Animal Microbiome

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Eukaryotic composition across seasons and social groups in the gut microbiota of wild baboons

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Abstract

Background Animals coexist with complex microbiota, including bacteria, viruses, and eukaryotes (e.g., fungi, protists, and helminths). While high-throughput sequencing is commonly used to characterize bacterial communities in animal microbiota, these methods are less often applied to gut eukaryotic composition. Here we used shotgun metagenomic sequencing to characterize eukaryotic diversity in the microbiomes of wild baboons and tested the degree to which eukaryotic community composition was predicted by host social group membership, sex, age, sequencing depth, and season of sample collection.

Results We analyzed a total of 75 fecal samples collected in 2012 and 2014 from 73 wild baboons in the Amboseli ecosystem in Kenya. DNA from these samples was subjected to shotgun metagenomic sequencing, revealing members of the kingdoms Protista, Chromista, and Fungi in 90.7%, 46.7%, and 20.3% of all samples, respectively (percentages indicate the percent of samples in which each kingdom was observed). Social group membership explained 11.2% of the global diversity in gut eukaryotic species composition, but we did not detect statistically significant effects of season, host age, or host sex. Across samples, the most prevalent protists were *Entamoeba coli* (74.66% of samples), *Enteromonas hominis* (53.33% of samples), and *Blastocystis subtype* 3 (38.66% of samples), while the most prevalent fungi included *Pichia manshurica* (14.66% of samples), and *Ogataea naganishii* (6.66% of samples).

Conclusions Protista, Chromista, and Fungi are common members of the gut microbiome of wild baboons. More work on eukaryotic members of primate gut microbiota is important for primate health monitoring and management strategies.

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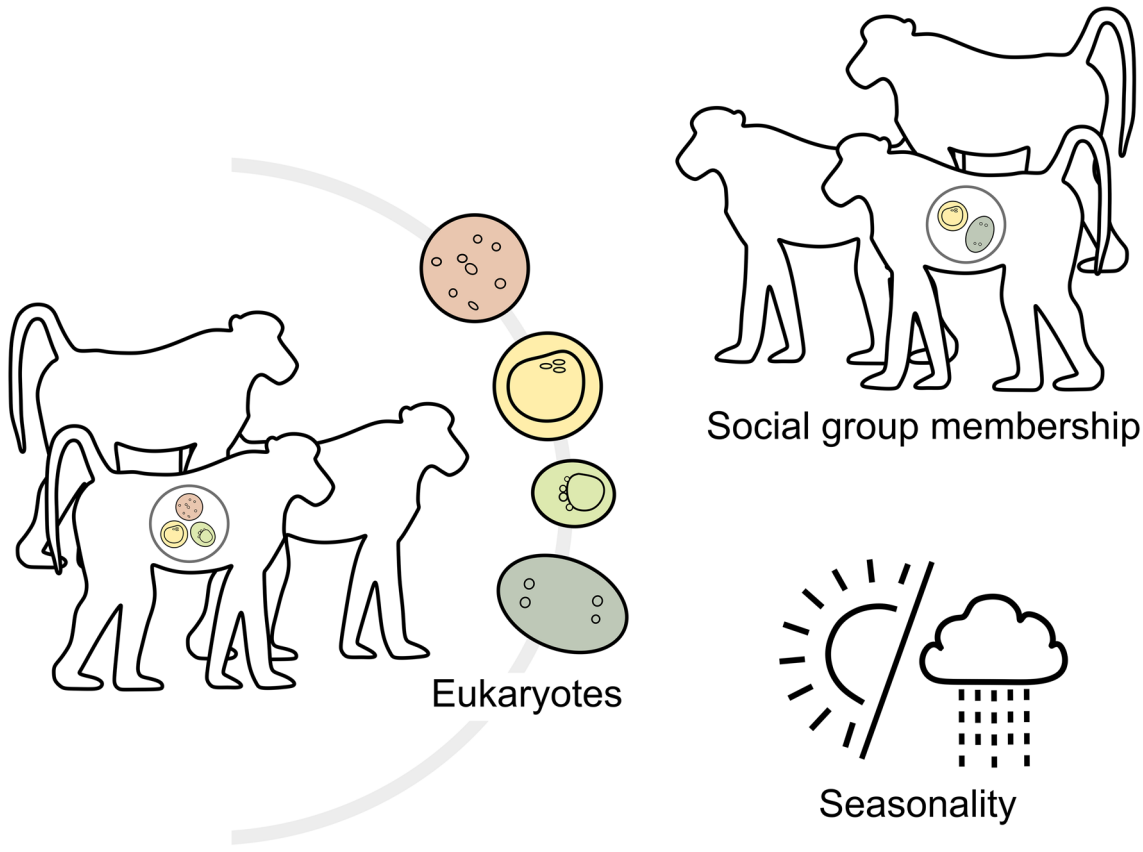
Graphical abstract



Keywords Eukaryotes, Gut Microbiome, Wild baboons, Fungi, Protists, Social groups

Introduction

Eukaryotes, including protists, metazoans, and fungi, are an integral part of mammalian gut microbial communities [1–7]. These taxa have complex interactions with other members of the gut microbiome and with mammalian hosts: they can degrade complex carbohydrates [8], serve as important agents of infectious disease [1, 9], and modulate host immune responses [4, 6, 10–14]. To date, gut eukaryotic diversity is best characterized in humans and domestic animals, with comparatively less research on wild mammals [15, 16]. Yet given the importance of gut eukaryotes to mammalian hosts, characterizing gut eukaryotic diversity in natural host populations is important to improve animal health and for developing a fundamental scientific understanding of gut microbial ecology.

For decades, gut eukaryotic communities have been most often characterized using microscopy and/or culturing techniques, and by amplifying taxon-specific marker genes [17–21]. These approaches are essential to characterizing gut eukaryotic diversity, but they have well-known limitations: microscopy is time consuming and can miss rare taxa; many gut eukaryotes cannot be cultured; and taxon-specific approaches will fail to detect other species [20, 22–24]. As a result, high-throughput sequencing-based approaches are becoming more common for gut eukaryotic detection [25–27]. These approaches include amplicon-based methods, such as amplifying 18S or ITS ribosomal genes to reveal drivers of eukaryotic and fungal diversity [16, 28–33]. However, amplicon-based approaches can have limited taxonomic resolution and sometimes struggle to distinguish between closely related species [34, 35]. Another option is to use shotgun metagenomic sequencing, which offers a non-targeted approach that captures all the accessible genomic DNA in a sample, including from bacteria, viruses, eukaryotes, the host, and host diet items [36–38]. These data are appealing because the same shotgun data set can be used to characterize multiple aspects of the gut microbiome or the host itself (e.g., the virome, fungal communities, host diet etc [39–41])., and may lend insight into functional genetic aspects of these

taxa (e.g., antibiotic resistance, pathogenicity etc [42–44]). However, the utility of shotgun metagenomic data typically depends on the breadth and quality of genomic reads in publicly available databases [41, 45–47]. Further, because gut eukaryotes represent a very small amount of the fraction of the reads generated in shotgun metagenomic data sets.

