Multiomics characterization of the zoo-housed gorilla gut microbiome reveals loss of bacterial diversity besides abundant fungal cellulose-degrading and archaeal methanogenic activity

Isabel Houtkamp[1], Martine van Zijll Langhout[2], Mark Bessem[3], Walter Pirovano[3,4] and Remco Kort[1,2,*]

[1]Amsterdam Institute of Life and Environment (A-LIFE), Vrije Universiteit Amsterdam, The Netherlands
[2]ARTIS Amsterdam Royal Zoo, The Netherlands
[3]BaseClear, Leiden, The Netherlands
[4]Department of Complex Trait Genetics, Center for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, The Netherlands

*Corresponding author: r.kort@vu.nl

Abstract

We carried out a comparative analysis between the microbiota composition of zoo-housed western lowland gorillas (*Gorilla gorilla gorilla*) and their counterparts living in the wild through 16s rRNA gene amplicon sequencing. In addition, we characterized the carbohydrate active potential and activity of the zoo-housed gorilla microbiome through shotgun metagenomics and RNA-seq. Alpha and beta-diversity indices significantly decreased in the zoo-housed gorilla microbiota compared to those of their wild fellow species. Abundant fiber-degrading native bacterial taxa of the wild gorilla gut, Chloroflexi and *Olsenella*, vanished, whereas oligosaccharide-consuming *Lactobacillus* species blossomed in presumably, the upper gastro-intestinal-tract of the zoo-housed gorilla. Metagenomic analysis of the CAZyome indicated predominant oligosaccharide-degrading activity, while RNA-sequencing revealed diverse cellulase and hemi-cellulase activities in the zoo-housed gorilla gut, contributing to a total of 268 identified carbohydrate active enzymes. We demonstrated for the first-time major activity of the cellulose-degrading fungi Neocallimastigomycetes and methanogenic archaea in the gorilla gut, the latter known to feed on hydrogen produced by the anaerobic fungi. We propose that the zoo-housed gorilla microbiome is adapted in composition and reduced in bacterial diversity due to lifestyle effects, while a previously unacknowledged fiber-degrading capacity is sustained by anaerobic gut fungi. Our study shows the added value of RNA-seq in a multiomics approach and highlights the contribution of eukaryotic and archaeal activity to the gorilla gut microbiome.

Keywords: *Gorilla gorilla gorilla*, primate microbiome, metagenomics, metatranscriptomics, high-fiber diet, Chloroflexi, *Olsenella*, *Treponema*, Neocallimastigomycetes, herbivore microbiome, CAZyome, rewilding.

List of abbreviations: CAZyome, collective of carbohydrate-active enzymes; MG, metagenome; MT, metatranscriptome; RNA-seq, RNA sequencing; WG, wild gorilla; ZHG, zoo-housed gorilla; ACE, abundance-based coverage estimators; GH, glycoside hydrolyase.

Introduction

Gut microbes are necessary to digest the complex polysaccharides in plants (fibers), as their own genomes harbor no enzymes for hydrolyzation and fermentation of plant material (Gomez, 2014). The gut microbiome has even allowed the evolution of herbivory as a dietary niche in this sense, and many mammals now completely rely on plant material as a dietary source (Moeller & Sanders, 2020). After allegedly millions of years of co-evolution, the gut microbiome is not only embedded in host metabolism, but also in the immune and endocrine systems, with hosts and microbiomes having shaped each other's genomes (Moeller et al., 2016). The host microbiome can adapt rapidly in composition and function, responding to dietary and other lifestyle changes. Although all mammals may experience such changes, the most frequent and drastic lifestyle transitions have occurred in human history, with industrialization as the most recent and fast-paced example. The exposure of humans to microbes drastically altered in industrialized societies because of antibiotics, higher sanitation levels, emergency C- sections, formula infant feeding and processed foods, which are low in dietary fiber (Sonnenburg and Sonnenburg, 2019a; Sonnenburg and Sonnenburg, 2019b). Comparisons between industrialized microbiomes with that of hunter-gatherer societies indicate that while the hunter- gatherer microbiome has a rich diversity of species and carbohydrate active enzymes, the industrialized microbiome has a lowered taxonomic diversity and is enriched for mucin-degrading enzymes. Increased abundance of mucin-degrading genes is a sign of low nutrient availability for the gut microbiome, allowing host mucin-degrading microbes to flourish. Whilst the microbiome may have adapted to industrialization, concerns are raised about host biology because of the 'asymmetric plasticity' between the rapidly adapting microbiome and the relatively stable mammalian genome. Sonnenburg and Sonnenburg (2019b) postulated that because of rapid lifestyle transitions, incompatibility of the microbiome with its host can arise, possibly leading to the emergence of non-communicable chronic diseases in industrialized societies.

