequence identity threshold of 90%, similar to other studies of tropical mammal diets via *trn*L, which
594 use thresholds between 90-95% (Hibert et al., 2013; Quéméré et al., 2013; Srivathsan et al., 2016). We
595 also set a relative abundance threshold to greater than 0.05% in at least one sample, to filter out potential
596 sequencing and PCR errors, chimeric sequences, etc. It is possible, particularly due to the small size
597 and high variability of the *trn*L locus, that our cut-offs removed some genuine dietary items.
598 Additionally, estimated abundances of the different plant taxa may not accurately reflect dietary
599 abundances (Deagle et al., 2019). DNA copy number can be biased by plant tissue type (*i.e.,* fruit, pith,
600 leaves, the latter of which contain more chloroplasts; Egea et al., 2010), relative digestibility (*i.e.,*
601 amount of fibre), and PCR amplification success (reviewed by Deagle et al., 2019). Some of these issues
602 also arise with other approaches. For example, accuracy of macroscopic faecal analysis depends on the
603 types of tissues consumed and the extent of digestion (King & Schoenecker, 2019), while observational
604 studies can overestimate the dietary importance of foods with longer handling times (Yamagiwa et al.,
605 2013).

606 Due to these limitations, it is important to establish how well the molecular results correspond
607 to traditional dietary analyses. The most abundant plant families detected in our study – Urticaceae,
608 Apocynaceae, Moraceae, Myristicaceae, and Primulaceae – were reported to be commonly consumed
609 by Grauer's gorillas in KBNP, based on feeding observations and faeces dissections (Yamagiwa et al.,
610 2005), and in NCA, based on feeding traces (van der Hoek, Pazo, et al., 2021). Vice versa, we detected
611 all top plant families reported in these observation-based studies, suggesting good correspondence
612 between our metabarcoding results and the literature on gorilla diet (van der Hoek, Pazo, et al., 2021;
613 Yamagiwa et al., 2005).

614 It is noteworthy that we have detected plants from which gorillas are known to primarily
615 consume either vegetative parts (*e.g.,* Basellaceae, Oleaceae, Poaceae in KBNP and Myristicaceae,
616 Marantaceae, Arecaceae in NCA) or fruits (*e.g.,* Moraceae, Euphorbiaceae, Sapindaceae, Piperaceae,
617 Primulaceae in KBNP and Vitaceae, Moraceae, Apocynaceae in NCA). This suggests that the *trn*L
618 approach can capture both vegetation and fruits in the diet. In concordance with previous investigations
619 of the diet of gorillas in KBNP (Yamagiwa et al., 2005), *Urera* sp. was the most common taxon in our
620 study (average abundance 21%). It seems likely that this taxon corresponds to *Urera hypselodendron*,
621 which gorillas in KBNP ate on 95% of days (Yamagiwa et al., 2005). However, *Urera* bark is very
622 fibrous, and an alternative explanation is that it preserves well in faeces and is thus easily detected.
623 On average, we detected 59 plant taxa per sample, more than the maximum number of plant
624 species Grauer's gorillas were reported to eat in one day in KBNP (17; Yamagiwa *et al.* 2005). It is
625 possible that gorillas have more complex diets than focal follows, direct observations (usually limited
626 to a few hours per day), or macroscopic faecal inspections suggest. Gorilla retention time can range
627 from 24 to 60 hours in zoos (Remis, 2000), and therefore varied feeding or movement through varied
628 habitats over several days could partially explain this discrepancy. Overall, the number of molecularly
629 identified plant taxa (120) is similar to previous studies, which reported 116 food plants in KBNP
630 (Yamagiwa et al., 2005) and over 100 in NCA (van der Hoek et al. 2021).
631
632 *Divergent dietary ecology between isolated populations*
633
634 We found that the dietary profiles of gorillas differed significantly by population and by social
635 group, with a unique suite of plants characterizing each population. Moreover, gorillas in NCA and
636 MNP tended to have more species-rich diets compared to KBNP. These two sites are 1500 m lower in
637 elevation than KBNP. Even within the KBNP population, we observed the highest dietary diversity in
638 the social group sampled at the lowest elevation (Namadiriri, 100 m below other social groups).
639 Globally, plant diversity tends to decrease with elevation (Rahbek, 1995), and this trend occurs within
640 KBNP too (Imani et al., 2016). Such an altitudinal diversity gradient is relevant for mountain gorillas,
641 who consume fewer species and spend more time feeding on herbaceous vegetation at higher elevation