Studies of wild non-human primates increasingly use high-throughput sequencing to characterize gut eukaryotes. For instance, metagenomic and 18S rRNA amplicon sequencing approaches have been used in a range of primates, often demonstrating greater sensitivity for detecting helminths, protists, and fungi than microscopy or culturing techniques [16, 28–30]. However, shotgun metagenomics remains underutilized for exploring the full diversity of primate gut eukaryotes, including non-parasitic fungi and protists [20].

Here we use shotgun metagenomic data from fecal samples to characterize eukaryotic members of the gut microbiota of wild baboons (*Papio* sp.) living in the Amboseli ecosystem in Kenya. This population has been the subject of continuous, individual-based research since 1971, providing detailed information on host environments, ranging patterns, social group membership, and social interactions [48]. Prior microscopy-based work in this population has identified several (eukaryotic) helminth taxa infecting the baboons, including *Trichuris trichiura*, *Streptopharagus pigmentatus*, *Strongyloides fullerborni*, *Abbreviata caucasica*, *Enterobius vermicularis*, and several strongyle species [21, 49–51]. In addition, we have used both 16S rRNA gene amplicon-based and shotgun metagenomic approaches to characterize the bacterial members of the baboon gut microbiome [52–56]. Together, these data revealed a variety of environmental and social factors that predict gut microbiome community composition. For instance, helminth diversity is highest for baboons in the dry season, and older animals exhibited higher diversity than younger animals [49, 50]. With respect to gut bacterial communities, we find strong effects of season and social group identity on gut bacterial composition [52–54]. Further, within social groups, baboons that are close grooming partners tend to have more similar gut bacterial communities than animals who rarely groom each other [52].

Building on prior work [49–52], here we used two shotgun metagenomic data sets from the Amboseli baboons to characterize gut eukaryotic diversity in this host population. One of these data sets was published previously by [52], described above, which found social effects on baboon gut bacterial communities. This data set consists of samples from 48 individual baboons living in two social groups in July and August 2012 null ([52] Bioproject PRJNA271618). The second data set is newly presented here and was designed to test the effects of season on eukaryotic diversity (the first data set cannot test seasonal effects because all samples were collected in the dry season). This "seasonal" data set includes samples from 27 individual baboons collected during the wet (April and May) and dry seasons (September) of 2014. Two individual baboons were included in both data sets so the total sample size is 75 samples from 73 individual baboons. We expected that gut eukaryotic communities would be influenced by similar factors to gut helminths and bacterial communities in this population. For instance, social group membership influences baboon ranging patterns, resource use, and social relationships, which might influence eukaryotic exposure and transmission. Seasonality also shapes baboon diet, water source use, and other aspects of the environment, all of which could influence exposure and susceptibility to eukaryotic taxa. Our results provide new insights into the gut eukaryotic composition of wild baboons, contributing useful information for understanding the biology and health of wild primates.

Materials and methods

Study subjects and sample material

The baboons sampled in this study were studied by the Amboseli Baboon Research Project (ABRP) in the Amboseli ecosystem in Kenya. Founded in 1971, the ABRP conducts longitudinal research on known individual baboons living in several social groups [48]. Members of this population are hybrids between yellow and anubis baboons (*Papio cynocephalus* and *P. anubis*) [57–59]. The Amboseli ecosystem experiences a 5-month dry season from June through October, followed by a 7-month wet season with highly variable rainfall [60].

Data on the Amboseli baboons is collected by experienced observers year-round during 5-hour monitoring sessions, six days per week. During these sessions, observers collect fecal samples opportunistically from study subjects, all of which are known to the observers from distinctive physical features. Specifically, samples are collected while observers move through the group collecting behavioral data, proceeding from subject to subject in an order determined by a randomized list, ensuring representative sampling of the entire social group. Thus, while observers are not able to collect all samples that are deposited in a given social group during an observation period, we consider the resulting sample collection to represent an unbiased sample of the social group with respect to key variables like health status and diet. For each sample, the baboon's social group membership is known from group censuses collected during each monitoring session. Age is known to within a few days' error for all individuals born into ABRP study groups ($n = 64$ baboons in this study) and estimated for other individuals based on observable morphological

characteristics and body condition ($n = 9$ baboons who were all immigrant males, as males but not females disperse from their natal groups in this population). Sex is known based on morphological differences. All fecal samples are collected within a few minutes of defecation, thoroughly mixed, and preserved in 95% ethanol (2:5 feces to ethanol).

We analyzed eukaryotic composition in two sets of fecal samples. The first set was used to test for social group effects on eukaryotic composition. This set included samples from 48 adult baboons living in two social groups: 'Mica's group' (11 females and 8 males), and 'Viola's group' (20 females and 9 males). These samples were collected within a one-month window during the dry season of 2012 (Fig. 1; Supplementary Table 1; an analysis of bacterial microbiome composition from these samples was published in Tung et al. [52]). The second dataset was used to test for seasonal differences in eukaryotic composition. It included samples from 27 baboons collected during the dry season in September 2014 ($n = 15$) and the wet season between April and May 2014 ($n = 12$) (Fig. 1; Supplementary Table 1). Host sex and age were known for individuals in both data sets and two individuals occurred in both data sets (Supplementary Fig. 1A-D).

Genomic DNA extraction and sequencing

Total DNA was extracted from the first (social group) set of fecal samples using the MO BIO Laboratories, Inc. (Carlsbad, CA) PowerSoil DNA Isolation kit. Samples for the second (seasonal) set were extracted using Qiagen's DNeasy PowerSoil kit (Venlo, Netherlands). Both protocols were performed according to the manufacturer's instructions. We included blank extractions for both data sets, which were found to contain undetectable DNA concentrations.