Interestingly, effects of industrialization on the microbiome are paralleled in zoo-born non-human primates. When zoo-housed, non-human primates are also exposed to higher sanitation levels, antibiotics, sometimes formula infant feeding, and most consistently, to a diet lower in plant complex carbohydrates than their wild fellow species. Efforts are made to understand the effects of captivity on the mammalian microbiome to gain a general understanding of lifestyle effects on the mammalian microbiome, but also to improve the health of zoo-housed animals and considering conservation efforts. Clayton et al. (2016, 2018) found that the microbiota of zoo-housed primates (doucs, *Pygathrix nemaeus*, and mantled howler monkeys, *Alouatta palliata*) are reduced in biodiversity and enriched in species that dominate the microbiome of industrialized humans, like *Bacteroides* and *Prevotella*. According to observations in North American Zoos, the gut microbiomes of chimpanzees, bonobos and gorillas contain 30% fewer genera than those of their wild fellow species, similar to the loss of genera in industrialized humans compared to humans living a hunter-gatherer lifestyle (Nishida & Ochman, 2021). Despite these effects, the microbiome of zoo-housed apes is still most similar to that of wild apes, and more similar to that of non-industrialized humans than that of westernized humans. This is attributed mainly to the presence of *Treponema* species, which are rarely present in industrialized human microbiomes, but are common members of the hunter-gatherer microbiome, and that of zoo-housed and wild great apes (Campbell et al., 2020). However, species that are considered characteristic for the wild great ape microbiome are often lost in zoo-housed microbiomes (Campbell et al., 2020; Clayton et al., 2016). The effect of lifestyle and diet on microbiome diversity and composition are thus evident both human and non-human primates. In gorillas, this is even visible to the naked eye. The

abdomens of wild gorillas (WG's) are bloated, a result of fermentation of plant material by the microbiome leading to gas production by methanogens. Zoo-housed gorillas' (ZHG's) abdomens often appear flat, indicating lower levels of fermentation of plant material (Masi, 2011). The WG's are herbivores and rely strongly on their microbiome to degrade plant material. Characteristic for the microbiome of western lowland gorillas is the high relative abundance of the phylum Chloroflexi compared to other great apes and humans, and the high abundance of the phylum Spirochaetes, which is only present at high abundance amongst other primates in chimpanzees (Hicks et al., 2018).

Most data on the non-human primate microbiome originates from 16S rRNA amplicon sequencing and is thus of a compositional nature. In human microbiome studies, shotgun metagenomics is now widely used, generating a taxonomic as well as a functional microbiome characterization (Zhang et al., 2019). For gorillas, such data is still limited, but its use is evident: previous studies utilized shotgun metagenomics to study the prevalence of antibiotic-resistance genes in non-human primate microbiomes (Clayton et al., 2016), or to show seasonal fluctuations in the abundances of functional pathways in the great ape microbiome (Hicks et al., 2018). Metagenomics studies can identify the potential of carbohydrate active enzymes in the gut microbiome, metatranscriptomics can reveal which genes are expressed under certain conditions (Zhang et al., 2019), and thus reveal the functional activity of the microbiome. To our knowledge, metatranscriptomics has never been used to study the gorilla microbiome so far. Here, we study the microbiome composition of the gorilla population of ARTIS, the Amsterdam Royal Zoo, through 16S rRNA amplicon sequencing. Furthermore, we present a metagenome and metatranscriptome analysis on the microbiome of the dominant male ('silverback') of this population. We aim to characterize the composition as well as the fiber-degrading potential and activity of the ZHG microbiome.

Methods

Diet of zoo-housed gorillas

The ARTIS gorillas are daily fed 3.6 kg crumble and gorilla pellets (Marion Leaf Eater Food, Plymouth, USA). The group receives 50 kg of a wide array of vegetables which differs each day of the week (rotated weekdays), including celeriac, endive, fennel, tomatoes, chicory and carrots. To increase the fiber content in this diet, branches, plants and flowers are added on a daily basis.

Comparative analysis of zoo-housed and wild gorilla microbiota

We utilized previously published 16s rRNA V1-V3 amplicon sequencing data (454 FLX-Titanium technology) of fecal samples from WG's, obtained by Gomez *et al.* (2015), for a comparative analysis in terms of diversity and composition with the microbiota data of ZHG fecal samples obtained in this study. The fecal samples from WG's were collected at the Dzanga-Ndoki National Park, Central African Republic, during November and December of 2009. This period corresponds to the dry season, characterized by low fruit availability and consumption of high-fiber fallback foods. Samples originate from four gorilla groups, of which two habituated to human presence, H1 (N = 11) and H2 (N = 11), and two unhabituated, U1 (N = 11) and U2 (N = 7). A more detailed description of this dataset can be found in Gomez *et al.* (2015). To the published OTU count data, the same preprocessing and analysis was applied as to the compositional data on the gut microbiota of ZHG's obtained in this study, as further described below.

Sample collection

A total of 15 fecal samples for 16S V3-V4 amplicon sequencing were collected from the ARTIS gorilla population between August 2019 and the end of January 2020. In July and August 2021, additional fecal samples from the silverback were collected in duplicate on two time points. Samples were collected within 30 minutes after defecation and frozen at – 60°C. These four samples were subjected to both shotgun metagenomics and RNA-seq.

DNA extraction, library preparation and Illumina sequencing

DNA was extracted from gorilla feces using the ZymoBIOMICS™ 96 MagBead DNA Kit (Zymo Research). DNA QC quantitation was performed with Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen) and agarose gel electrophoresis for DNA integrity. The DNA served as a template for PCR amplification of part of 16S rRNA genes and subsequent analysis by next generation sequencing using Illumina MiSeq. In short, amplicons of V3-V4 regions of 16S rRNA genes were generated by PCR with primers 341F and 785R which generated a ~630 bp amplicon (Klindworth et al., 2013) complemented with standard Illumina adapters. Unique Index Primers were attached to amplicons in each sample through a second PCR cycle. PCR products were purified using Agencourt© AMPure® XP (Becker Coulter) and DNA concentration was measured by fluorometric analysis (Quant-it, Invitrogen). Subsequently, PCR amplicons were pooled using equimolar quantities, followed by sequencing on an Illumina MiSeq with the paired-end 300 cycles protocol and indexing. For samples subjected to shotgun-metagenomics and RNA-seq, ZymoBIOMICS™ ZymoD4300 kit was used to extract DNA and ZymoBIOMICS™ kit Zymo R2040 to extract RNA. Library preparation was performed using Illumina RNA and DNA library preparation kits (20037135, 20020595, 20022371, FC-131-1096). Sequencing was performed with Illumina Novaseq 6000 (Paired End 150 run).

