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

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

<sup>1</sup>Animal Ecology, Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

<sup>2</sup>Department of Anthropology, University of California, Davis, California, USA

<sup>3</sup>Department of Human Behavior, Ecology and Culture, Max-Planck Institute for Evolutionary Anthropology, Leipzig, Germany

<sup>4</sup>Conservation Ecology Group, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands

<sup>5</sup>Plant Ecology, Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

<sup>6</sup>The Dian Fossey Gorilla Fund International, Kinshasa, Democratic Republic of the Congo

<sup>7</sup>Institut Congolais pour la Conservation de la Nature, Kinshasa, Democratic Republic of the Congo

<sup>8</sup>Department of Environmental Biology, Sapienza University of Rome, Rome, Italy

<sup>9</sup>Institute of Ecology and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

## Correspondence

Alice Michel, Department of Anthropology, University of California, Davis, CA, USA.

Email: [aljmicel@ucdavis.edu](mailto:aljmicel@ucdavis.edu)

Katerina Guschanski, Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, UK.

Email: [katerina.guschanski@ed.ac.uk](mailto:katerina.guschanski@ed.ac.uk)

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## Abstract

The animal gut microbiome has been implicated in a number of key biological processes, ranging from digestion to behaviour, and has also been suggested to facilitate local adaptation. Yet 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 gut microbiome from the same sample, despite their probable interdependence. Here, we investigate the interplay between diet and gut microbiome in three geographically isolated populations of the critically endangered Grauer's gorilla (*Gorilla beringei graueri*), which we show to be genetically differentiated. We find population- and social group-specific dietary and gut microbial profiles and covariation between diet and gut microbiome, despite the presence of core microbial taxa. There was no detectable effect of age, and only marginal effects of sex and 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 plant availability constraining dietary choices. The observed pattern of covariation between diet and gut microbiome is probably a result of long-term social and environmental factors. Our study suggests that the gut microbiome is sufficiently plastic to support flexible food selection and hence contribute to local adaptation.

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## KEYWORDS

16S rRNA, critically endangered, faecal DNA, genetic diversity, metabarcoding, *trnL*

## 1 | INTRODUCTION

The ranges of many species span ecologically diverse habitats, leading to some degree of adaptation to the predominant local condition. Our view of how species adapt has recently expanded beyond the organismal level, to also include natural selection acting on the community of associated microorganisms, the microbiome (Rosenberg & Zilber-Rosenberg, 2016). In animals, the gut microbiome plays a critical role in key biological processes such as digestion, health, behaviour and even host genomic evolution (Agranyoni et al., 2021; Davidson et al., 2020; Moran et al., 2019; 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 been shown to possess unique gut microbiomes (Bueno de Mesquita et al., 2021; Couch et al., 2020; Uren Webster et al., 2018). Other studies 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 respond quickly to changes in environmental conditions (Koskella et al., 2017), supporting their role in host local adaptation (Alberdi et al., 2016). Experimental work inspecting the directionality of the diet-microbiome link suggests 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, changes in the gut microbiome itself can alter dietary choice (Trevelline & Kohl, 2022). In the wild, it is possible that the microbiome may impact dietary choices by modulating host behaviour, for example, by constraining selection to similar foods even in different habitats or by promoting dispersal decisions that reduce environmental change ("natal habitat-biased dispersal").

Here, we investigate geographical variation of the gut microbiome and its potential role in local dietary adaptation by jointly analysing dietary and gut microbial diversity and composition in several isolated populations of the critically endangered Grauer's gorilla (*Gorilla beringei graueri*; Plumptre et al., 2016). This gorilla subspecies is endemic to the eastern Democratic Republic of Congo (DRC). Our study populations occupy the ecological extremes of the species' range, approximated here by altitude (600m above sea level [asl] and 2500m asl). Grauer's gorillas are herbivores, consuming a large diversity of plants and plant parts (Yamagiwa et al., 2005). However, due to political instability throughout their range, very little is known about the ecology of different populations (but see van der Hoek, Binyinyi, et al., 2021a; van der Hoek, Pazo, et al., 2021b).

Using faecal DNA metabarcoding and host genotyping, we first investigated whether isolated and genetically differentiated gorilla populations show dietary similarities. As plant communities differ considerably by altitude throughout the region (Imani et al., 2016), the presence of shared food taxa across populations would be indicative of restrictive dietary selection (a core Grauer's gorilla diet). If such a pattern of food selection occurs at least in part via gut microbial influence over foraging, we also expect to find a conserved set of gut microbial taxa (a core microbiome). In contrast, if plasticity of the gut microbiome confers dietary flexibility, potentially facilitating local adaptation, we expect diet and the microbiome to differ significantly among populations, with strong covariation between them and little evidence for conserved dietary and microbial taxa.

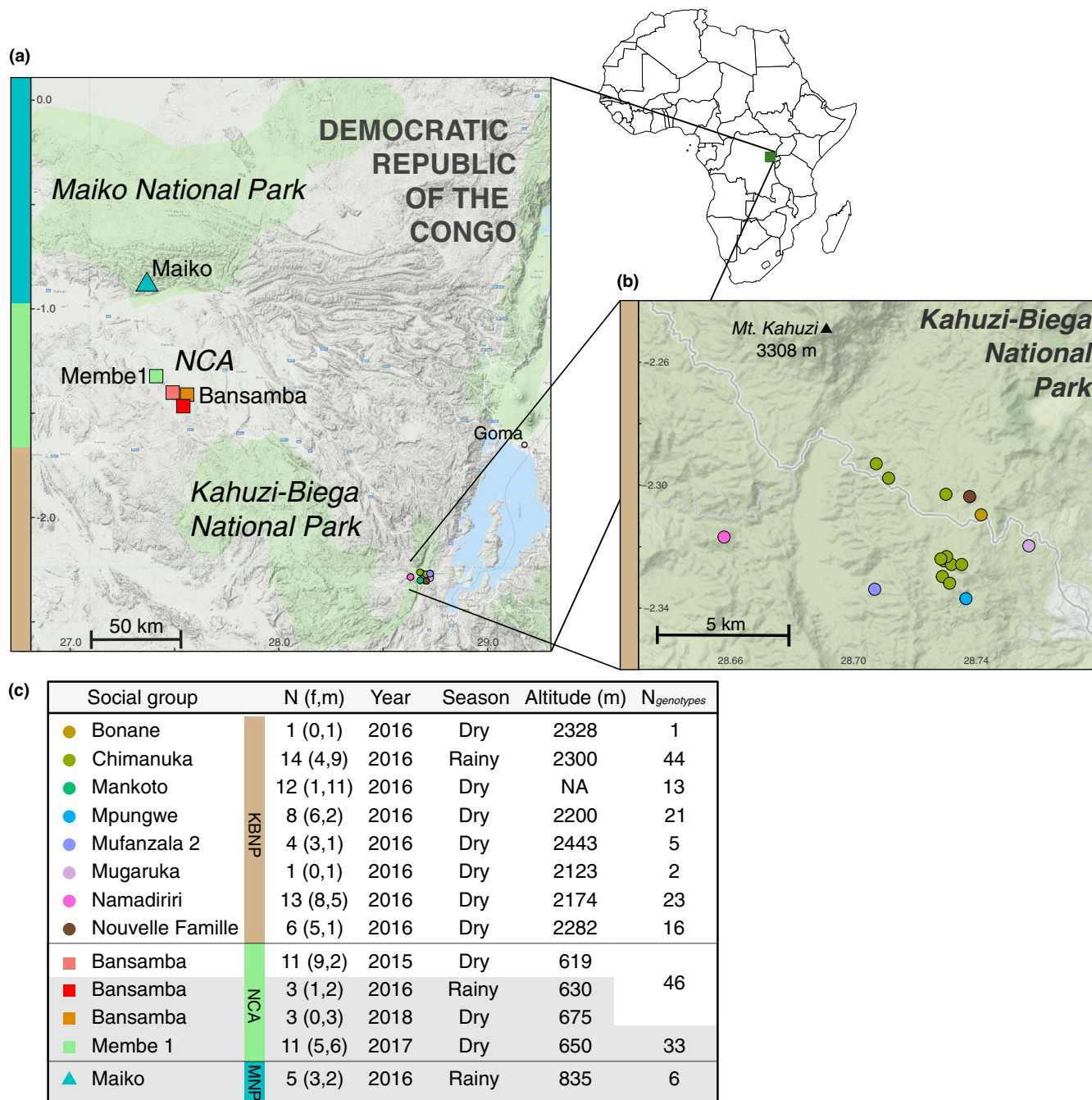
## 2 | MATERIALS AND METHODS

### 2.1 | 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 noninvasively, without disturbing the animals.

### 2.2 | Sample collection

Grauer's gorilla faecal samples ( $n = 220$ ) were opportunistically collected in eastern DRC between 2015 and 2018 at three sites: Kahuzi-Biega National Park (KBNP, 2.32°S, 28.72°E; 2500m asl), Nkuba Conservation Area in Walikale territory, North Kivu (NCA, 1.38°S, 27.47°E; 600m asl) and Maiko National Park (MNP, 0.87°S, 27.35°E; 830m asl; Figure 1). Nine different gorilla social groups (six in KBNP, two in NCA, one in MNP) and two solitary male gorillas (in KBNP) were sampled. Only one group, Chimanuka in KBNP, was habituated to human presence, and samples from this group were collected from identified individuals after defecation. All other samples were collected from night nests without knowledge of individual identity following the two-step collection method (Nsubuga et al., 2004). Geographical location and altitude were recorded using handheld GPS devices for all sampling sites except for the Mankoto group in KBNP, for which this information was not recorded in the field. We assigned age classes based on dung diameter, as follows: infant <4 cm, sharing a nest with an adult; juvenile/subadult 4–5 cm, own nest; and adults >5 cm (McNeilage et al., 2006; Schaller, 1963). For the Chimanuka group, age classes of identified individuals were known from observations.