For samples in the first (social group) data set [52], 200 ng of DNA were prepared for metagenomic sequencing on an Illumina HiSeq 2500, using Kapa Biosystems Library Preparation Kits (Kapa Biosystems, Wilmington, MA). While our DNA extraction kits included a bead-beating step, no other modifications were made to our protocol (e.g., targeted enrichment) to isolate helminth or other parasite eggs. The DNA samples, including the blank extraction, were sheared to an average size of 400 base pairs, followed by ligation to barcoded adapters. The libraries were subjected to 100 bp paired-end sequencing at the UCLA Neuroscience Genomics Core. In total, 1.4 billion raw, paired-end Illumina sequences were generated, with a mean ± SD of 14.4 ± 13.7 million read pairs per sample for the social group dataset (the blank sample contained fewer than 3,000 reads). The raw metagenomic sequencing data are deposited in the NCBI's Short Read Archive (BioProject PRJNA271618).

For samples in the second (seasonal) data set, 200 ng of DNA were prepared for metagenomic sequencing on the Illumina NovaSeq X, utilizing the SeqWell purePlex DNA Library Preparation Kit (SeqWell, Beverly, MA). Samples, including the blank DNA extraction, were subjected to 150 bp paired-end sequencing at the University of Minnesota Genomics Core. In total, 2.54 billion raw, paired-end Illumina sequences were generated, with a mean ± SD of 94 ± 31.2 million read pairs per sample for the seasonal dataset (as before, the blank sample contained < 3000 reads). All the raw reads for the second dataset ($n = 27$) are deposited in NCBI's Short Read Archive (BioProject PRJEB81717).

Identifying dominant eukaryotic members of the baboon gut microbiome

The raw data were processed using FastQC [61] to assess the quality of the reads. Duplicate reads were removed using FastUniq [62] and trimmed to remove adapter sequences and low-quality bases (PHRED score < 20) using Trimmomatic (v0.39) in paired-end mode [63]. We also removed read pairs where one read was shorter than 75 base pairs after trimming, as EukDetect currently only supports analysis of reads that are over 75 bp long. This step ensures that all the retained reads are of sufficient length for reliable mapping to the reference marker database, hence reducing alignment errors and false positive detections. To remove host-derived sequences, high-quality reads were aligned to the Papio anubis reference genome (GCF_008728515.1_Panubis1.0_genomic.

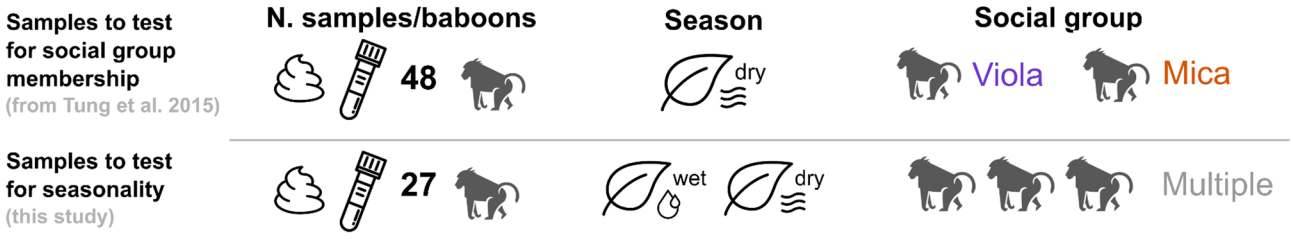


Fig. 1 Schematic of the two sets of fecal samples investigated in this study (Supplementary Table 1). Shotgun metagenomic data for the first set of samples on social group membership was published in 2015 by Tung et al. [52]; these data are publicly available in the NCBI's Short Read Archive (Bioproject PRJNA271618). Shotgun metagenomic data for the second set of samples on seasonality were generated for the present study; these data are publicly available on NCBI's Short Read Archive (Bioproject PRJEB81717). Data on host sex and age were available for samples in both data sets

fna) [64], using samtools [65] and Bowtie2 v2.5.1 [66], and host-aligned reads were filtered out. In contrast, the unaligned reads were retained for downstream analysis (Supplementary Fig. 1E-F).

We then used two previously-developed pipelines to further filter our sequences and identify the presence/absence of eukaryotic taxa in each sample: EukDetect [27] (https://github.com/allind/EukDetect) and its descendent pipeline gutprotist-search (https://github.com/allind/gutprotist-search). In brief, EukDetect aligns reads to a database consisting of conserved eukaryotic marker genes from curated whole genome assemblies [27]. Gutprotist-search, developed alongside EukDetect, complements the approach in EukDetect by using a database of NCBI sequences for particular taxonomic identifiers for eukaryotic taxa that lack genome assemblies. Because genome sequences are unavailable for many gut eukaryotes, gutprotist-search helps identify taxa that might otherwise go undetected.

Both pipelines were run using the recommended Snakemake workflow engine [27, 67] with default parameters. The metagenomic reads were aligned to the EukDetect marker database and the gutprotist-search database using Bowtie2 [66], followed by stringent quality filtering based on mapping quality to retain reads with a PHRED score ≥ 30, ensuring high base-call accuracy. Sequence complexity was assessed using a complexity score threshold of ≥ 0.5 to retain only high-complexity reads, reducing spurious alignments due to low-complexity regions. Additional filtering as described in EukDetect protocol extended these steps to refine taxonomic assignments by addressing off-target al.ignments that can generate false positives. Specifically, reads mapping to multiple species within a genus were compared for sequence identity and coverage, with lower confidence species excluded, and the ETE3 toolkit [68] was used to validate alignment accuracy. Following recommended practice, only taxa with more than four reads aligning to at least two distinct marker genes were retained, but unfiltered results were also explored for low-abundance species as recommended by the authors [27]. For these cases, we considered the taxa present if alignments met a minimum percent identity threshold of 95%. The EukDetect and gutprotist-search pipelines output a list of the eukaryotic taxa identified in the samples and associated relevant statistics such as the number of observed marker genes per sample per taxon, number of reads mapping to the marker genes, total marker gene coverage, and percentage identity. We combined the results of these pipelines for each sample into a single table for our analyses (Supplementary Table 2). In this table, taxa were marked present (1) in a given sample if they were detected in one or both pipelines and absent (0) from a sample if they were not detected by either pipeline. We chose to analyze our data based on presence/absence (as opposed to relative abundance) because sequencing reads from eukaryotic taxa are relatively rare and hence abundance estimates are likely to be imprecise. Finally, the insect metazoan *Callosobruchus maculatus* was detected in 1 sample, but excluded from downstream analyses as it was likely acquired from food, and therefore not part of the gut eukaryotic community.