Data preprocessing and quality control

Paired-end sequence reads were generated using the Illumina NovaSeq 6000 system. FASTQ read sequence files were generated using fastq version 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an

inhouse filtering protocol of Baseclear BV, Leiden, the Netherlands. In addition, reads containing (partial) adapters were clipped up to a minimum read length of 50 bp. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8 Andrews (2010).

Classification of operational taxonomic units

Paired-end sequence reads were collapsed into pseudoreads using sequence overlap with USEARCH version 9.2(Edgar & Bateman, 2010). Classification of these pseudoreads is based on the results of alignment with SNAP version 1.0.23 against the RDP database version 11.5 for bacterial organisms (Cole et al., 2014). All amplicon sequencing data analyses were performed using RStudio Statistical Software version 4.1.3. For compositional analysis of the ARTIS gorilla microbiome, rare OTUs were filtered out: OTUs not observed more than three times in at least 20% of the samples were removed. Subsequently, total sum scaling was applied to obtain relative abundances. For analysis and visualization, RStudio was employed (R version 4.1.3, phyloseq_1.38.0), using phyloseq (McMurdie & Holmes, 2013), vegan (Dixon, 2003) and metacoder 0.3.5 (Foster et al., 2017) packages. Since limited data on the gorilla microbiome is available, we employ a previously published dataset by Gomez *et al.* (Gomez et al., 2015), We utilize the OTU tables and assigned taxonomies as originally published. Both ZHG and WG OTU count data were subsampled to the minimum observed sequencing depth in all samples before calculating diversity metrics, to account for large differences in sequencing depth between both datasets. Significance of differences in diversity metrics between groups was determined using Wilcoxon rank sum test, with Benjamini-Hochberg *p*-value correction for multiple testing, executed in RStudio.

Taxonomic assignment of DNA and RNA reads

We used Kraken2 (2.1.1)/Bracken (2.6.1) to estimate taxonomic composition of the metagenomes and metatranscriptomes utilizing an inhouse Kraken/Braken database which we extended with the addition of 6 Neocallimastigomycetes genomes*: Anaeromyces sp. S4 v1.0* (GenBank: GCA_002104895.1), *Neocallimastix sp. G1 v1.0* (GenBank: GCA_002104975.1), *ASM1694683v1* (GenBank: GCA_016946835.1), *Orpinomyces* sp. strain C1A (GenBank: GCA_000412615.1), *Piromyces* sp. finnis v3.0 (GenBank: GenBank: GCA_002104945.1) and *Piromyces sp.* E2 v1.0 (GenBank: GCA_002157105.1). Assignments to the order of Primates were filtered out and total sum scaling was applied, as they are considered contamination. Heat trees were created with metacoder v0.3.5 in RStudio.

Functional profiling of metagenome and metatranscriptome

Shotgun metagenomics reads were filtered for polyG tails, a known artefact of the Illumina NextSeq two-color dye technology, using fastp version 0.23.1 (Chen et al., 2018). Reads were filtered for host mRNA using STAR aligner version 2.7.9a (Dobin et al., 2013) and the Kamilah_GGO_v0 reference genome (Refseq GCF_008122165.1). The RNA-seq reads were subjected to the same preprocessing, but additionally filtered for ribosomal RNA with sortmerna version 4.3.4 (Kopylova et al., 2012) by aligning reads against all SILVA databases of release 138.1, which contain small (16S/18S, SSU) and large (23S/28S, LSU) ribosomal subunits of Archaea, Bacteria and Eukarya (Quast et al., 2013). Preprocessed DNA and RNA reads were annotated with HUMAnN3.0 version 3.0.0, with uniref90 (version 20191b_full) as a reference database. To increase mapping rates, reads were assembled with MegaHit (1.2.9) (Li et al., 2015), genes were clustered using CD-HIT (4.8.1) (Fu et al., 2012), annotated with prokka (v1.14.5) (Seemann, 2014) and EGGNOG5.0 (Huerta-Cepas et al., 2019) using eggnog mapper (v2.0.0) (Cantalapiedra et al., 2021). Subsequently, the original preprocessed reads were realigned against the deduplicated contigs with bowtie2 v2.4.2 (Langmead & Salzberg, 2012) and subjected to a second analysis with HUMAnN3.0.0, using the contig KEGG and COGG annotation as custom mapping files, to obtain relative abundances of these functional groups. Normalization

was performed using HUMAnN3's normalization function. Analyses were executed with Nextflow version 21.10.5 (build 5658) (di Tommaso et al., 2017) for reproducibility.