642 (Ganas et al., 2004). Taken together, our data thus support the theory that feeding in eastern gorillas is
643 at least partially determined by plant availability, with larger dietary repertoire at lower elevations.
644 Another plausible interpretation is that gorillas in low altitude NCA and MNP have greater daily ranges.
645 This could be driven by the search for fruit, suggested in NCA (van der Hoek, Binyinyi, et al., 2021).
646 Or, gorillas may travel further to acquire sufficient nutrition due to potential degradation of habitat in
647 the low-elevation sites, relative to protected KBNP (Maldonado et al., 2012). Such increased mobility
648 could place individuals at even greater risk to poaching.

649 We identified 21 taxa that have not previously been reported in the Grauer's gorilla diet. Some
650 of these occur in KBNP (*e.g.*, various Apocynaceae tribes, non-native Cactaceae, and Laurales like
651 *Ocotea usambarensis*; Spira et al., 2018; Yumoto et al., 1994), and some are eaten by mountain gorillas
652 (*e.g., Solanum,* Solanoideae; Rothman et al., 2014; Watts, 1984) or western lowland gorillas (*e.g.,*
653 *Beilschmiedia*, Laruales; Remis et al., 2001). Others, such as *Gnetum sp.* and Humiriaceae sp., have not
654 been recorded in KBNP, but are western lowland gorilla foods (Rogers et al., 2004; Takenoshita &
655 Yamagiwa, 2008). These taxa were significantly more common in the understudied NCA and MNP
656 sites, suggesting that gorillas incorporate locally available plants into their diet. Another possibility is
657 that these taxa are environmental "contaminants", such as building material of night nests from which
658 samples were collected.

659 Other studies have documented the importance of season in gorilla diet, as gorillas seasonally
660 increase fruit consumption (van der Hoek, Pazo, et al., 2021; Yamagiwa & Basabose, 2006). Our ability
661 to evaluate the effect of season was limited by sampling. For example, gorillas in MNP had a
662 compositionally distinct diet, and these samples were collected later in the year. Still, the two
663 populations sampled at similar timepoints, NCA and most of KBNP, also diverged in diet and
664 microbiome. It is thus possible that the divergences that we observed are partially due to seasonal
665 factors, but further sampling is necessary to elucidate the effect of season.

666

667 *The gut microbiome of Grauer's gorillas resembles that of other gorillas*

668

669 In general, the microbial families and phyla dominating the Grauer's gorilla gut microbiome
670 were similar to those reported in other great apes (top phyla: Firmicutes, Bacteroidetes, Spirochaetes,
671 Chloroflexi, Actinobacteria, Proteobacteria; Campbell et al., 2020; Gomez et al., 2016; Hicks et al.,
672 2018; Nishida & Ochman, 2019). Like other gorilla subspecies, the abundance of Chloroflexi and
673 Spirochaetes in the wild Grauer's gorilla gut microbiome is strikingly high compared to Old World
674 monkeys and humans (Gomez et al. 2016, Hicks et al. 2018, Campbell et al. 2020). However, there
675 were differences in the relative abundance of each of the major phyla compared to wild western lowland
676 gorillas, with the Grauer's gorilla gut microbiomes showing proportionally fewer Actinobacteria and
677 Chloroflexi, but more Firmicutes, Bacteroidetes, and Spirochaetes (Campbell et al., 2020; Fontsere et
678 al., 2021; Hicks et al., 2018). Specifically, the relative abundance of Firmicutes in Grauer's gorilla
679 microbiomes was higher (64.7% average) compared to western lowland (42-53%; Campbell et al.,
680 2020; Fontsere et al., 2021; Hicks et al., 2018) and mountain gorillas (34%, Gomez et al. 2016). The
681 abundance of Bacteroidetes (21.0%) was intermediate between western lowland (10-19%; Campbell et
682 al., 2020; Fontsere et al., 2021; Hicks et al., 2018) and mountain gorillas (30%, Gomez et al. 2016).
683 Higher ratios of Firmicutes-to-Bacteroidetes are thought to indicate greater energy extraction potential
684 (Mariat *et al.* 2009) and decline in captivity (Clayton et al. 2018). Thus, the relatively high F:B ratio in
685 Grauer's gorillas here might facilitate efficient metabolism of their fibrous food resources [log(F:B) =
686 1.12 in this study compared to log(F:B) < 1 in wild western lowland gorillas, Hicks et al. 2018]. Further,
687 we detected higher gut microbiome alpha diversity in Grauer's gorillas (13 phyla/sample) than reported
688 for western lowland gorillas (9), chimpanzees (9), and humans (<5, Hicks et al*.,* 2018), suggesting either
689 higher diversity of Grauer's gorilla microbiomes or better characterisation of rare phyla in our study
690 (*e.g.,* Patescibacteria, Epsilonbacteraeota).