Grooming network and microbiome correlation analysis

Following [52], we tested whether grooming-based social networks predicted gut eukaryotic similarity by constructing grooming networks based on observations of grooming interactions collected in the year prior to (and including) the period of microbiome sampling for the social group data set (8 August 2011 to 8 August 2012: 1648 total grooming interactions, with 667 in Mica's group and 981 in Viola's group). Grooming interactions were collected during regular monitoring visits to these social groups (2–4 times per week; 5 h per visit). From these data, we calculated a count of observed grooming interactions between all adult dyads present in each social group (range = 0 to 41 interactions per dyad).

Statistical analyses

All statistical analyses were conducted in the R statistical environment (R version 4.3.3) [69]. We began our analyses by reporting the eukaryotic taxa that we identified across both sets of samples ($N = 75$ samples; 48 from the first (social) set and 27 from the second (seasonal) set).

Next, to test whether eukaryotic species richness differed between baboon social groups (first sample set) or season (second sample set), we calculated alpha diversity using taxonomic richness, defined as the number of unique eukaryotic taxa present in each sample using the package *vegan* [70]. We then tested for differences in richness as a function of social group and season, using linear models. We fit separate linear models for each data set, with taxonomic richness as the response variable. The predictor variables were either the social group the baboon belonged to or the season of sample collection (wet or dry), host sex, host age in years, and sequencing depth. These models were implemented using the lm function from the *stats* package [69].

To test for compositional differences between eukaryotes as a function of baboon social group or season, we calculated Jaccard distances between the samples within each data set using the vegdist function in *vegan* [70] (Jaccard distances measure dissimilarity between samples based on the presence/absence of taxa in each sample). To visualize how eukaryotic species composition varied between social groups or seasons, we used non-metric multidimensional scaling (NMDS). To determine the proportion of variance in community composition

attributable to social group or season, we conducted a permutational multivariate analysis of variance (PERMANOVA) on Jaccard dissimilarities between samples. For the social group dataset, the variables in the model included: the social group in which the baboon lived, the baboon's sex, the baboon's age in years, and sequencing depth. The seasonal dataset model had the same structure, but the social group variable was replaced with the season of sample collection (wet or dry). These models were implemented in the adonis function in *vegan*, with 10,000 permutations [70].

To test the effect of social group and season on the presence of each eukaryotic species within each data set, we ran a series of binomial generalized linear models (GLM) implemented in the *stats* package, where the response variables were the presence (1) or absence (0) of a given eukaryotic taxon. The fixed effects were either the social group in which the baboon lived or the season of sample collection, the baboon's sex, the baboon's age in years (as a continuous variable), and sequencing depth (as a continuous variable). All the linear predictors were z-transformed prior to the analyses. For the heatmap visualizations, age was grouped into categorical intervals (1–5 years, 5–10 years, and over 10 years) to differentiate eukaryotic species abundance patterns across the defined age classes. We prioritized variables based on previous published work. For example, variables such as social group and season were prioritized given their known effects on the gut microbial composition of primates [52, 53, 71–73], while variables like age, sex, and read count, which have previously shown minimal to no impact at the microbial level were placed last in the formula [52, 54, 73–75] (note, however, that the order we entered variables in the model did not affect their effect size or p-values).

To test whether grooming-based social networks predicted gut eukaryotic similarity, we used grooming interaction data to construct a matrix of grooming relationship strength by scoring the strongest dyadic grooming relationship in each group as a 1 and weighting all other dyadic relationships relative to this strongest grooming bond. We then used Mantel tests (implemented in the *vegan* package in R) to test the strength of the correlation between group-specific grooming networks and group-specific Jaccard distance matrices. The grooming network analysis was performed on the data set used to test social group membership but not on the data set used to test seasonality, as the latter did not have sufficient number of individuals to perform this analysis.

Ethics statement

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Notre Dame under protocol number 22-05-7259 and adhered to the laws of Kenya.

Results

Metagenomic analysis reveals diverse gut eukaryotic communities in wild baboons

Across both the social ($n = 48$) and seasonal ($n = 27$) data sets, we found genetic evidence for 21 eukaryotic species (Supplementary Fig. 2; Supplementary Table 3; range = 0 to 7 taxa per sample). The mean number of eukaryotic taxa per sample across both data sets was 3.10 (SD = 1.60). Within each data set, the average number of eukaryotic taxa present per sample was 3.09 (SD = 1.47) in the social group data set and 3.12 (SD = 1.84) in the seasonal data set. Information on the potential transmission mode and health relevance of each taxon is in Supplementary Table 4. Additionally, we detected genetic evidence for the metazoan arthropod, *Callosobruchus maculatus* in 1.33% ($n = 1$ of samples). Because baboons frequently ingest insects, these sequences may come from a closely-related insect in the baboon diet and are not members of the microbiome community. Sequences attributed to *C. maculatus* are removed from our analyses.

Overall, Protista was the most well-represented kingdom, found in 90.7% of samples (68 of 75 samples), followed by Chromista (46.7%; 35 of 75 samples), and Fungi (29.3%; 22 of 75 samples; Supplementary Fig. 2). Over half of the samples in the data set ($n = 56$ samples; 74.7%) contained at least two detectable eukaryotic taxa, while 2.7% ($n = 2$ samples) contained 7 eukaryotic taxa, the maximum number we observed. Only 2 samples (2.67%) had no detectable eukaryotes (one in the social group data set and one in the seasonal data set). Across all samples, the five most prevalent species were *Entamoeba coli* ($n = 56$ samples; 74.7%), *Enteromonas hominis* ($n = 40$ samples; 53.3%), *Blastocystis subtype 3* ($n = 29$ samples; 38.7%), *Iodamoeba sp* ($n = 27$ samples; 36%), and *Chilomastix mensnili* ($n = 26$ samples; 34.7%; Supplementary Table 3). We also detected a set of species found in one or only a handful of samples, including (*Candida blattae*, *Malassezia restricta*, *Ogatea naganashii*, *Preussia sp.*, and *Aspergillus sydowii* (see Supplementary Table 3). We did not detect sequencing reads that aligned to any of the helminth taxa found in previous studies in this population via microscopy [21, 49–51].