Annotation of Carbohydrate Active Enzymes

To quantify the relative abundances of Carbohydrate Active enzyme (CAZyme) families we ran HUMAnN3.0 on the read-contig alignments with a custom id-mapping file containing the corresponding CAZyme family for each contig. To create this file, clustered contigs were aligned to the dbcan2 version of the CAZy database (version 10: CAZyDB.09242021.fa) using run_dbcan (v2.0.11) (Zhang et al., 2018), employing the option for alignment with HMMER in metagenome mode. Normalization was performed using HUMAnN3.0's recommended normalization function.

Sensitive detection of fungal genes and transcripts

After preprocessing, ShortBRED was used for sensitive detection of proteins of interest. We created markers for several proteins originating from Neocallimagistomycotes species, downloaded from UniProt release 2022_01. Preprocessed DNA and RNA reads were searched against several markers for proteins of interest. A first set of markers contained all Neocallimastigomycetes sequences in UniProt annotated with the subcellular localization 'hydrogenosomal'. A second set of markers consisted of all malic enzyme sequences (Malate dehydrogenase (decarboxylating) EC 1.1.1.39) assigned to Neocallimastigomycetes, and the last sets of GH48 and GH6 proteins. Clustid was set to 85% and minimum marker length to 8 amino acids, based on the authors of ShortBREDs own parameter optimization, except for the marker set of malic enzymes, for which the clustid option was set to 100% because of high sequence similarity. A workflow overview of shotgun metagenomics and RNA-seq data analysis is included in the Supplemental Figure S1.

Ethics approval

This study was approved by the ARTIS Amsterdam Royal Zoo Ethical Committee. All methods were noninvasive and part of routine preventative health monitoring.

Data availability

All sequence data has been deposited in the Sequence Read Archive (SRA) with BioProject number PRJNA827108. All scripts utilized for data analysis are available in a dedicated GitHub repository (Houtkamp, 2022) .

Results

Microbiome diversity is reduced in zoo-housed gorillas compared to wild gorillas

In this study, we first obtained the composition of the ZHG microbiome through 16S rRNA V3-V4 amplicon sequencing on 15 fecal samples of a group of western lowland gorillas in ARTIS, the Amsterdam Zoo. Since microbiome data from wild gorillas (WG's) is rare, we utilize compositional data of four groups of wild western lowland gorillas obtained by pyrosequencing the V1-V3 amplicon with 454 FLX-Titanium technology, as opposed to Illumina Miseq technology, for comparison. Figure 1 presents a rarefaction curve for all samples of zoo-housed gorillas (ZHG's) and four groups of WG's.