**FIGURE 1** (a) Map of Grauer's gorilla faecal 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 colours designate social groups, coded as in (c). Note that multiple circles are present for the Chimanuka group, consistent with opportunistic sampling of identified individuals. Geographical 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 are available for samples shaded in grey. The 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

### 2.3 | DNA extraction

Faecal samples were exported to Uppsala University, Sweden, for molecular analysis. DNA was extracted from 50mg of material using

the DNeasy PowerSoil DNA Extraction Kit (Qiagen) in a dedicated primate faecal extraction laboratory, with the following modifications to the manufacturer's protocol: samples were incubated under shaking (500rpm) in the C1 solution overnight at 23°C, transferred

into a heating block and incubated at 65°C for 10 min, followed by bead beating on a vortex at maximum speed for 1 h at room temperature. Incubation in C2 and C3 solution was on ice, and in C6 solution at room temperature for 5 min before elution.

## 2.4 | Gorilla genotyping, individual identification, relatedness and population differentiation analyses

We genotyped all 220 samples at 12 microsatellite loci (vWF, D1s550, D4s1627, D5s1457, D5s1470, D6s474, D6s1056, D7s817, D8s1106, D10s1432, D14s306 and D16s2624) following the two-step multiplex protocol (Arandjelovic et al., 2009) and sexed them with the amelogenin assay (Bradley et al., 2001). Up to four loci were pooled, based on fluorophores and product sizes, and run on an ABI GeneAnalyzer (ThermoFisher Scientific). We scored genotypes manually in GENEMAPPER version 5.0 (Chatterji & Pachter, 2006) and identified individuals in CERVUS version 3.0.7 (Kalinowski et al., 2007). Samples were considered to originate from the same individual if their genotypes matched at five or more loci without mismatches, with the probability of identity assuming full-sibling relationship (PID<sub>sib</sub>) < .05. We manually generated consensus individual genotypes from matching samples, taking into account the time and place of sample collection and the presence of other individuals from the same group.

We tested for deviations from Hardy–Weinberg equilibrium, heterozygote deficiency and linkage disequilibrium at each locus in GENEPOP version 4.7.5 (Raymond & Rousset, 1995; Rousset, 2008). Genetic population structure was assessed using STRUCTURE version 2.3.4 (Porrás-Hurtado et al., 2013) with 20 independent runs for  $K = 1–11$  (11 social groups), a 100,000-iteration burn-in, and data collection for 1,000,000 runs, assuming population admixture and correlated allele frequencies (Falush et al., 2003). Results from different runs of  $K$  were merged in CLUMPP (Jakobsson & Rosenberg, 2007; Kopelman et al., 2015), and analysed and visualized in “pophelper” in R version 4.2.0 (Francis, 2017; R Core Team, 2022). The most probably value of  $K$  was determined using  $\Delta K$  (Evanno et al., 2005). We used the “adegenet” R package for principal coordinate analysis (PCoA) of genotypes (Jombart, 2008). Population differentiation statistics  $F_{ST}$  and  $F'_{ST}$  (Meirmans & Hedrick, 2011) were calculated in GENODIVE version 3.04 (Meirmans, 2020), and significance assessed with 9999 permutations. We compared genetic relatedness between populations and social groups using an AMOVA in the R package “poppr” (Kamvar et al., 2014) and calculated pairwise relatedness ( $r$ ) between all individuals within KBNP and NCA separately in ML-RELATE (Kalinowski et al., 2006).

## 2.5 | Characterization of gorilla diet

We characterized the diet of 92 individuals identified by genotyping (see Results; Tables S1 and S2). We aimed to analyse one nest site per group, but also included additional nest sites of the same group collected during the same year and season to maximize the

number of studied individuals (Table S2). A single sample per individual was studied. The majority of our samples were collected during the dry season, but we also included some samples, social groups (Chimanuka) and one population (MNP) that were collected during the rainy season (Table S2). We present our analyses with and without these samples.

We amplified the P6 loop of the *trnL* chloroplast intron (Taberlet et al., 2007), a locus typically used for herbivore dietary metabarcoding, and for which a large database of tropical plants is available (Mallott et al., 2018). We used the standard *trnL* g and h primers (Table S3), tagged with 96 8-bp barcodes. Each barcode differed from all others at a minimum of three positions. DNA amplifications were carried out in 20  $\mu$ l reactions containing 2  $\mu$ l faecal DNA extract, 1 U Platinum II Taq Hot-Start DNA polymerase, 1 $\times$  Platinum II Buffer, 0.2 mM each dNTP, 2 mM MgCl<sub>2</sub> and 1  $\mu$ M each primer. Each DNA sample was amplified twice. The duplicates were placed randomly on different polymerase chain reaction (PCR) plates to avoid potential batch effects and biases due to cross-contamination of sample and/or barcoded primers (Table S1). We included one PCR-negative, two to three empty wells per plate and five DNA extraction blanks to check for contamination (Taberlet et al., 2018). PCR conditions consisted of 2 min denaturation at 94°C followed by 35 cycles of 94°C for 30s, 51°C for 30s and 68°C for 15s, without final extension. PCR products were visualized on a 2% agarose gel to confirm amplification without contamination.

The barcoded PCR products were pooled column-wise (16  $\mu$ l for each sample, duplicates in separate pools), mixed with 640  $\mu$ l PB Buffer and purified using MinElute columns (Qiagen), eluting in 50  $\mu$ l EB buffer. Double-indexed next-generation sequencing libraries (Kircher et al., 2012) were prepared as detailed previously (Brealey et al., 2020; Rohland et al., 2015) but using nonbarcoded incomplete adapters after blunt-end repair. Two library preparation blanks were carried through all steps. Each pool was quantified using quantitative PCR (qPCR) with PreHyb primers (Table S3; Rohland et al., 2015) and amplification settings as in Brealey et al. (2020).

Each sample pool and both library blanks received a unique combination of indices (Table S1) using the reaction mixture and cycling conditions in Brealey et al. (2020) for indexing PCR. The number of cycles ranged from eight to 10, depending on the copy number estimated from qPCR (Table S1). Library preparation blanks were amplified for 10 cycles to maximize capture of potential contaminants. We performed MinElute purification and quantified indexed pools with qPCR (as above), using primers i7 and i5 (Rohland et al., 2015; Table S3). Indexed sample pools were combined in equimolar amounts, except for library preparation blanks, of which we added 0.5  $\mu$ l each, corresponding to the lowest amount added for any sample. The final sequencing pool was subjected to two-sided size selection with AmPure XP beads (Beckman Coulter) (0.5 $\times$  followed by 1.8 $\times$ ), which is optimized for *trnL* amplicons (~10–150 bp in length + 148 bp of barcoded and indexed adapters) and eluted in 30  $\mu$ l EB buffer. The cleaned library pool was sequenced at the Uppsala Science for Life Laboratory on a single MiSeq lane with 150-bp paired-end sequencing with version 2 chemistry.

Using OBITOOLS version 1.2.13 (Boyer et al., 2016), paired reads with quality scores >40 and overlap >10bp were retained and merged. Sample identity for each read was established through its index and barcode, requiring an exact sequence match. Sequences were clustered into molecular operational taxonomic units (MOTUs), each representing a unique plant taxon (Valentini et al., 2009). A large number of MOTUs had fewer than 10 sequences across all samples and were removed, as recommended (e.g., Shehzad et al., 2012). We also removed sequences that differed by exactly one nucleotide from a more abundant sequence and had a total count less than 5% of the more abundant sequence, following Boyer et al. (2016). Taxonomic assignment used a custom-made reference database (see below). Based on the frequency of identity to the reference database (Figure S1) and similar to other *trnL*-based studies of primate diet (e.g., Quéméré et al., 2013), we removed sequences below an identity threshold of 0.90, which are probably chimeric and enriched in sequencing or PCR errors.