Social group membership predicts gut eukaryotic diversity and composition

In the first (social group membership) data set, we found significant differences in the gut eukaryotic alpha diversity for baboons in different social groups (linear model, $p = 5.83 \times 10^{-4}$; Fig. 2A; Supplementary Table 5). Samples from baboons living in Viola's group exhibited higher

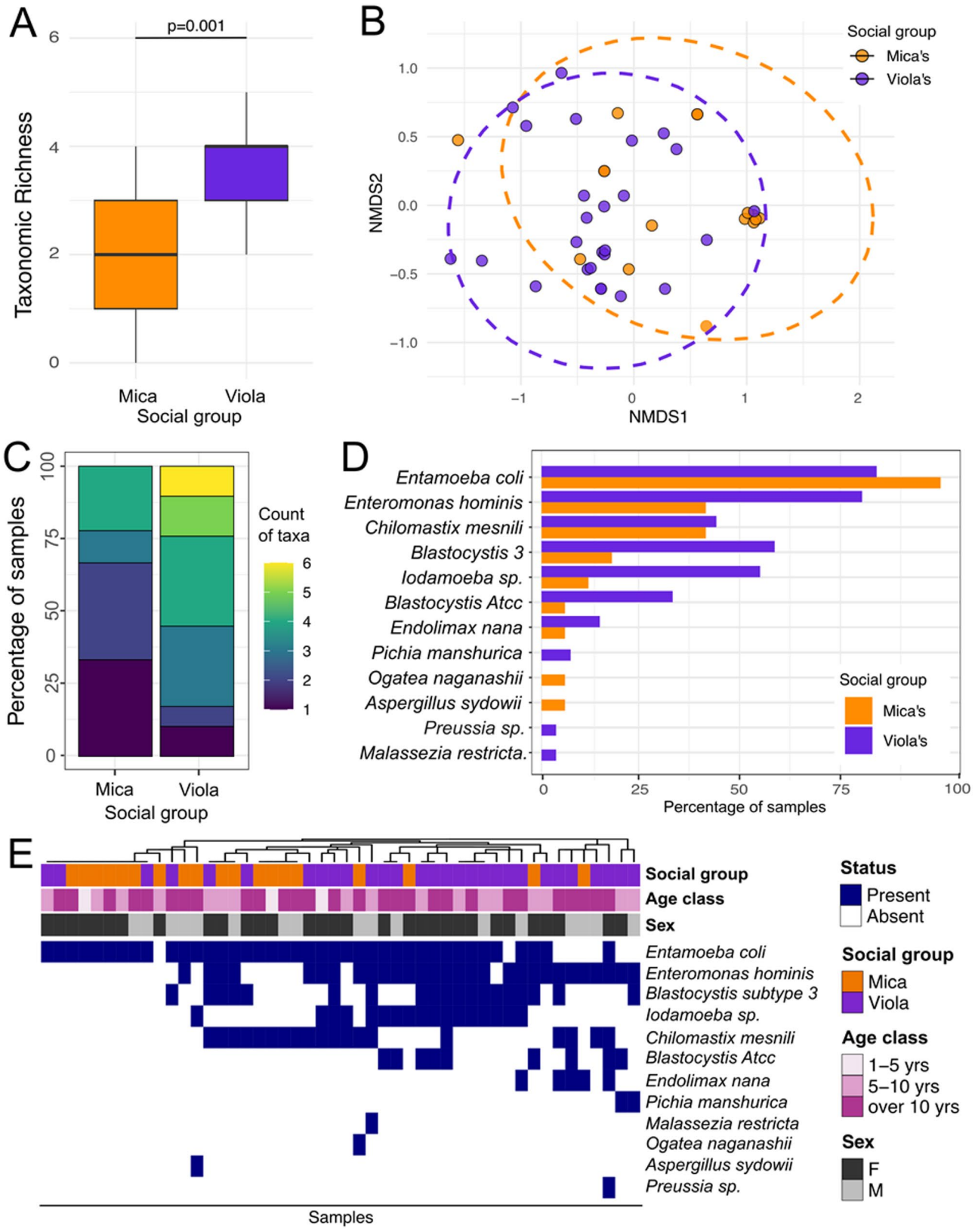


Fig. 2 (See legend on next page.)

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Fig. 2 Social group membership is correlated with baboon gut eukaryotic composition. (**A**) Eukaryotic species richness in Viola's and Mica's social groups. A linear model was used to calculate statistical significance. (**B**) Non-metric multidimensional scaling (NMDS) plot of gut eukaryotic composition as measured using Jaccard index dissimilarity matrices. (**C**) Number of eukaryotic taxa per sample in Mica and Viola's social groups and (**D**) prevalence of eukaryotic taxa in the two social groups. (**E**) Heatmap of the eukaryotic taxonomic composition of samples used to test for social group membership. Each column represents one fecal sample and its associated metadata on social group, age class, and sex of the host. Samples are clustered using Euclidean distance of eukaryotic community composition

taxonomic richness compared to those living in Mica's group (Fig. 2A).

Eukaryotic composition was also significantly different between Viola's and Mica's groups, explaining 11.2% of the variation in eukaryotic composition (PERMANOVA: $r^2 = 0.111$ $p = 1 \times 10^{-3}$; Fig. 2B; Supplementary Table 6). Because some samples only contained one eukaryotic taxon, we repeated this analysis, subsetting to only samples where we detected at least two eukaryotic taxa. We continued to detect a significant effect of social group in this reduced data set (PERMANOVA: $r^2 = 0.08186$ $p = 1.3 \times 10^{-2}$; Supplementary Fig. 3A). We also found a trend such that the number of paired-end sequences (i.e., sequencing depth) in each sample had a small effect on our estimates of gut eukaryotic composition ($r^2 = 0.045$; $p = 0.05$). By contrast, host age and sex did not make statistically significant contributions to gut eukaryotic composition (age, $p = 0.45$; sex, $p = 0.504$; see Supplementary Table 6).