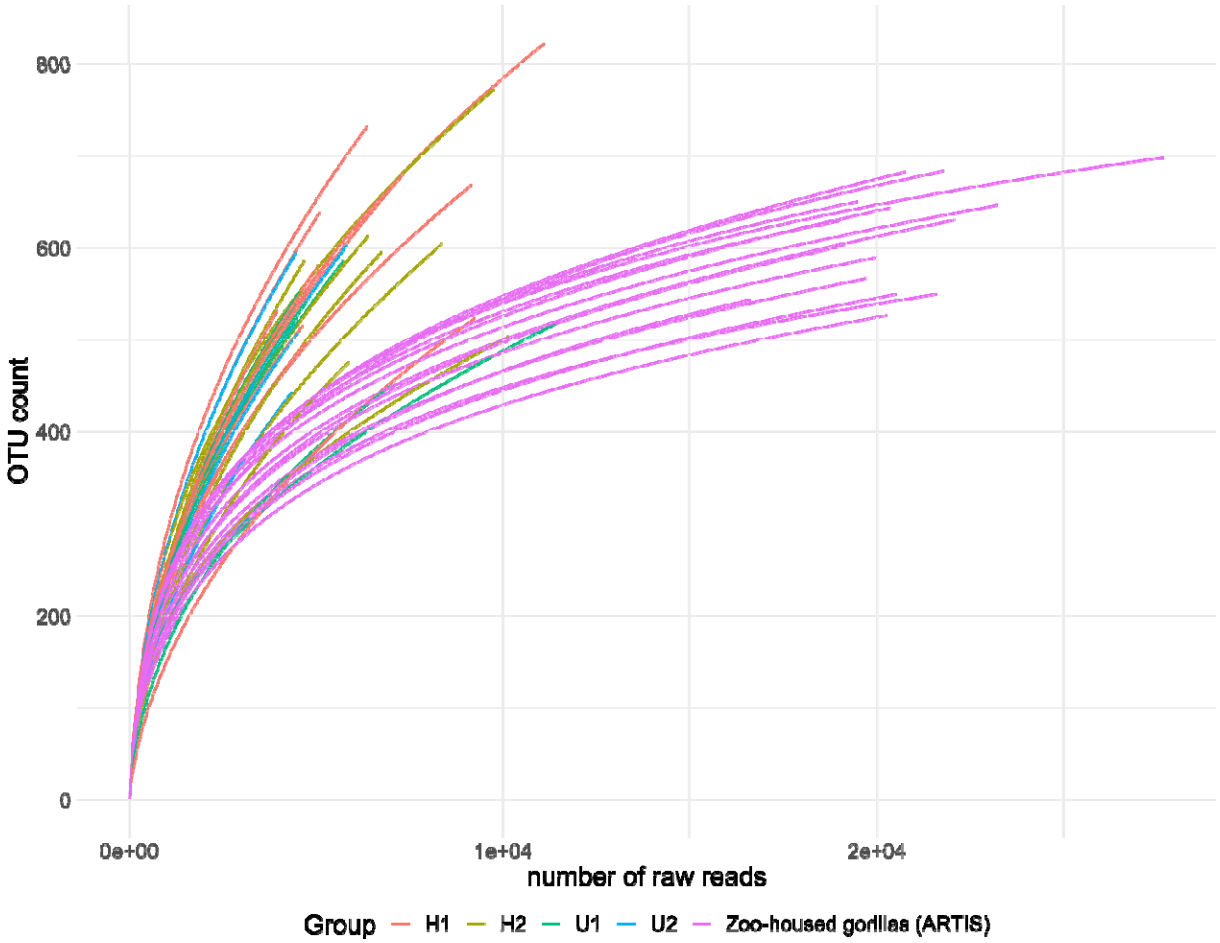


Figure 1 Rarefaction curve of wild and zoo-housed gorilla 16s rRNA gene profiling data. Rarefaction curves for the 16s rRNA gene profiling data of fecal samples from four groups of WG's from the Central African Republic [13], including habituated groups (H1, red; H2, olive green) and unhabituated groups (U1, green; U2, blue) and for 15 fecal samples (purple) from ZHG's in ARTIS analyzed in this study. The number of OTUs (operational taxonomic units) is presented relative to the number of raw reads in a rarefaction analysis.

The rarefaction curve (Figure 1) shows that the sequencing depth of the ZHG samples largely exceeds that of the WG samples, resulting in a higher count of total observed raw counts in ZHG samples. However, species richness per number of reads is lower in ZHG samples when compared to WG samples. After rarefaction to minimal observed sampling depth across ZHG and WG data, Chao1, ACE and Fisher diversity indices were significantly decreased in ZHG, but no significant difference was found for the Shannon diversity index

between groups, as calculated by the Wilcoxon rank sum test, and Benjamini-Hochberg correction for multiple testing (Figure 2). The statistics are listed in Supplementary data sheet 1. Chao1 and ACE and Fisher are known to be mainly sensitive to species richness, whereas Shannon's diversity index also reflects species evenness. Therefore, we conclude that species richness was decreased in the ZHG samples, in line with the rarefaction analysis. The Bray-Curtis dissimilarity between sample pairs was lowered within ZHG samples compared to WG samples, indicating that beta diversity is lowered in ZHG samples as well (Supplemental Figure S2).