691 We identified eleven microbial taxa present in every individual in this dataset. As these are
692 shared by individuals living up to 175 km apart, they may be functionally important. Many of the these
693 are carbohydrate- and fibre-degraders related to common members of western lowland gorilla
694 microbiomes that were much more common in wild vs. captive individuals, e.g. *Flexilinea* (Chloroflexi;
695 Anaerolineaceae)*, Prevotella 7* (Bacteroidetes; Prevotellaceae)*,* and *Ruminococcus* (Firmicutes;
696 Ruminococcaceae) (Campbell et al., 2020; Hicks et al., 2018; Narat et al., 2020). However, some of

697 these core taxa were not particularly common in previous studies of gorilla microbiomes, *e.g.,*
698 *Senegalimassilia* (Actinobacteria; Eggerthellaceae) and *Sutterella* (Gammaproteobacteria;
699 Burkholderiaceae), though they are common in the gut of baboons (Grieneisen et al., 2021), geladas
700 (Baniel et al., 2021), white-faced capuchins, (Mallott, Amato, et al., 2018), and woolly monkeys
701 (Quiroga-González et al., 2021). The gut microbiome of Grauer's gorillas appeared to be relatively
702 stable through time, as evidenced by greater similarity amongst samples from the same individuals
703 collected almost one year apart compared to other individuals from the same social group, a finding
704 generally in agreement with studies on the human gut microbiome (Björk et al., 2019; Faith et al., 2013).
705
706 *Gut microbiome differs between populations and social groups*
707
708 The Grauer's gorilla gut microbiome differed between populations separated by large
709 geographic and genetic distances and also across social groups. As we found no effect of genetic
710 relatedness, it is likely that contact and social association with groupmates explains why individuals
711 from the same social group show greater similarity in their gut microbiome than to individuals from
712 other social groups within the same population. Time spent in close proximity offers opportunities for
713 transfer of gut microbiota, such as through grooming and coprophagy (Graczyk & Cranfield, 2003). An
714 effect of social association on the gut microbiome has previously been identified for a range of primates,
715 including chimpanzees (Degnan et al., 2012; Moeller et al., 2016), baboons (Tung et al., 2015), colobus
716 monkeys (Wikberg et al., 2020), black howler monkeys (Amato et al., 2017), sifakas (Perofsky et al.,
717 2017, 2021), and humans (Dill-McFarland et al., 2019), as well as other group-living animals, *e.g.*
718 bighorn sheep (Couch et al., 2020). However, this does not preclude an effect of heritability.
719 Longitudinal studies in Amboseli baboons have shown that there is a high heritability of the primate
720 gut microbiome, which cannot easily be detected in shorter-term studies (Grieneisen et al., 2021).
721 Alternatively, it may not be social interaction *per se* but rather shared ecological factors within
722 groups that drive the observed patterns. While diet was less important than population and social group
723 identity in explaining variation in the gut microbiome, it is possible that dietary similarity among
724 members of the same group drives convergence in the microbiome over longer time periods. This would