## 2.6 | Compiling plant *trnL* reference database

We built a local DNA barcoding reference library by downloading all 324,502 available sequences from NCBI GenBank using the search query: "(trnL[All Fields] OR complete genome[All Fields]) AND (plants[filter] AND (chloroplast[filter] OR plastid[filter]))" (last accessed December 2, 2021). In OBITOOLS, the sequence list was annotated with taxonomy information downloaded from NCBI (<ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz>, last accessed December 3, 2021). To complete the database of *trnL* genes, we used the same *trnL* g-h primers as in the wet laboratory to extract *trnL* variants in silico in the program ECOPCR version 2.1 (Ficetola et al., 2010), following an established protocol (Boyer et al., 2016). We kept sequences 10–230 bp long with at most three primer mismatches in total (Taberlet et al., 2018). The final database contained 21,308 *trnL* in silico amplicons, in 608 families and 5662 genera.

To evaluate the resolution of our reference database with respect to local plant diversity, we compared plant taxa present in it to a list of plants known to occur in the Kahuzi and Itembero regions of KBNP (Yumoto et al., 1994) that we first updated to reflect current taxonomic classification using the Global Biodiversity Information Facility. The updated list contained 328 taxa, in 81 unique families and 234 genera. Of these, all families and 77.4% of genera were present in our *trnL* database.

## 2.7 | Characterization of gorilla gut microbiome

We characterized gut microbial composition in 70 individuals in KBNP and NCA populations using a single sample per individual (Table S2). We selected the same sample used for dietary analyses and only dry season samples from the Bansamba group in NCA. To quantify possible contamination, we carried nine random extraction blanks through the entire data processing pipeline.

The V4 region of the 16S rRNA gene was amplified with primers 515F/806R (Table S3) for each sample in duplicate. The 20- $\mu$ l PCR contained 2  $\mu$ l of extracted DNA, 5  $\mu$ M each of the forward and reverse primer, 1 $\times$  Phusion High-Fidelity Buffer, 0.02 units Phusion HF DNA polymerase (2 U  $\mu$ l<sup>-1</sup>), 0.012 mg DMSO and 0.05  $\mu$ M (each) dNTPs. Thermal cycling conditions were: 30 s at 98°C, 25 cycles of 98°C for 10 s, 52°C for 20 s and 72°C for 20 s, and 10 min at 72°C. PCR cycles were limited to 25 to minimize the risk of unspecified products and chimeras. Duplicate reactions were pooled and cleaned with AmPure beads (Qiagen).

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

After demultiplexing sequencing reads and removing adapters (Brealey et al., 2021), we estimated microbial amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016), which avoids biases of arbitrary similarity thresholds (Edgar, 2018). Forward and reverse reads were truncated to 200 and 150 bp, respectively, after which read quality scores dropped below 35. We merged paired-end reads, requiring  $\geq 12$  bp overlap, and removed sequences outside the range 250–256 bp and those with any barcode mismatch, as recommended (Callahan et al., 2016).

Taxonomy was assigned using the SILVA 132 reference database, released in December 2017 (Quast et al., 2012). Species-level assignment required a strict 100% match (Edgar, 2018). We removed singletons and ASVs labelled "Unassigned," "Eukaryota," "mitochondria" or "chloroplast," but retained Archaea. Although archaeal amplification from the V4 region of the 16S rRNA gene is limited (Raymann et al., 2017), within-data set comparisons are nonetheless informative.

## 2.8 | Statistical analyses of *trnL* and 16S data sets

To examine dietary and microbiome diversity, we analysed the *trnL* and 16S rRNA metabarcoding data sets, after first filtering out rare sequence variants of <0.5% relative abundance in every sample, as suggested (Deagle et al., 2019). We evaluated sampling effort and sequencing depth accumulation curves in the R packages "vegan" (Oksanen et al., 2020) and "ranacapa" (Kandlikar et al., 2018), respectively. We checked whether the predicted number of taxa (asymptote of the sequencing accumulation curve) minus actual number of taxa (richness) related to any of the considered biological variables

or sequencing depth (read count) using a generalized linear model (GLM) with quasi-Poisson error distribution in the R package “lme4” (Bates et al., 2015).

We calculated two alpha diversity metrics for each data set: richness, or the number of taxa, and Shannon's diversity index, or evenness (Chao et al., 2014). As recommended by McMurdie and Holmes (2013), we did not rarefy our data but included read number as the first factor in all models. 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) comparisons between levels of significant categorical variables ( $\chi^2$  test with Bonferroni correction; Lenth et al., 2021).

To assess trends in diet and microbiome composition (beta diversity), 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 centred and log-ratio (CLR) transformed each data set using the R packages “zcompositions” (Palarea-Albaladejo & Martín-Fernández, 2015) and “compositions” (van den Boogaart & Tolosana-Delgado, 2008). For the microbiome data set, we also computed compositional abundance at phylogenetic balances using phylogenetic isometric log-ratio transform (phILR; Silverman et al., 2017) by aligning sequences to the Greengenes 13\_5 mega-phylogeny (DeSantis et al., 2006) in SEPP using default parameters (Mirarab et al., 2012). We evaluated sources of variation in diet and microbiome beta diversity using PERMANOVA, via the function *adonis2* in “vegan” (Anderson & Walsh, 2013), modelling Aitchison's dissimilarity (Euclidean distance between CLR or phILR values; Aitchison et al., 2000) as a function of ecological and biological variables. The predictor variables were: sequencing read count as first variable, even if  $p > .05$ ; population; social group; sex; age class; and altitude. We evaluated the marginal effect of each predictor variable (*by* = “margin” argument), such that the sequential order of variables did not affect variable significance or effect size. Post hoc comparisons between levels of overall significant variables were done using “pairwiseAdonis” with Bonferroni correction (Arbizu, 2020).

We estimated the covariance between diet and microbiome composition using a co-inertia analysis 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 geographical distance, altitude difference, diet composition (Aitchison distance), and social group and population as binary (same, 0, or different, 1). The influence of genetic distance (1 – genetic relatedness) was also modelled only within KBNP, since individual genetic relatedness cannot be accurately measured across isolated populations. Significance was assessed using 999 matrix permutations. To identify dietary and

microbial taxa that may be responsible for compositional differences between populations and social groups, we used the R package “ALDEx2” and focused on differences with effect sizes  $>1$  (Wilcoxon rank sum test with correction for false discovery rate  $p < .05$ ; Gloor et al., 2017).

## 3 | RESULTS

### 3.1 | Study populations of Grauer's gorillas are genetically differentiated

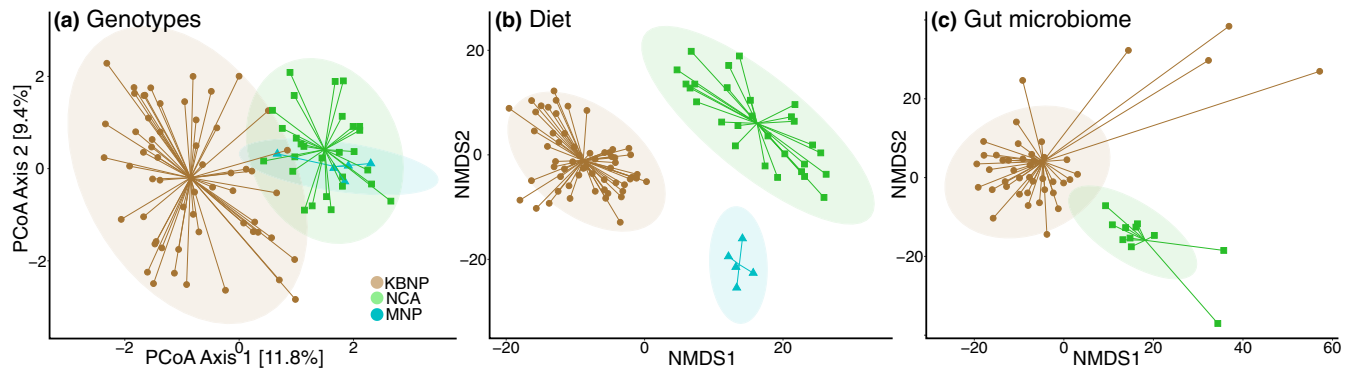
We identified 92 unique individuals in the three study populations: 59 in KBNP, 28 in NCA and five in MNP (Figure 1; Table S2). Individuals belonged to six different social groups and two solitary adult males in KBNP, two social groups in NCA and one group in MNP. Each individual was sampled 1–13 times, with 4–17 individuals per social group.