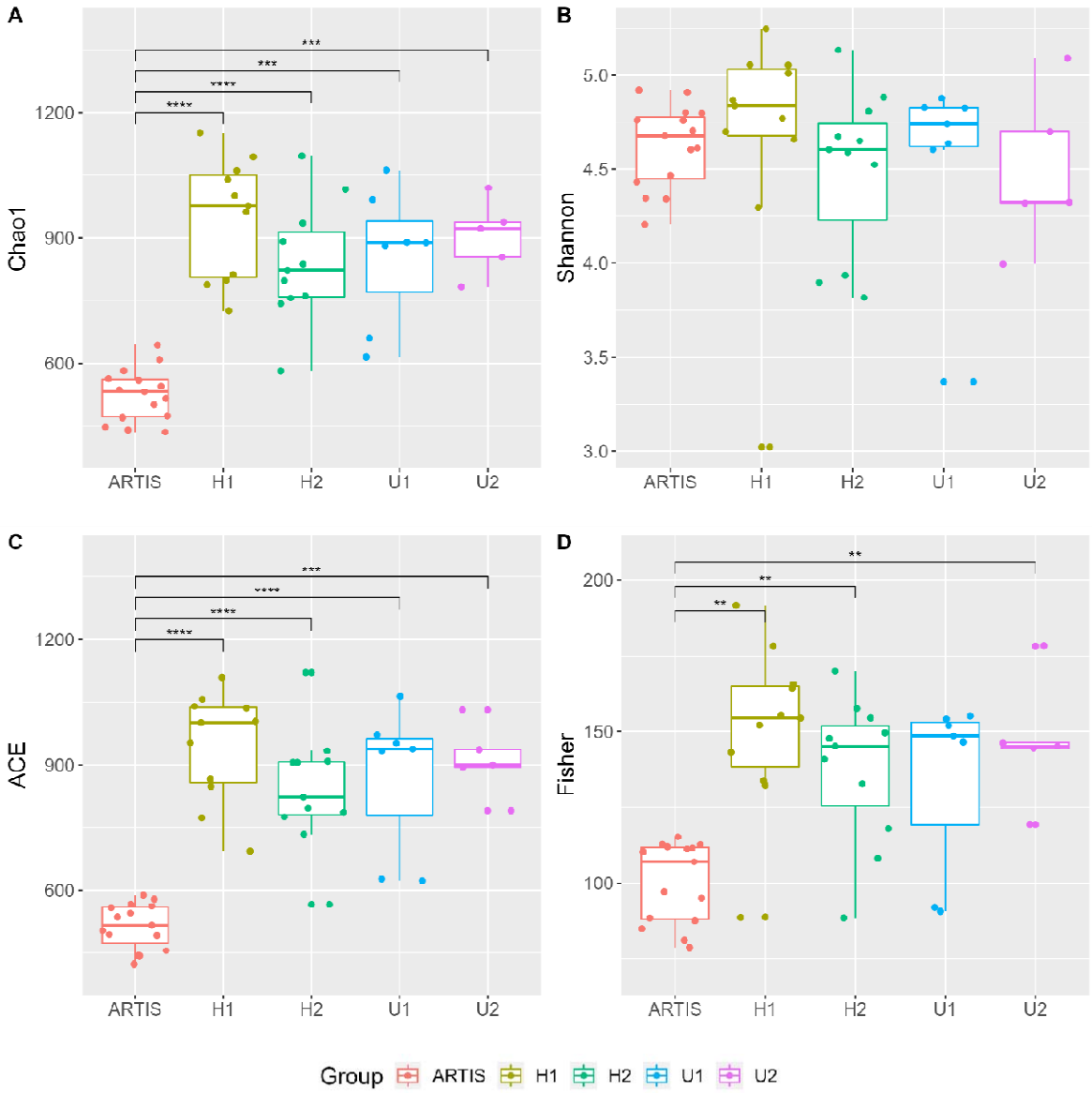
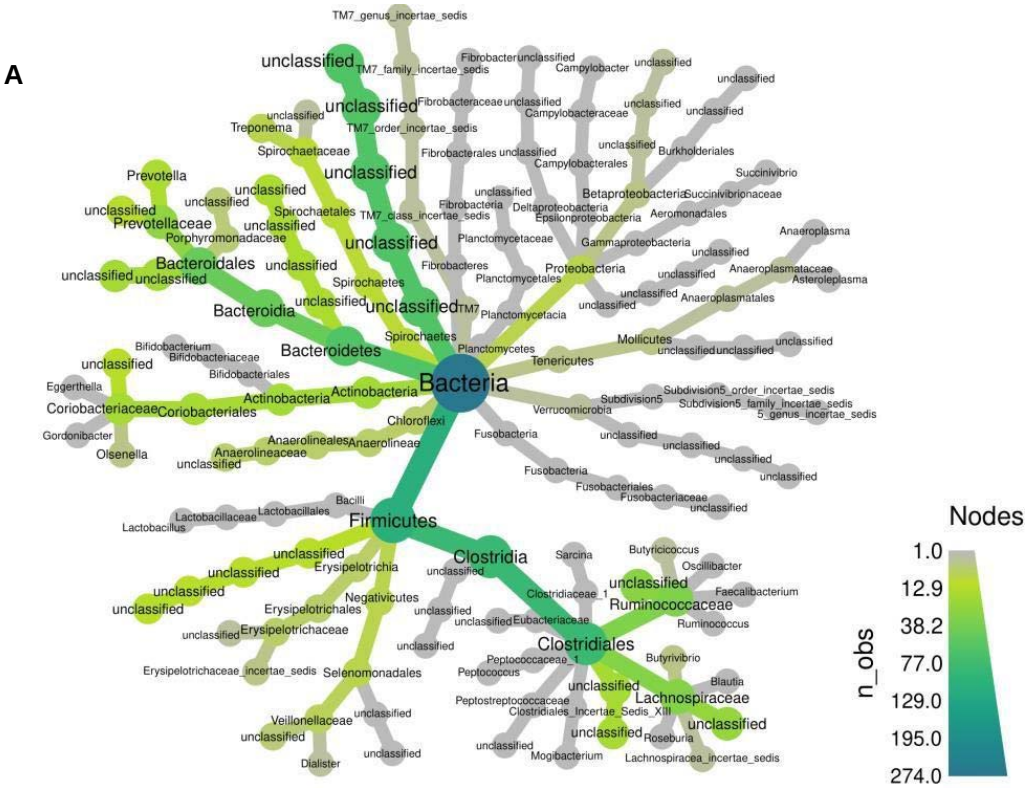