On average, 50% of Viola's and 12.5% of Mica's samples had at least 3 eukaryotic taxa (Fig. 2C). In support of the patterns of alpha diversity we observed (Fig. 3A), most taxa were more prevalent in Viola's group as opposed to Mica's group, including *Blastocystis subtype* 3 (coefficient: -2.03, $p = 0.01$; Supplementary Table 7), *Blastocystis. ATCC* (coefficient: -1.99, $p = 0.08$), *Enteromonas hominis* (coefficient: -1.77, $p = 0.01$), *Iodamoeba sp.* (coefficient: -2.21, $p = 0.01$), and *Endolimax nana* (coefficient: -1.82, $p = 0.20$). Only one taxon, *Entamoeba coli*, was more prevalent in the Mica's group compared to the Viola's group (Fig. 2D-E). *Ogataea naganishii* and *Aspergillus sydowii* were found at low prevalence (2%) only in samples from Mica's group, while *Preussia sp. BSL10* and *Malassezia restricta* were exclusively found in samples from Viola's group (2%, Fig. 2D-E). Finally, we assessed whether grooming interactions predicted eukaryotic composition and found no significant relationship between these two variables (Mantel test: Mica's group: r=-0.188, $p = 0.964$; Viola's group: r=-0.032, $p = 0.748$).

Season did not predict gut eukaryotic diversity and composition

We found no significant differences in the taxonomic richness of the eukaryotes between samples collected in the dry season compared to the wet season (linear model, $p = 0.856$; Fig. 3A). Likewise, season did not explain a statistically significant fraction of variance in community composition, nor was community composition explained by host age, host sex, or sequencing depth (PERMANOVA, $r^2 = 0.036$; $p = 0.48$; age, $p = 0.20$; sex, $p = 0.64$, number of paired-end sequences, $p = 0.13$; Fig. 3B and Supplementary Fig. 3B).

On average, 25.9% of samples collected in the dry season and 22.2% of samples collected in the wet season contained at least 3 detectable eukaryotic taxa (Fig. 3C). In line with our results on the social group dataset, *Entamoeba coli* emerged as the most prevalent and dominant species ($n = 16$ samples; 59.3%), followed by *Enteromonas hominis* ($n = 11$ samples; 40.7%), *Blastocystis* subtype 3 and *Iodamoeba* sp. ($n = 10$ samples each; 37% each), *Pichia manshurica* ($n = 9$ samples; 33.3%), *Chilomastix mesnili* ($n = 7$ samples; 25.9%), *Ogatea naganishii* ($n = 4$ samples; 14.8%), *Blastocystis* ATCC ($n = 3$ samples; 11.1%) and *Byssochlamys* sp. AF001 ($n = 2$ samples; 7.4%; Fig. 3D-E).

Discussion

We used shotgun metagenomic data from fecal samples to characterize the composition and diversity of the eukaryotic communities inhabiting the gastrointestinal tract of wild baboons. We found that host social group membership, but not season, predicted community structure. Here we discuss: (1) the diversity of eukaryotic taxa we detected; (2) how the forces that shape gut eukaryotic communities compare to those that shape gut helminth and bacterial communities; and (3) future prospects for using shotgun metagenomic approaches to detect gut eukaryotic taxa in fecal samples.

Diversity of eukaryotic taxa in shotgun metagenomics data from baboons

We detected genetic evidence for several eukaryotic taxa, spanning 3 kingdoms (Fungi, Protista, and Chromista in order of prevalence), revealing 21 species across both data sets. Most of these taxa are considered non-pathogenic commensals and have been previously found in human populations across the world [76]. The most prevalent taxa was *Entamoeba coli* (74.6% of samples). Using microscopy and targeted sequencing techniques like 18S rRNA amplicon sequencing, *Entamoeba coli* has been identified in several non-human primates, including mountain gorillas (*Gorilla beringei beringei*) [77], western lowland gorillas (*Gorilla gorilla gorilla*) [78], red colobus (*Procolobus badius tephrosceles*) [79, 80], red-tailed