None of the 12 microsatellite loci deviated from Hardy-Weinberg equilibrium after Bonferroni correction for multiple testing ( $p > .1$ ). On average, there were 6.1 alleles per locus (Table S2). The average observed and expected heterozygosities were 0.66 and 0.68, respectively. The test for global heterozygote deficiency was not significant overall ( $p = .6$ ) or in any population ( $p > .4$ ). The linkage disequilibrium test was not significant for any locus pair ( $p > .1$ ). Thus, we considered all loci in further analyses.

Analysis of the three populations using STRUCTURE (Porrás-Hurtado et al., 2013) revealed two distinct genetic groups (optimal  $K = 2$  according to  $\Delta K$ ; Evanno et al., 2005; Figure S2). The clusters differentiated gorillas in high-altitude KBNP (2500m asl) from those in low-altitude NCA and MNP (600–830m asl) (Figure S3), consistent with PCoA (Figure 2a). All three populations were significantly differentiated from one another ( $F'_{ST} = 0.26–0.45$ ;  $p < .001$ ; Table S4A), with largest differences between MNP and KBNP, which are furthest apart geographically (215 km). Individuals within social groups were more closely related than individuals in different groups in the same population (AMOVA  $\phi = 0.12$ ,  $p < .001$ ; Table S4B), consistent with gorilla social structure (Harcourt & Stewart, 2013).

### 3.2 | Negative controls in *trnL* and 16S rRNA metabarcoding

To quantify contamination in the diet (*trnL*) and the gut microbiome (16S rRNA) data sets, we analysed DNA extraction blanks, PCR-negative controls, unused barcode combinations, and library preparation negative controls (for diet; Tables S2, S5 and S6). In the diet data set, the extraction and PCR negatives contained 16 *trnL* reads in total, identified to 12 plant taxa. Each taxon had one to three reads summed across all negative controls, yet up to 3620–154,357 reads per sample (Table S5). There were no reads with unused barcode combinations, suggesting that barcode cross-contamination was negligible. In the microbiome data set, four extraction blanks had



**FIGURE 2** (a) PCoA 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)

**TABLE 1** Most prevalent dietary plant taxa by population

ID	NCBI-based finest taxonomic identity	Distribution-refined probable identity	Mean abundance KBNP	Mean abundance NCA	Mean abundance MNP	KBNP rank <sup>a</sup>	NCA rank <sup>a</sup>	MNP rank <sup>a</sup>
1	<i>Urera</i> sp.	<i>Urera hypselodendron</i>	35.1%	0.2%	0.1%	1	14	19
2	Apocynaceae sp.	<i>Taccazea apiculata</i>	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.	<i>Pycnanthus</i> , <i>Staudtia</i> or <i>Afradisia</i> sp.	0.05%	14.5%	8.1%	13	1	5
5 <sup>b</sup>	Apocynoideae sp.	<i>Baisea</i> , <i>Funtumia</i> or <i>Motandra</i> sp.	4.6%	8.9%	14.7%	4	2	3
25	<i>Megaphrynium macrostachyum</i>	<i>Megaphrynium macrostachyum</i>	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.	<i>Strophanthus</i> sp.	0.02%	0.2%	12.9%	29	26	2
14 <sup>b</sup>	<i>Ficus</i> sp.	<i>Ficus</i> sp.	2.7%	5.6%	1.9%	8	6	14

<sup>a</sup>Each taxon was first ranked by its relative abundance per sample, then taking the mean across all samples in a population to obtain population-level ranks. This measure thus reflects the average abundance rank of a given taxon across all samples in a population. The top three ranking taxa per population are shown, as well as all taxa present in every sample above the relative abundance threshold of 0.5% (shaded rows).

<sup>b</sup>These taxa had greater than 1% relative abundance in all three populations.

90 reads in total, whereas the remaining five had none (Table S6). These mapped to eight 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 2244–14,307 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).

### 3.3 | Diet of Grauer's gorillas

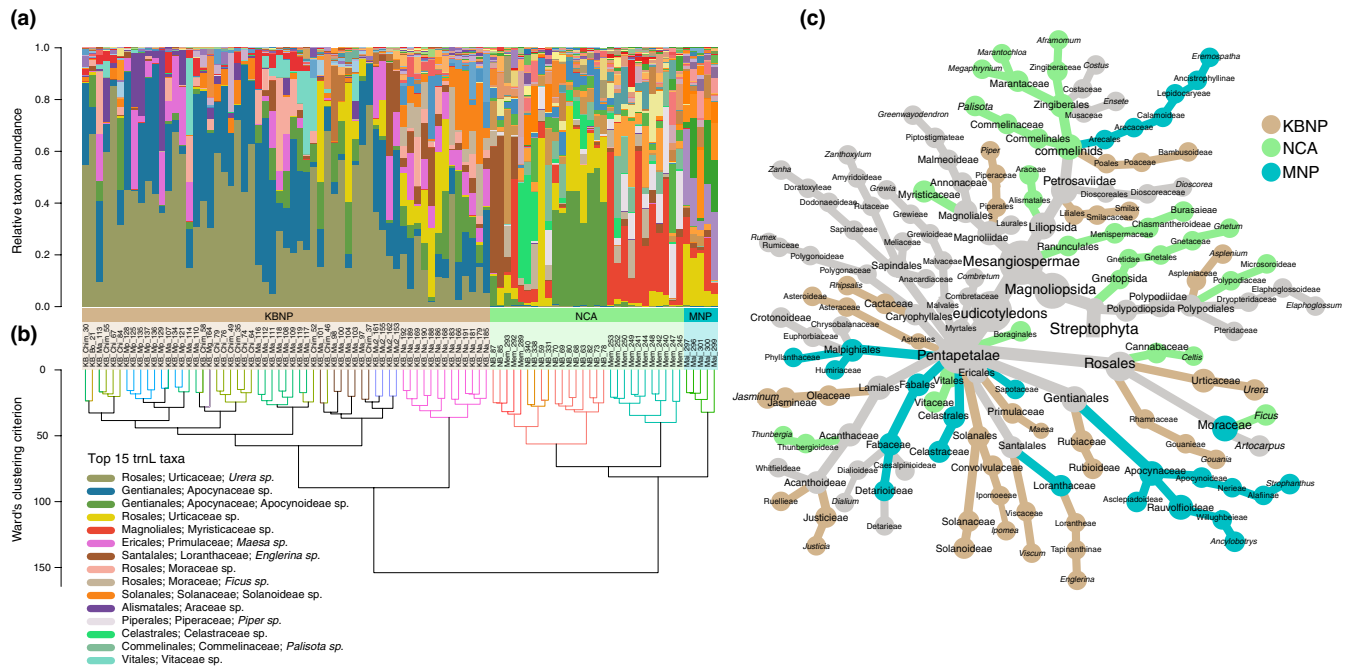
We characterized the diet of 92 Grauer's gorilla individuals (Table 1) using the chloroplast *trnL P6 loop* locus. After data filtering, we retained 5,367,160 *trnL* sequencing reads (45% of raw reads) belonging to 120 unique taxa (Table S7A, S8). PCR replicates were more similar

to each other than to other samples in alpha and beta diversity ( $p < .001$ , Figure S4), and hence their sequencing data were pooled. Sample size and sequencing depth were sufficient to capture dietary diversity in KBNP and NCA, but not in MNP, where only five individuals were sampled (Figure S5).

Of the 120 detected dietary plant 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) level (Table S8). All but 21 taxa have previously been recorded in the Grauer's gorilla diet in KBNP, NCA, and Mt. Tshiaberimu (Kambale, 2018; van der Hoek, Pazo, et al., 2021b; Yamagiwa et al., 1994, 2005 Yumoto et al., 1994; Table S8, columns S and T; Figure S6). These 21 taxa are, however, present in the region (Spira et al., 2018).

Each Grauer's gorilla faecal sample contained 36–80 *trnL* taxa (mean  $58.52 \pm 10.83$ ; Figure 3a), with each population showing





**FIGURE 3** (a) Plants consumed by Grauer's gorillas in KBNP, NCA and MNP. The 15 most abundant taxa across all samples are shown. Populations are designated with coloured bars below (MNP cyan, NCA green, KBNP 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. Branches are coloured by social group, following the code in Figure 1. (c) Plant taxa in Grauer's gorilla diet, coloured by the population in which they are significantly more abundant (ALDEX2 Wilcoxon test  $p < .05$ ). For taxa that differ between two or more population pairs, the colour corresponds to the population with greatest effect size. Grey taxa do not differ significantly in abundance between populations. Branch lengths do not reflect phylogenetic distance. Diagram generated with the "metacoder" package in R (Foster et al., 2017)

a different set of most abundant and prevalent plants (Table 1; Table S8). Five plant taxa were found in every sample collected during the dry season in KBNP and NCA, even though they showed very low abundance in some samples (0.1%). Only two plant taxa had abundances over 1% in all three populations (Table 1).