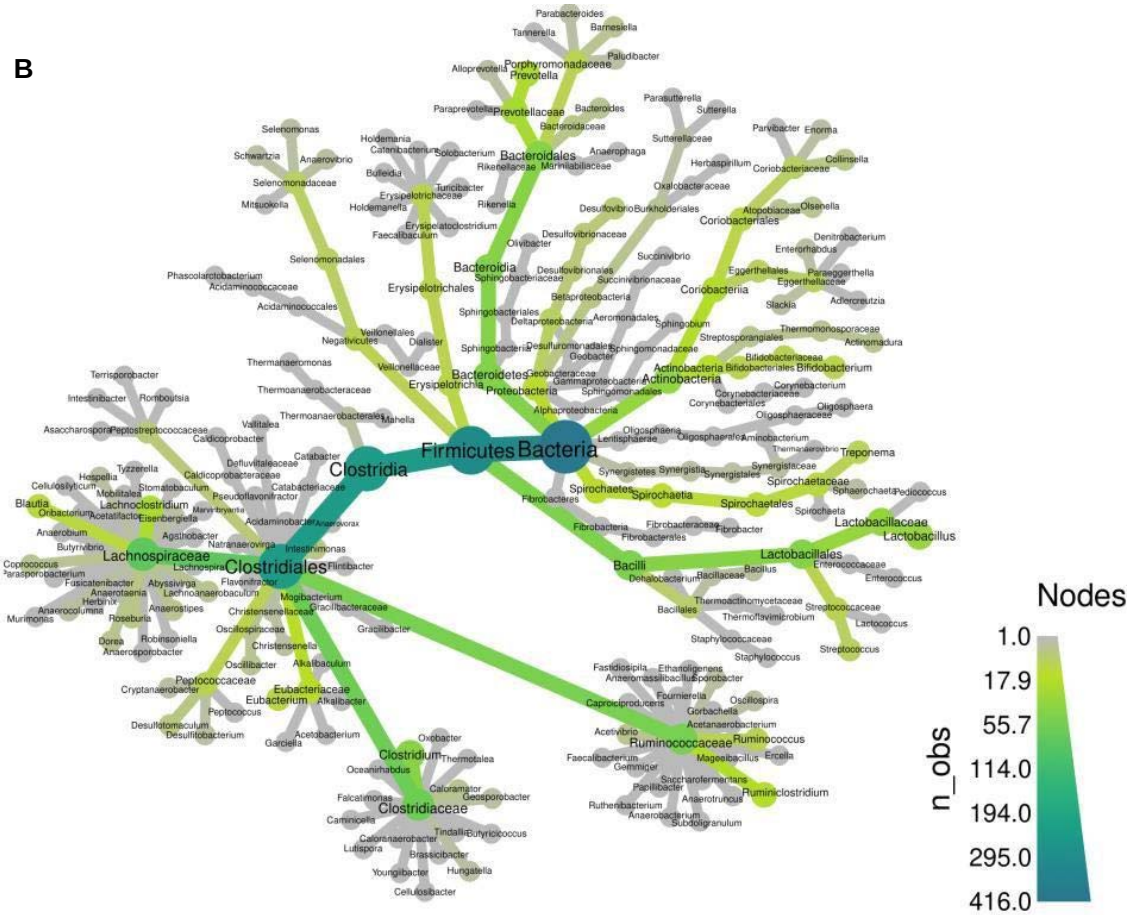
Figure 2 – Alpha diversity metrics of the zoo-housed and wild gorilla gut microbiome. The alpha diversity metrics for ZHG's (red) and four groups of WG's, including two habituated groups H1 (olive green), H2 (green) and two unhabituated groups U1 (blue) and U2 (purple). The diversity indices include **A)** Chao1, **B)** Shannon, **C)** abundance-based coverage estimators index (ACE), and **D)** Fisher. Metrics were calculated on rarefied data to account for differences in sequence depths between groups. The indicators of significance levels: **** = $p < 0.0001$, *** = $p <$

0.001, ** = $p < 0.01$, * = $p < 0.05$ were based on Wilcoxon rank sum tests with Benjamini-Hochberg correction for multiple testing.

Characteristic members of the wild gorilla microbiota are lost in zoo-housed gorillas

The taxonomic composition of WG and ZHG samples is portrayed in the heat trees in Figure 3. In heat trees size and color of nodes and edges are correlated with the abundance of organisms in each community (Foster et al., 2017).WG samples were dominated by Firmicutes (mean = 38.0%), Bacteroidetes (mean = 23.5%), Actinobacteria (mean = 14.4%), unclassified bacteria (mean = 13.6%) Chloroflexi (mean = 5.8%) and Spirochaetes (mean = 1.82%). Dominance of these five phyla was also found in a study of 87 western lowland gorillas (Hicks et al., 2018), where Firmicutes and Bacteroidetes dominate the microbiome, followed by Chloroflexi, Spirochaetes and Actinobacteria. Chloroflexi were previously found to be characteristic of the gorilla microbiome, degrading carbohydrates of high-fiber fallback foods consumed during dry seasons (Hicks et al., 2018). At phylum level, the ARTIS gorilla microbiome is dominated by Firmicutes (71.7%), Bacteroidetes (15.7%) and Actinobacteria (7.9%). Chloroflexi species are not found in the ZHG samples at all after removal of singletons. Also, Spirochaetes, to which the genus *Treponema* belongs, are marker species in the native gorilla microbiome, being the fourth most dominant phylum in the microbiome of western lowland gorillas and chimpanzees, but not found in western humans. In a previous study, they were found to be characteristic of the gorilla microbiome during wet periods, when fruit availability is high (Hicks et al., 2018). Spirochaetes are conserved in the ZHG microbiome (mean Spirochaetes WG = 1.8%, mean Spirochaetes ZHG = 1.6%, mean *Treponema* WG 1.4%, mean *Treponema* ZHG = 0.86%). At genus level the wild microbiome is dominated by *Olsenella* (mean WG = 10.9%). But like Chloroflexi, the genus *Olsenella* (phylum Actinobacteria) is lost in the zoo-housed microbiome. Notably, *Bifidobacterium*, a well-known bacterial genus in the human gut, belonging to the same phylum of Actinobacteria, has strongly increased in ZHG (mean = 1%). *Lactobacillus* (mean = 15.0%) is the foremost dominant genus in ZHG samples. Most abundant *Lactobacillus* species are *Lactobacillus acidophilus* (mean = 6.2%), followed by *Lactobacillus gorillae* (4.4%) and *Lactobacillus animalis* (1.3%). The genus *Lactobacillu*s only contributes 0.27% in WG samples. An overview of the top 10 genera in both ZHG and WG samples is given in Supplemental Table S1.