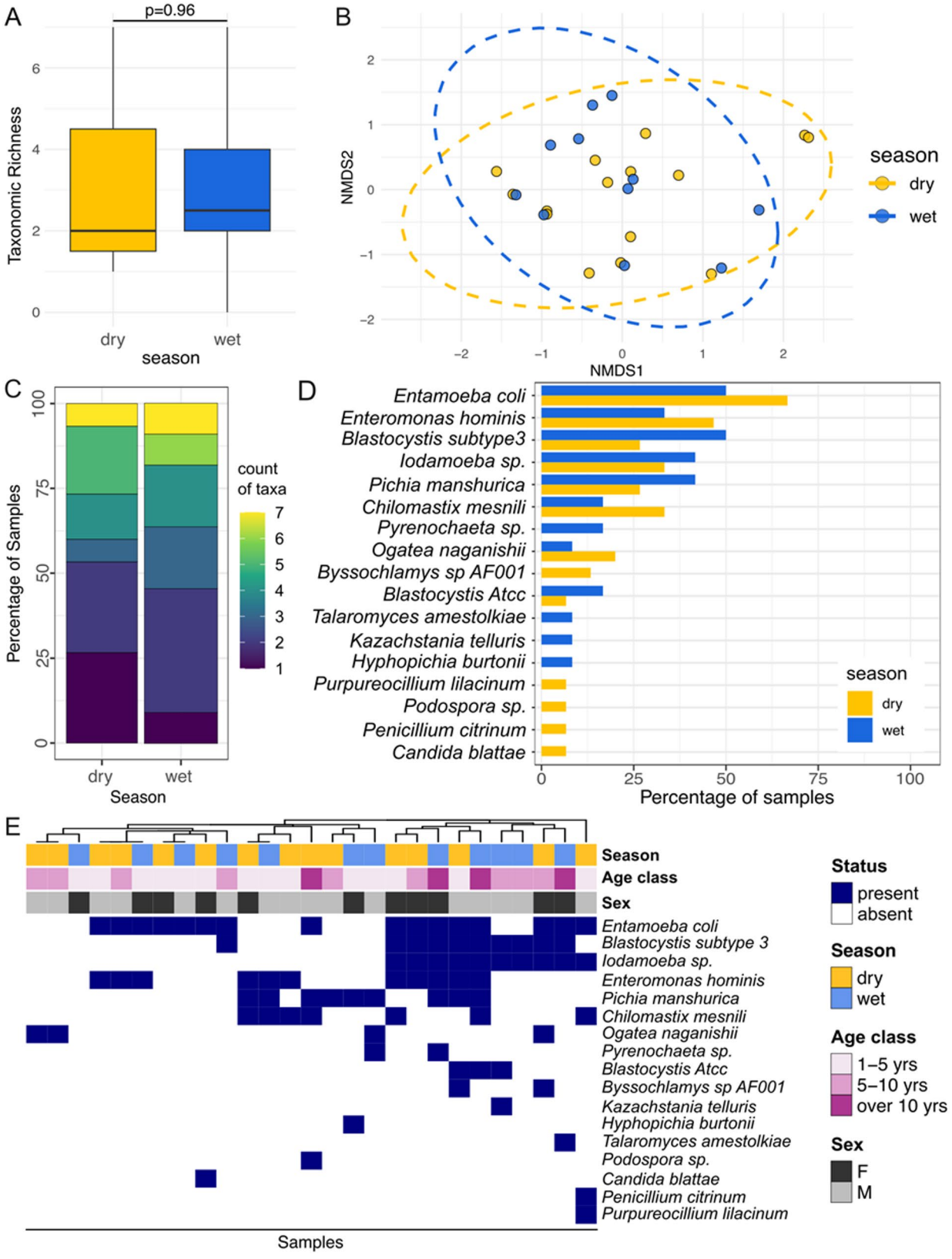

Fig. 3 (See legend on next page.)

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Fig. 3 Season did not predict baboon gut eukaryotic diversity and composition. (**A**) Eukaryotic species richness in the wet and dry seasons. A linear model was used to calculate statistical significance. (**B**) Non-metric multidimensional scaling (NMDS) plot of gut eukaryotic microbiome composition as measured using Jaccard index dissimilarity matrices. (**C**) Number of eukaryotic taxa per sample in the dry and wet season. (**D**) prevalence of eukaryotic taxa in the wet and dry seasons. (**E**) Heatmap of the eukaryotic taxonomic composition of samples used to test for the effects of seasonality, with metadata on season, age class, and sex of the host. Each column represents one fecal sample; samples are clustered using Euclidean distance of eukaryotic community composition

monkeys (*Cercopithecus ascanius schmidti*) [79, 80], vervet monkeys (*Cercopithecus aethiops pygerythrus*) [79, 80], baboons (*Papio anubis*), and chimpanzees (*Pan troglodytes*) [79, 80]. The high prevalence of *Entamoeba coli* among non-human primates might be attributable to their social behavior and ranging patterns, as *Entamoeba* species are transmitted by intake of mature cysts through contaminated water or food, or direct oral-fecal contact [81, 82]. *Blastocystis subtype 3* and *Enteromonas hominis* are also prevalent in other non-human primates [16, 78, 83, 84].

Blastocystis was also very prevalent (17-38.7% of samples, depending on the species). *Blastocystis* is a common chromista found in humans and non-human primates such as baboons, gorillas, chimpanzees, and other mammals, as well as in birds [15, 85, 86]. Whether *Blastocystis* is commensal or pathogenic is still under debate [1]. In humans, members of the *Blastocystis* genus are common and stable colonizers of the gut of healthy individuals [87, 88]. However, *Blastocystis* has also been associated with some gastrointestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) in humans [89], indicating possible pathogenicity. The role of *Blastocystis* in non-human primates remains unclear [15]. However, the high prevalence of *Blastocystis* among the baboons in this study, which had no observable gastrointestinal symptoms, suggests that *Blastocystis* might be a commensal member of the gut microbiome of wild baboons. More research is necessary to understand potential links between the presence of specific eukaryotic taxa and health status in wild baboons.

The most common *Blastocystis* identified in the gut of the baboons in our study was *Blastocystis* subtype 3, found in 38.7% of the samples. *Blastocystis* subtype 3 occurs in humans and livestock [90, 91]. In Amboseli, wild baboons and livestock share water holes, and because *Blastocystis* are commonly transmitted through shared water sources, it is possible that baboons and livestock transmit *Blastocystis* to each other. However, further research is warranted to test this possibility.

Other taxa were less common in our samples (found in less than 10.7% of samples), and included *Candida blattae*, *Malassezia restricta* (which is a member of the normal primate skin microbiota, occasionally contributing to skin conditions), *Ogatea naganashii*, *Preussia sp.* (a non-pathogenic genus), and *Aspergillus sydowii* (which can cause respiratory infections in immunocompromised individuals; Supplementary Table 4). The latter was exclusively found in Mica's group at low prevalence (2%), suggesting that, in contrast to recent observations in wild macaques (*Tibetan macaque*, *Macaca fascicularis*, and *Macaca namestrina*) [92, 93], *Aspergillus* is not a common member of the wild baboon gut microbiome.

Importantly, we did not detect any genetic evidence for helminth taxa that have been identified in earlier studies in this population via microscopy. These species include *Trichuris trichiura*, *Streptopharagus pigmentatus*, *Strongyloides fullerborni*, *Abbreviata caucasica*, *Enterobius vermicularis*, and several strongyle species [21, 49–51]. We speculate about the reasons for this failure in the section below on prospects for using shotgun metagenomics to detect eukaryotes in fecal DNA.

We did, however, detect a metazoan arthropod *Callosobruchus maculatus* (the cowpea weevil) in one baboon sample—a species that is not a resident of the gut microbiome. *C. maculatus* oviposits its eggs on legume seeds and is relatively labile in its use of legume species [94]. Its presence in the baboon gut sample is likely a result of direct consumption of food, most likely the seeds of *Vachellia spp*., but possibly legumes that occur naturally in Amboseli. Baboons in Amboseli are known for their eclectic but selective feeding habits. While their diets mainly consist of vegetation, including leaves, grasses, fruits, and seeds (including from legumes), they supplement these food items with insects (including beetles) and, on some occasions, vertebrates [74]. The presence of *C. maculatus*, therefore, highlights the need to consider dietary contamination in the future studies, as detection of non-endogenous taxa in fecal samples could reflect transient dietary components instead of true resident members of the microbial or eukaryotic communities.

Forces shaping gut eukaryotic communities

Recent work by Mann et al. [16], found that the forces that shape gut eukaryotic communities differ somewhat from those that shape gut bacterial communities. Our results support this perspective. For instance, consistent with prior work on the gut bacterial communities in this host population [52], we found that social group membership explained a substantial fraction of eukaryotic community composition. Also similar to prior work on gut bacterial communities in the Amboseli baboons [95] and in geladas [96], we found that larger social groups (Viola's) had greater eukaryotic diversity than the smaller

group (Mica's). This pattern may be because baboons in larger groups have larger home ranges than smaller groups, a pattern often observed in primates [97, 98]. Viola's home range was bigger than Mica's at the time the samples we analyzed were collected [52]. Hence, Viola's group may have been exposed to a wider variety of water sources, foods, and substrates, leading to different patterns of gut eukaryotic colonization and diversity.