### 3.4 | Geography, altitude and social group influence dietary diversity and composition in Grauer's gorillas

Dietary richness and evenness differed significantly by population and social group ( $p < .001$ , Table S9). Both were significantly higher in low-altitude populations (NCA and MNP) than in high-altitude KBNP (mean richness:  $66.8 \pm 7.5$  taxa in MNP,  $65.6 \pm 6.1$  in NCA vs.  $54.5 \pm 10.4$  in KBNP,  $p < .001$ ; evenness:  $10.0 \pm 1.3$  in MNP,  $8.4 \pm 2.5$  in NCA vs.  $5.4 \pm 2.6$  in KBNP,  $p < .001$ ; Figure S7). Altitude was also inversely related to dietary richness and evenness in KBNP ( $p < .001$ ; Figure S8). In contrast, neither sex nor age (age class available for 70 individuals) had an effect on dietary richness or evenness ( $p > .3$ ; Table S9). We obtained qualitatively similar results when analysing only dry season samples (i.e., excluding Chimanka group, three individuals from the Bansamba group, and MNP; Table S9), with the exception that dietary richness did not significantly change with altitude in KBNP ( $p = .2$ ).

Hierarchical clustering of dietary composition first separated high-altitude (KBNP) from low-altitude (NCA and MNP) locations (Figure 3b), even though MNP samples were collected during the rainy season. Within populations, individuals clustered by social group. Nonmetric multidimensional scaling (NMDS) ordination showed a similar pattern (Figure 2b). After accounting for sequencing depth, dietary composition was significantly influenced by population ( $p < .001$ , explaining 27.9% of the variance) and social group ( $p < .001$ , explaining an additional 21.6%) but not by sex ( $p = .7$ ) or age ( $p = .2$ ; Table 2). All social groups differed significantly from each other ( $p < .05$ ; Table 2), except for some comparisons involving the Mufanzala2 group, which had a small sample size ( $n = 4$ ). Restricting the analysis to two similarly sized social groups from each of the NCA and KBNP populations collected during the dry season, we confirmed the presence of significant between-group and between-population diet differences (Table S10), demonstrating that our results are robust to differences in sample size and season.

Using ALDEX2, we identified differentially abundant dietary taxa across populations. These taxa were among the most abundant in each population (Table 1), most of which were absent or present at very low abundance in other populations (Figure 3c; Table S11). Out of the 21 previously undescribed food items, 13 were significantly more abundant in low-altitude populations compared to the high-altitude population KBNP (Tables S8 and S9; Figure S6). Within

TABLE 2 PERMANOVA model of factors influencing dietary composition

Variable	Df	R <sup>2</sup>	F	p	Post hoc tests	
					Significant pairwise comparisons	P <sub>Bonferroni</sub>
Read count	1	.01	1.73	.05	-	
Population	2	.279	17.80	<.001	NCA – KBNP KBNP – MNP <sup>a</sup> MNP – NCA	<.001 <.001 <.001
Social Group	6	0.216	4.56	<.001	<b>KBNP</b> Chimanuka <sup>a</sup> – Nouvelle Famille Chimanuka <sup>a</sup> – Mankoto Chimanuka <sup>a</sup> – Mpungwe Chimanuka <sup>a</sup> – Mufanzala2 Chimanuka <sup>a</sup> – Namadiriri Nouvelle Famille – Mankoto Nouvelle Famille – Mpungwe Nouvelle Famille – Namadiriri Mankoto – Mpungwe Mankoto – Mufanzala2 Mankoto – Namadiriri Mpungwe – Namadiriri Mufanzala2 – Namadiriri <b>NCA</b> Membe1 – Bansamba <sup>(a)</sup>	.02 .005 .005 .02 .005 .02 .02 .005 .005 .03 .005 .005 .02 .005
Sex	1	.005	0.82	.6	-	
Age class <sup>b</sup>	2	.022	1.28	.1	-	

Note: PERMANOVA implemented using the *adonis2* function (Oksanen et al., 2020) testing the marginal effect of each predictor variable.

<sup>a</sup>Samples from MNP, Chimanuka group in KBNP, and three out of 17 individuals from Bansamba group in NCA were collected during the rainy season, whereas all other samples were collected during the dry season. Removal of these individuals from this analysis did not affect the results (Table S10).

<sup>b</sup>Age class (Infants [*N* = 7], Juveniles/subadults [*N* = 21], Adults [*N* = 42]) was modelled separately using a reduced data set, since only 70 of the 92 samples had age estimates. In this model the other predictor variables had estimates similar to those of the complete data set.

populations, each social group consumed between two and 32 differentially abundant taxa (mean = 11.3 ± 11.6).

### 3.5 | Gut microbiome of Grauer's gorillas in Kahuzi-Biega National Park and Nkuba conservation area

We characterized 16S rRNA diversity in 70 individuals for which we also had dietary data (Figure 1c; Table S2), using the same samples as for diet. Two samples had low read counts (five and 348, compared to the mean 43,611 ± 11,357) and were excluded. Our final data set consisted of 68 unique individuals and contained 2,965,516 reads in 417 unique microbial taxa (Table S12).

The sample accumulation analyses suggested that additional sampling of faeces from more individuals could uncover novel gut commensals at the population level (Figure S9A). However, per-sample sequencing depth was sufficient to obtain a good representation of host microbiome diversity (Figure S9B). We detected 16 different phyla and 48 different families of microorganisms in the

gut microbiome of Grauer's gorillas. All taxa were identified at least to the family level, 309 taxa to the genus level and 17 to the species level (Table S12). None were closely related to dominant soil microorganisms (Delgado-Baquerizo et al., 2018). We detected seven Archaea ASVs, belonging to the families Methanomethylphilaceae and Methanobacteriaceae. Each faecal sample contained on average 200.29 ± 19.6 taxa (min = 160, max = 237), each with average abundance of 0.2 ± 0.4%. Eleven taxa were present in every gorilla faecal sample from both populations (the core gut microbiome); however, populations differed in the most abundant taxa (Table 3). In accordance with previous studies on great apes (Campbell et al., 2020; Gomez et al., 2016; Hicks et al., 2018; Nishida & Ochman, 2019), Grauer's gorilla gut microbiomes were dominated by the phyla Firmicutes (65.6% in KBNP, 60.0% in NCA), Bacteroidetes (20.7% in KBNP, 23.1% in NCA), 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).

TABLE 3 Most prevalent gut microbiome taxa by population

ASV	NCBI-based finest taxonomic identity	Mean abundance KBNP	Mean abundance NCA	Rank KBNP <sup>a</sup>	Rank NCA <sup>a</sup>
3 <sup>b</sup>	Bacteria; Firmicutes; Clostridia; Clostridiales; Family XIII; AD3011 group	2.40%	2.49%	1	3
6 <sup>b</sup>	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; UCG-004	2.32%	1.16%	2	17
4	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; <i>Faecalibacterium</i>	2.78%	0.84%	3	39
5 <sup>b</sup>	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; RC9 gut group	1.61%	6.36%	7	1
2 <sup>b</sup>	Bacteria; Firmicutes; Clostridia; Clostridiales; Christensenellaceae; R-7 group	2.90%	3.97%	4	2
1 <sup>b</sup>	Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae; <i>Flexilinea</i>	2.72%	4.27%	6	4
22	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; <i>Prevotella 7</i>	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; <i>Oribacterium</i>	0.96%	0.27%	19	61
30	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; <i>Sutterella</i>	0.95%	0.14%	16	86
33	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; <i>Ruminiclostridium 9</i>	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; <i>Senegalimassilia</i>	0.32%	0.48%	67	40
152	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae	0.15%	0.11%	100	123

<sup>a</sup>Each taxon was first ranked by its relative abundance per sample, then taking the mean across all samples in a population to obtain population-level ranks. This measure thus reflects the average abundance rank of a given taxon across all samples in a population. The top three ranking taxa per population are shown, as well as all taxa present in every sample above the relative abundance threshold of 0.5% (shaded rows).

<sup>b</sup>These taxa had greater than 1% relative abundance in all three populations.

### 3.6 | 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;  $p = .02$ ; evenness: 83.7 ± 17.8 vs. 74.2 ± 12.8,  $p = .01$ ), the opposite trend to diet, although neither microbiome richness nor evenness were related to altitude within KBNP ( $p = .07, .9$ ; Table S13; Figure S11). While richness of the microbiome did not differ by sex ( $p = .2$ ), females had more even microbiomes than males (85.7 vs. 78.5,  $p = .002$ ). There were no differences by age (richness  $p = .3$ ; evenness  $p = .1$ ). The gut microbiome alpha diversity differed significantly by population even after removing rainy season samples (i.e., excluding Chimanuka group; richness  $p = .001$ , evenness  $p = .008$ ; Table S13).