725 imply limited between-group homogenization in diet, which could occur if groups have limited range
726 overlap or restrict their foraging to particular areas. Since plant diversity in KBNP varies with altitude
727 (Imani et al., 2016), this is possible, particularly if gorilla groups forage only within their preferred
728 altitudinal range, similar to the proposed patterns for female dispersal in the Bwindi mountain gorillas
729 (Guschanski et al., 2008).
730
731 *The gorilla gut microbiome is intertwined with diet*
732
733 Experimental work in captive settings has identified diet as one of the most important factors
734 determining microbiome diversity and composition (Clayton et al., 2018). Studies of wild primate
735 populations frequently use proxies for diet, such as seasonal changes or environmental variables (e.g.
736 forest type, habitat degradation) (Amato et al. 2019; Baniel et al. 2021; Bueno de Mesquita et al. 2021;
737 Clayton et al. 2018; Correa et al. 2021; Gomez et al. 2015, 2016; but see Hicks et al. 2018 for joint diet
738 and gut microbiome study). We add to this work by simultaneously characterizing diet and gut
739 microbiome in two populations of wild Grauer's gorillas. In accordance with expectations from
740 experimental work, we indeed detect high co-inertia and correlation between diet and microbiome
741 composition.
742 Despite this global trend, population and social group membership were the only significant
743 predictors of microbiome composition. This could indicate that microbial changes reflect long-term
744 dietary patterns more consistent with social and ranging patterns than with diet at the precise time of
745 sampling. Other local variables, such as abiotic and biotic environmental factors and the social
746 environment may have additional effects on the gut microbiome. If diet and microbiome are linked, a
747 more varied diet might be expected to select for a more complex gut microbiome. However, in contrast
748 to findings in healthy humans (Heiman & Greenway, 2016), the low-altitude population that had higher
749 dietary richness did not show higher gut microbial richness than the high-altitude population. In fact,
750 the population with greater dietary evenness, NCA, had significantly lower gut microbial evenness. To
751 understand if this relative lack of microbial diversity indicates nutritional redundancy in the diet of
752 NCA gorillas requires further investigation into the nutritional diversity of the plant species consumed.

753 Divergence by population and social group in Grauer's gorilla gut microbiomes tended to
754 derive from differences in specific taxa within universally common bacterial families, especially
755 Lachnospiraceae and Rikellenaceae. This observation emphasizes the trend towards consistency of
756 digestive function across populations of the same species (Amato et al., 2019). There were, however,
757 several family-level differences. For instance, *Treponema* (Spirochaetaceae) were more abundant in
758 NCA, whereas *Prevotella* (Prevotellaceae) were more abundant in KBNP. Hicks et al. (2018) found
759 *Treponema* in western gorillas associated with high-fibre fallback foods, like Marantaceae, Moraceae,
760 and Zingeraceae. The genus *Prevotella*, meanwhile, was enriched during periods of high fruit
761 abundance (*e.g.,* Annonaceae, and Urticaceae) (Hicks et al., 2018). *Prevotella* is common in
762 carbohydrate-rich diets in other studies as well, and is sometimes associated with captivity (Campbell
763 et al., 2020; Clayton et al., 2016; Hicks et al., 2018; Precup & Vodnar, 2019). Consistent with these
764 reports, we found greater abundance of Marantaceae, Moraceae, Commelinaceae, and Zingeraceae in
765 diets of NCA Grauer's gorillas, which had more *Treponema*, and greater abundance of Urticaceae,
766 Primulaceae, Sapindaceae, and Piperaceae in KBNP, where *Prevotella* was more abundant. While
767 speculation about functional correlations is possible, alternate approaches, such as shotgun
768 metagenomics coupled with deeper characterization of global bacterial diversity and metabolic function
769 would further enhance our understanding of the relationship between diet and microbial communities.
770

771 **Conclusions**

772 We provide evidence that fragmented and genetically differentiated populations of critically
773 endangered Grauer's gorillas differ in dietary and gut microbiome composition, suggesting that they
774 might be locally adapted. We emphasize the utility of faecal sampling for minimally-invasive
775 population monitoring of different aspects of endangered species biology, from genetics to ecology to
776 foraging behaviour. Despite its limitations, a molecular approach can reveal otherwise clandestine
777 insights into the ecology of elusive animals and is particularly powerful when combined with traditional
778 field observational methods. Our results stress the importance of protecting geographically-isolated
779 wildlife populations, as safeguarding ecological and genetic biodiversity is the objective of species
780 conservation.

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Author contribution

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

Data availability

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

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