Direct transmission between hosts could also explain differences in eukaryotic communities between social groups because baboons largely interact with members of their own group, and animals in larger groups have direct contact with more conspecific hosts. While the current study did not directly assess transmission, previous research shows that gut parasites like *Entamoeba*, *Giardia*, *Blastocystis*, and *Balantidium coli* spread within and among primate social units via physical contact as well as shared environmental sources [52, 99–102]. Further, in the Amboseli baboons, Tung et al. [52] showed that grooming networks correlate with gut bacterial composition. However, we found no correlation between baboon grooming networks and gut eukaryotic composition. While this pattern suggests that contact networks may not shape overall eukaryotic community structure, our study is somewhat hampered by the relatively low representation of eukaryotes among the gut microbiota and the relatively small sample size. Nonetheless, our study establishes a starting point for examining potential eukaryotic transmission events in social groups and detecting shifts in their composition due to health issues and or environmental stressors.

In addition to social effects, prior work in Amboseli also found profound effects of season on gut bacterial communities [53]. However, we found little evidence for this pattern in gut eukaryotic communities. Specifically, there were no strong differences in gut eukaryotic diversity or composition for samples collected from the wet versus dry seasons. This result contrasts with previous studies that identified seasonality as important in shaping the bacterial component of the gut microbiome of human [72] and animal populations [71, 103, 104], including baboons [71, 72, 74, 103]. However, we note that the sample size for the seasonal data set was small ($n = 27$ samples, with 15 samples collected during the dry season and 12 samples collected during the wet season), and the individuals lived in multiple social groups. Given that social group membership had relatively strong effects on eukaryotic communities in our other dataset, this confound may have hampered our ability to detect seasonal effects on gut eukaryotic composition. Likewise, the social group membership sample set was collected entirely in the dry season and could not be used to assess seasonal variability. As a result, we were unable to test the importance of these two variables (social group and season) in relation to each other. As the sampling of these individuals is ongoing, we envision future studies will be able to overcome these limitations.

Other variables such as host sex and host age were less important in explaining eukaryotic diversity and composition in our sample (1.6% and 1.7%, respectively). This pattern contrasts with previous studies of bacterial communities that reported both host sex and host age to be drivers of microbial community variation [30, 105–107], but is consistent with prior work on gut bacterial communities in the Amboseli baboons, which find no effects of host sex on gut microbiota [52, 95]. The absence of sex effects could reflect similar exposure to bacterial and eukaryotic patterns for males and females via shared sources of food and water. Combined, these results suggest that social group membership plays an important role in the ability of eukaryotes to colonize hosts and are in line with previous studies that reported a clear association between social group membership and the composition of bacterial communities in the gut of humans, non-human primates, carnivores, rodents, insects, and birds [52, 107–115]. Physical contact, shared environment, and group behaviors might therefore influence both prokaryotic and eukaryotic diversity and composition via partially shared mechanisms. Further studies are needed to establish to what extent bacterial and eukaryotic microbiomes may be governed by the same ecological constraints in primates.

Prospects for using shotgun metagenomics to detect gut eukaryotes in feces

Eukaryote detection using shotgun metagenomic approaches is constrained by the taxa present in reference databases. This bias may result in the exclusion or under-detection of novel, rare, or uncultured eukaryotes, since they are often inadequately represented or excluded from the current genomic databases [27, 116]. Furthermore, shotgun metagenomic methods will also often fail to detect rare taxa in the gut microbiome because these organisms often do not contribute enough DNA to be detected, unless the shotgun sequencing is very deep. Both of these limitations may explain why we failed to detect helminths known to infect the Amboseli baboons [21, 49, 50]. Genomic resources for helminths are underdeveloped, and multicellular helminths contribute very little DNA to fecal samples as compared to bacteria and viruses [117]. As a result, they may fall below the detection threshold in metagenomic studies [118]. Future studies may benefit from integrating complementary methods such as DNA enrichment, targeted PCR, or improving the reference databases to better capture the gut eukaryote diversity [22, 119, 120].

While we failed to detect some taxa, it is worth noting that other aspects of our methods did not have a strong

impact on the eukaryotes we found. While we used different DNA isolation kits and cell lysis methods in constructing our social group and seasonal datasets (the DNeasy PowerSoil kit from Qiagen for the seasonal dataset and the MO BIO Laboratories, Inc. PowerSoil DNA Isolation kit for the social dataset), the kit formats are identical (Qiagen purchased MO BIO and re-branded the kit under their name, but the kit components are the same). Hence, we do not believe this contributed to differences between the two data sets: both kits recovered the same eukaryotic species, ensuring the validity of our conclusions regarding community composition.

Finally, while the samples included in this study were collected over a limited period of time and more than a decade ago (in 2012 and in 2014), these constraints do not detract from the overall contributions of the study, which represents one of the first to use high-throughput sequencing methods to characterize gut eukaryotic composition in a wild primate population. Future studies should collect samples longitudinally within hosts, spanning both different seasons and including hosts from different social groups. Targeting deeper sequencing depth and leveraging long-read sequencing platforms will provide further insight into the rare microbial and eukaryotic taxa inhabiting the gut microbiome of wildlife, with the ultimate goal of informing conservation efforts, and improving the management of wild baboon populations.

Conclusions

To our knowledge, this is the first shotgun metagenomic study to characterize the eukaryotic gut microbiota of wild baboons. Our results confirm that eukaryotes are an important part of the microbial communities inhabiting the gut of wild baboons and indicate that social group membership plays a role in shaping gut eukaryotic composition and diversity over time. Understanding how social factors affect microbiome composition could therefore be informative about the evolution of social behavior and its health implications, and reinforces the importance of considering social dynamics in microbiome research.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Conceptualization, EAA, MNC, MYA, JT; Sample and metadata collection, EAA, JT, SCA; Samples processing and data analysis, MNC, SW, PF; Writing – original draft, MNC.; Writing – review & editing, MNC, PF, SW, EAA, MYA, RM, GO, JK, JT, SCA; Supervision, RWM, GO, MYA, EAA; Funding acquisition, EAA, JT, SCA. All authors read and approved the final manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession codes PRJNA271618 and PRJEB81717.

Declarations

Competing interests

The authors declare no competing interests.

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