Gut microbiome composition differed between the two populations (KBNP and NCA) (Figure 2c) and among social groups (Figure 4a,b), with population explaining 10.5% of the total variance, and social group in KBNP explaining an additional 17.8% ( $p < .001$ ; Table 4). Intergroup differences were significant, including among groups collected during the dry season (Table 4). Overall, gut microbiome dissimilarity was largest between individuals from different

populations, followed by individuals from different social groups, and smallest between individuals from the same social group (Figure 5c). Altitude explained 12.7% of the variance across populations ( $N = 56$ ,  $p < .001$ , altitude range 1824 m) and accounted for 3.8% in KBNP ( $N = 45$ ,  $p = .01$ , altitude range 320 m). Genetic distance among gorillas was not a significant predictor of gut microbiome composition in NCA ( $N = 11$ ,  $\rho = -0.08$ ,  $p = .7$ ), whereas in KBNP it had a weak effect ( $N = 57$ ,  $\rho = 0.06$ ,  $p = .02$ ) that disappeared when social group was also considered ( $N = 57$ ,  $\rho = 0.015$ ,  $p = .3$ ; Figure S12). Microbiome composition did not differ by sex or age within social groups ( $p > .05$ , Table 4), but sex was a weak but significant predictor of microbiome composition when social group was not considered ( $R^2 = .03$ ,  $p = .005$ ; Figure S13). Results using only dry season samples (Table S14A) and phylogeny-informed (phILR) distances were qualitatively similar (Table S15).

We identified 42 taxa that differed significantly in abundance between NCA and KBNP ( $p < .05$ , effect size  $> 1$ ; Table S16). In KBNP, gorilla gut microbiomes had a higher abundance of Muribaculaceae and Erysipelotrichaceae, whereas in NCA the families Spirochaetaceae and Christensenellaceae were more abundant. At a finer phylogenetic level, the relative abundance of specific ASVs in shared families such as Rikenellaceae, Lachnospiraceae and Ruminococcaceae differed by population.

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	.019	1.68	.05	–	
Population	1	.105	7.97	<.001	KBNP – NCA	<.001
Social Group	5	.178	2.18	<.001	Chimanuka <sup>a</sup> – Nouvelle Famille	.004
					Chimanuka <sup>a</sup> – Mankoto	.01
					Chimanuka <sup>a</sup> – Mpungwe	.007
					Chimanuka <sup>a</sup> – Namadiriri	.004
					Nouvelle Famille – Mankoto	.02
					Nouvelle Famille – Namadiriri	.01
					Mankoto – Mpungwe	.007
					Mankoto – Namadiriri	.004
Sex <sup>b</sup>	1	.014	1.18	.2	–	
Age class <sup>c</sup>	2	.031	1.22	.2	–	

Note: PERMANOVA implemented using the *adonis2* function (Oksanen et al., 2020) testing the marginal effect of each predictor variable.

<sup>a</sup>Samples from Chimanuka group in KBNP were collected during the rainy season, whereas all other samples were collected during the dry season. Results were similar when removing Chimanuka (Table S14A).

<sup>b</sup>Although not significant in this full model, sex had a weak but significant influence on gut microbial composition in a model containing population but not social group ( $R^2 = 0.02$ ,  $p = .03$ ) and when excluding both social group and population ( $R^2 = 0.03$ ,  $p = .005$ ).

<sup>c</sup>Age class (Infants [ $N = 3$ ], Juveniles/subadults [ $N = 21$ ], Adults [ $N = 38$ ]) was modelled separately using a reduced data set, since only 62 of the 68 samples had age estimates. In this model the other predictor variables had similar estimates as in the complete data set.

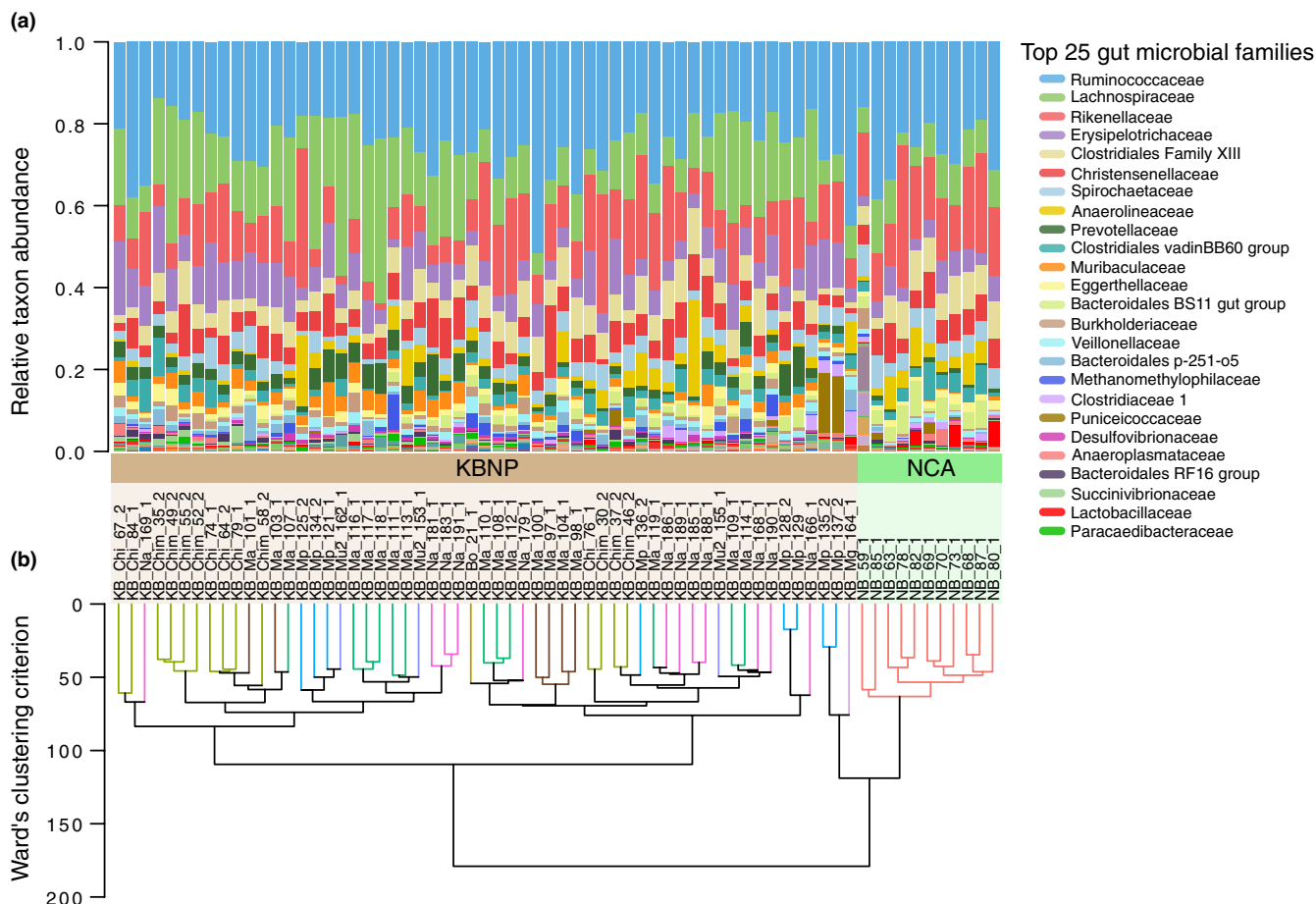
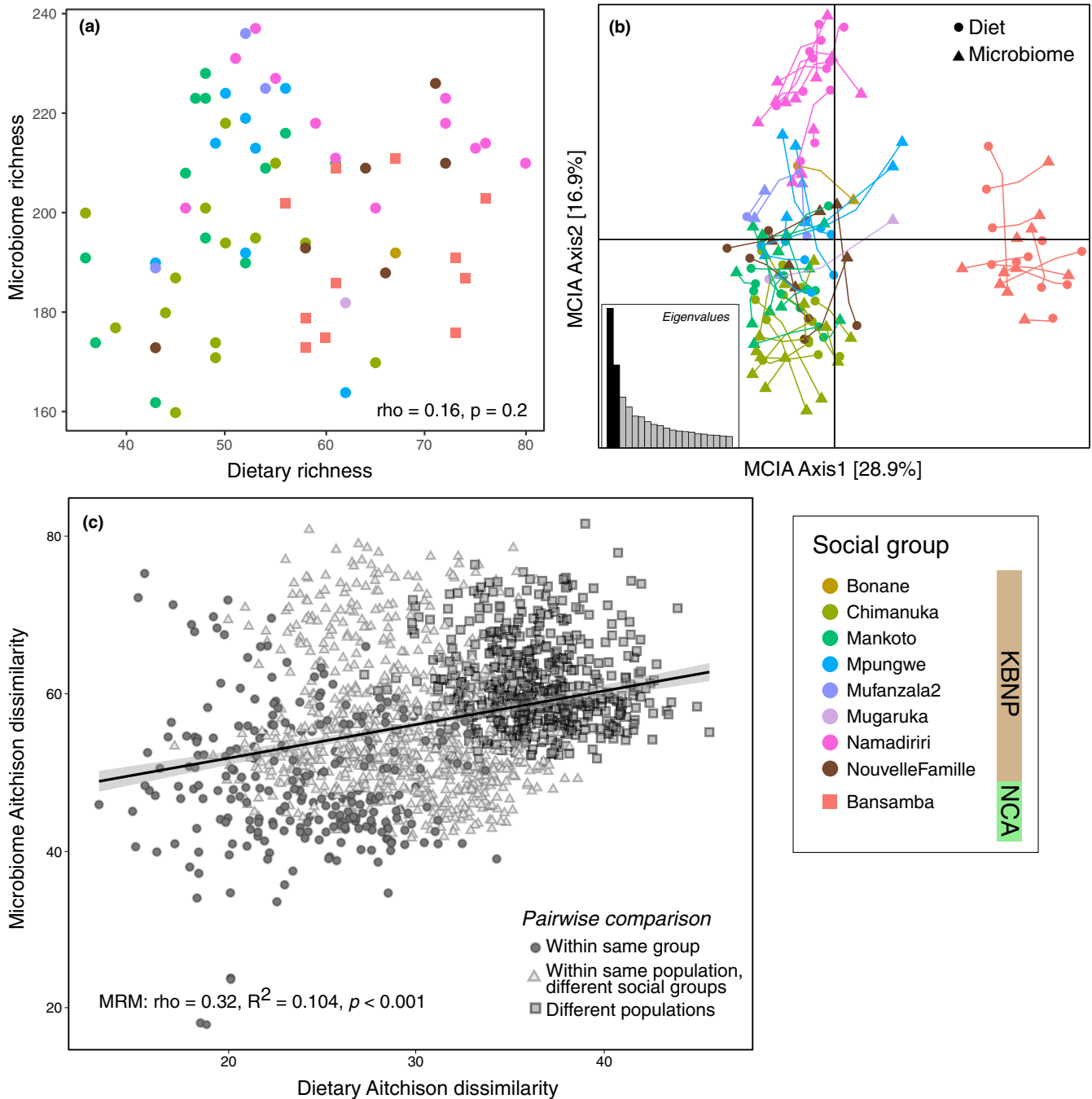


FIGURE 4 Gut microbiome composition (a) at the family level and (b) showing population clustering in composition, using CLR Aitchison distances dendrogram based on Ward's clustering criterion (Murtagh & Legendre, 2014). Branches are colored by social group, following the code in Figure 1.



**FIGURE 5** Relationship between diet and gut microbiome. (a) Microbiome and dietary richness, assessed as per-sample total sequence count, are not correlated ( $p = .2$ ). (b) High multiple co-inertia (MCI) between microbiome and diet composition in CLR-transformed space with Aitchison distance ( $RV = 55.7\%$ ,  $MC p < .001$  based on 999 permutations). (c) Compositional differences (Aitchison distances) in diet and microbiome between samples (i.e., individual gorillas) are correlated in matrix regression

### 3.7 | Diet and gut microbiome covary across studied populations

Compositional differences in dietary and gut microbial profiles showed significant co-inertia ( $RV = 0.557$ ,  $p < .001$ ; Figure 5b) and were correlated ( $\rho = 0.32$ ,  $p < .001$ ; Figure 5c), even after removing the rainy season samples ( $RV = 0.599$ ,  $p < .001$ ;  $\rho = 0.35$ ,  $p < .001$ ). We detected no correspondence between dietary and

gut microbial richness ( $p = .2$ ; Figure 5a) or evenness ( $p = .1$ ). In our data set, population and social group were significantly correlated with gut microbiome composition, whereas dietary composition, geography and genetic relatedness had no effect after accounting for social group and population (Tables 5, S14B and S17; Figure S12). As with other analyses, data set subsampling indicated that results were robust to sample size differences between populations (Table S18).

TABLE 5 MRM model comparing the effects of geography, diet and sociodemographic factors on Grauer's gorilla gut microbiome composition<sup>a</sup>

Gut microbiome composition across populations			MRM model statistics		
Explanatory variable	Spearman's $\rho$	$p$	$N$	$R^2$	$F$ -statistic
Geographical distance	0.07	.4	56	.278	98.35
Altitudinal difference	-0.08	.6			
Diet composition <sup>b</sup>	-0.19	.07			
Population	0.64	<.001			
Social group	0.45	<.001			

<sup>a</sup>Microbiome and diet composition in Aitchison distances.

<sup>b</sup>Model results without Chimanku are shown in Table S14B.

## 4 | DISCUSSION

In this study, we applied faecal genotyping and DNA metabarcoding to characterize the diet and gut microbiome of individually identified Grauer's gorillas in three genetically differentiated populations (Baas et al., 2018), one of which (MNP) has not been previously studied. These populations span the Grauer's gorilla altitudinal range, which is the widest of all gorilla taxa (Plumptre et al., 2016), providing us with the opportunity to test for dietary and gut microbial codifferentiation. In particular, we set out to investigate if the gut microbiome may facilitate local adaptation by supporting digestion of diverse foods. Alternatively, the presence of conserved dietary patterns across populations along with a core gut microbiome would indicate a stabilizing role of gut microorganisms, possibly limiting ecological adaptation. In Grauer's gorillas, some differences in diet between populations have been suggested previously (van der Hoek, Pazo, et al., 2021b; Yamagiwa et al., 2005), but the gut microbiome has never before been assessed.

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

### 4.1 | New insights into Grauer's gorilla diet and feeding behaviour

Grauer's gorillas in the three study populations consumed 120 different plant taxa (Table S8), similar to reported dietary composition and diversity from observational studies (116 and 100 different

plants; van der Hoek, Pazo, et al., 2021b; Yamagiwa et al., 2005; respectively). Low-altitude populations consumed a greater diversity of plants than high-altitude populations (Figures 3 and S7; Table S9), consistent with the elevational biodiversity gradient (Imani et al., 2016; Rahbek, 1995). We documented 54–66 different plant taxa in each faecal sample, which is considerably more than the number of plants consumed based on behavioural observations (17 plant taxa per day on average in KBNP; Yamagiwa et al., 2005). In captivity, gorilla gut retention time is 24–60h (Remis, 2000). Therefore, each faecal sample may represent plants consumed over several days, with some items digested faster than others. Alternatively, our method could capture taxa that are missed in observational studies because they are consumed infrequently, in small quantities, at times of the day that are rarely observed (early in the morning or late in the evening), or which may be contaminants, nest building material or involved in play or display and unrelated to diet.

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

Grauer's gorillas in different populations consumed distinct diets (Figures 2 and 3; Tables 1 and 2), with only two taxa shared across all three populations at an average abundance of >1% per sample: *Ficus* sp. and *Apocynoideae* sp. (probably *Baisea* sp., *Funtumia* sp., or *Motandra* sp. based on plant distribution; Spira et al., 2018). At broader taxonomic scales, all individuals consumed four plant families (Urticaceae, Apocynaceae, Moraceae and Vitaceae), but the relative abundances varied considerably across populations, from less than 1% to up to 42%. The detection of shared taxa suggests that the same plants or their close relatives are present in all three study sites. However, the pronounced differences in their relative abundance suggest either that (i) their availability differs, and gorilla dietary choice is essentially passive and primarily based on food

availability, or (ii) that gorilla dietary choice is strongly determined by social factors, and food selection is a result of variation in culturally transmitted feeding preferences that differ across populations and social groups. Comparative vegetation surveys in different gorilla populations could help distinguish between these possibilities. Higher dietary diversity of low-altitude populations supports the notion of rather opportunistic consumption of available plants. However, we also uncover dietary differences among social groups from the same population, suggesting that social factors also play a role. Since gorilla groups show extensive range overlap, they would be well suited for future investigations into the role of cultural vs. ecological factors affecting dietary choices by evaluating if group-specific dietary patterns persist even when different social groups use the same habitat.

## 4.2 | The role of Grauer's gorilla gut microbiome in ecological adaptation

In accordance with previous studies (Amato et al., 2019; Campbell et al., 2020; Gomez et al., 2016; Moeller et al., 2014), we detect evidence for a Grauer's gorilla core gut microbiome (present in every sample after removing low-abundance taxa, Methods; Table 3, Figure 4). We identified 11 taxa belonging to carbohydrate-degrading clades that were present in all study samples. Many of the same phyla, families and genera are also common in other great ape gut microbiomes, including western lowland gorillas, chimpanzees and humans (Campbell et al., 2020; Fonsere et al., 2021; Gomez et al., 2015, 2016; Hicks et al., 2018; Nishida & Ochman, 2019). Nevertheless, gut microbiome composition in Grauer's gorillas differed by population and to a lesser extent by social group, though considerably less so than dietary composition. This could be explained by functional constraints placed on the gut microbiome, with key taxa required to perform essential digestive functions, whereas other taxa vary and codiversify with the host. Indeed, interspecies studies find a strong effect of host evolutionary relationships on primate gut microbiome structure and composition (Amato et al., 2019) and we expected to detect similar, albeit less pronounced differences across isolated populations.

Our results indicate that dietary choice is not constrained by the gut microbiome. This is consistent with many studies showing that animals, including many primates, experience seasonal dietary changes, which are also accompanied by gut microbiome changes (Baniel et al., 2021; Gomez et al., 2016; Hicks et al., 2018; Orkin et al., 2019; Sharma et al., 2020). Here we detect differences between isolated populations sampled during the same season. This is probably the result of microbiome–host codiversification coupled with plasticity of the gut microbial community that may facilitate local adaptation to different environmental conditions. Interpopulation differences tended to derive from differential abundance of specific taxa within common bacterial families, such as Lachnospiraceae and Rikellenaceae. An example of potentially adaptive population differences is *Treponema* (ASV322, Spirochaetaceae), which

was significantly less abundant in KBNP compared to NCA. Hicks et al. (2018) found a correspondence between *Treponema* and plant taxa Marantaceae and Zingiberaceae in western lowland gorillas and suggested that it was due to the high fibre content of these fallback foods. We find the same relationship, with significantly higher abundances of Marantaceae and Zingiberaceae in NCA, which are important for Grauer's gorillas at low elevation (van der Hoek, Pazo, et al., 2021b).

We did not detect strong effects of genetic relatedness or geographical distance on gut microbiome composition after accounting for population and social group identity, despite clear group-specific microbiome patterns. It is noteworthy that social group identity, geographical and genetic distance are all strongly correlated with each other (Table S17). Our findings thus support previous studies that show the influence of sociality on gut microbiome composition in primates (chimpanzees, Degnan et al., 2012; Moeller et al., 2016; baboons, Tung et al., 2015; colobus monkeys, Wikberg et al., 2020; black howler monkeys, Amato et al., 2017; sifakas, Perofsky et al., 2017, 2021; Rudolph et al., 2022; humans, Dill-McFarland et al., 2019) and other group-living animals (e.g., bighorn sheep, Couch et al., 2020). Members of the same social group travel together and experience the same environments over extended periods of time, which could synchronize their diet and also their microbiome. The gut microbiome may in addition be directly influenced by social interactions, such as grooming and coprophagy (Amato et al., 2016; Archie & Tung, 2015; Graczyk & Cranfield, 2003). However, this does not mean that host genetics are unimportant, as longitudinal studies in Amboseli baboons have shown that the primate gut microbiome is highly heritable, which cannot easily be detected in shorter-term studies (Grieneisen et al., 2021).

The plasticity of the gut microbiome supports its potential role in facilitating adaptation to different ecological conditions, which has important consequences for species evolution, dispersal and conservation. Adaptation to changes in ecological conditions as a result of climate change, range expansion or dispersal into novel habitats may be supported by the ability to digest diverse foods. Habitat-biased dispersal, with dispersal decisions probably driven by the availability of familiar foods, has been reported in mountain gorillas (Guschanski et al., 2008). If the microbiome restricted dietary choice, we would expect much greater consistency of dietary items across populations than observed here, particularly as similar food plants appear to be available in different regions. This means that gut microbiome flexibility may facilitate adaptation to novel habitats during translocations of individuals or populations, which is an open question in conservation management (West et al., 2019). Nevertheless, the gut microbiome may impose constraints on diet by driving selection of foods of similar nutrient content, even if they differ taxonomically. For example, giant pandas have typical carnivore gut microbiomes despite being bamboo specialists, because the macronutritional value of consumed bamboo is similar to that of meat (Nie et al., 2019). Similarly, the gut microbiome of wild rhesus macaques is strongly correlated to seasonal patterns of macronutrient intake, but not food type (fruit, leaves, etc.; Cui et al., 2021).

Metabolic dietary analyses, as conducted for other gorilla species (e.g., Gomez et al., 2015; Rothman et al., 2008), will enable investigations of whether nutritional values are conserved in different populations.

### 4.3 | Understanding diet and ecology of wild animals requires a combination of approaches

As with every method, the metabarcoding approach to diet and microbiome faces limitations, specifically in marker gene selection, reference database bias, threshold decisions and interpretation of abundance. While Grauer's gorillas predominantly feed on plants, they occasionally consume insects and fungi (van der Hoek, Pazo, et al., 2021b; Yamagiwa et al., 1991), which are not characterized using a chloroplast gene, *trnL*. For dietary analysis of omnivorous species, expanding to multiple loci will be necessary (e.g., ITS for fungi, CO1 for insects; Taberlet et al., 2018). Further, metabarcoding studies are limited by the content of reference databases, which may be incomplete for biodiversity-rich or extreme habitats and unstudied microbiomes (Hird, 2017; Taberlet et al., 2018). Similarly, genuine dietary or microbial taxa could be removed depending on the chosen thresholds for sequence identity and relative abundance. We used conservative values similar to other studies of diet and gut microbiomes (Deagle et al., 2019; Hibert et al., 2013; Quéméré et al., 2013; Srivathsan et al., 2016). However, genuine taxa could still have been removed, particularly for dietary characterization due to the small size and high variability of the *trnL* locus. Additionally, abundance estimates may not be an accurate reflection of reality (Deagle et al., 2019; Gloor et al., 2017). DNA copy number can be biased by plant tissue type (i.e., fruit vs. leaves, with leaves containing more chloroplasts; Egea et al., 2010), the copy number of the rRNA locus, relative digestibility (i.e., amount of fibre) and PCR amplification success (reviewed by Deagle et al., 2019). However, other methods for dietary characterization also face biases. For example, accuracy of macroscopic faecal analysis depends on the types of tissues consumed and the extent of digestion (King & Schoenecker, 2019). Observational studies can overestimate the importance of foods with longer handling times (Matthews et al., 2020) and require habituating study animals, which may make them more vulnerable to poaching and increase exposure to human-transmitted diseases (Green & Gabriel, 2020). Hence, understanding ecological and particularly dietary diversity of different animal species would benefit from a combination of approaches. Molecular methods are particularly suited for the study of unhabituated animals, in regions where tracking over a long time period is not feasible or desirable.

The use of shotgun metagenomics will ameliorate many of the limitations described above and also allow for more complete interpretation by enabling functional characterization of gut microbial communities. It would thus be feasible to test if the gut microbiome differs in functional profiles as a result of dietary differences across populations, or if functions remain conserved, suggesting that nutritional values of different diets are indeed similar. With the decrease

in sequencing costs and massive growth of whole genome reference databases (Formenti et al., 2022; Lewin et al., 2018), the use of shotgun metagenomics will increase in the coming years, fuelling the application of the hologenomic framework to wild animal populations.

## 5 | CONCLUSIONS

Our results suggest that the animal gut microbiome may contribute to adaptation to new environments, while retaining a core set of potentially essential constituents. We provide evidence that this microbial plasticity is associated with dietary flexibility, and as such the gut microbiome may enable the host to exploit new resources, a precursor to local adaptation. If so, the microbiome may indirectly encourage subsequent cultural adaptation to feeding on new dietary items. We emphasize the utility of faecal sampling for minimally invasive population monitoring of different aspects of endangered species biology, from genetics to ecology and foraging behaviour. A molecular approach can reveal otherwise clandestine insights into the biology of elusive animals and is particularly powerful when combined with traditional observational methods. Our results highlight the importance of incorporating multiple axes of population differentiation into studies of endangered animals, since safeguarding ecological and genetic biodiversity is the primary objective of species conservation.

### AUTHOR CONTRIBUTIONS

A.M., R.M. and K.G. planned the study design. A.M. generated dietary data and performed all analyses. R.M. generated gut microbial data. P.N., Y.L., M.A.G. and J.S. generated gorilla genotyping data. K.N. and L.P. provided reagents and expertise for dietary analyses. N.I., A.P., U.N., E.B., R.N.P., D.C. and K.G. collected faecal samples and provided support in the field. D.C., L.P. and K.G. provided project supervision. K.G. supervised the experiments and data analyses and acquired the funding. A.M. and K.G. wrote the manuscript, with contribution from all authors. All authors reviewed and approved the final manuscript.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

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

### OPEN RESEARCH BADGES



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. Sequences and associated metadata for the gut microbiome and diet generated in this project have been uploaded to the European Nucleotide Archive (ENA) under Accession no.: PRJEB49814.

### BENEFIT-SHARING

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

### ORCID

Alice Michel  <https://orcid.org/0000-0002-0273-4097>

Katerina Guschanski  <https://orcid.org/0000-0002-8493-5457>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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