A
Bacteria
Firmicutes
Bacteroidetes
Bacteroidia
Bacteroidales
Clostridia
Clostridiales
Ruminococcaceae
Lachnospiraceae
Nodes
n_obs
1.0
12.9
38.2
77.0
129.0
195.0
274.0


B
Bacteria
Firmicutes
Bacteroidetes
Bacteroidia
Bacteroidales
Clostridia
Clostridiales
Lachnospiraceae
Ruminococcaceae
Clostridiaceae
Lactobacillales
Lactobacillaceae
Nodes
n_obs
1.0
17.9
55.7
114.0
194.0
295.0
416.0

Altered composition and substantial fungal and archaeal activity in the zoo-housed gorilla gut microbiome

We proceeded our investigation with a metagenomics (MG) and metatranscriptomics (MT) analysis of four new samples from two time points of the dominant male of the ARTIS gorillas. We used Kraken/Braken2 to determine the taxonomic origin of metagenomic and metatranscriptomics reads. Bacteria accounted for 98.9% of the gorilla gut metagenome. Eukaryotes (0.07%) Archaea (0.4%) and viruses (0.01 %) thus together accounted for roughly 1% of the remaining genetic material in the gorilla gut. Interestingly, in the gut metatranscriptome only 60.1% of transcripts was of bacterial origin. Eukaryotic transcripts made up 20.1% of the metatranscriptome, and 19.3% of transcripts originated from Archaea. A remaining 0.03% of transcripts was attributed to viruses in MT samples. The taxonomy assignment to MG and MT samples is in accordance with the 16s rRNA amplicon sequencing results. Firmicutes and Bacteroides dominate the bacterial microbiome. Based on the 16S rRNA sequencing results, the phylum Chloroflexi and the genus *Olsenella* are considered lost in the zoo-housed microbiome. Chloroflexi contributed to 0.13% of MG reads and 0.03% of MT reads according to the Kraken2/Bracken taxonomy assignment. To *Olsenella,* 0.13% of MG and 0.01% of MT reads were assigned. The taxonomy assignment thus confirmed the reduced abundance of Chloroflexi and *Olsenella*. The true abundances of these taxa in the microbiome probably fluctuate around the methodological detection limit (reads assigned to Chloroflexi and *Olsenella* were present in the 16s rRNA data before removal of rare OTUs, see methods). The high relative abundance of *Lactobacillus* is reflected in the MT and MG samples: 10.5% of MG reads was assigned to *Lactobacillus*, as was 14.6% of MT reads.

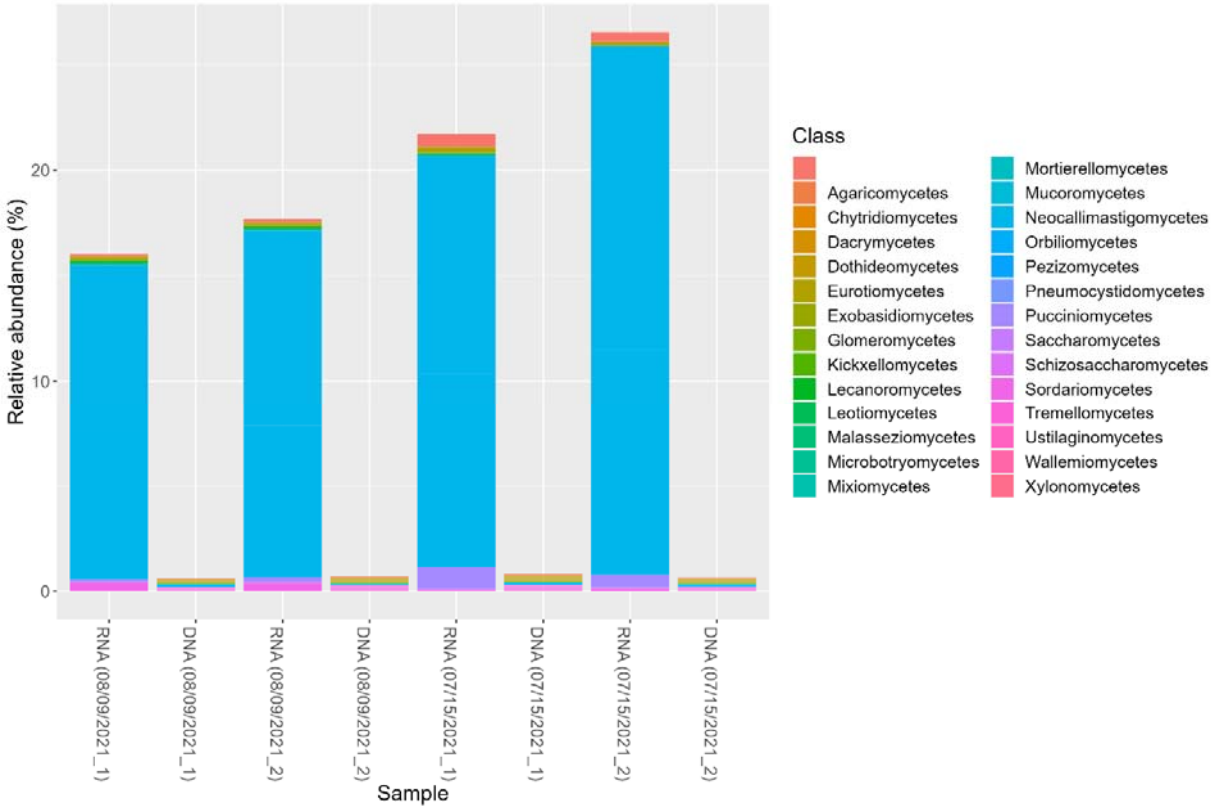


Figure 4 – Relative abundance of eukaryotic DNA and RNA reads originating from the gut microbiome of a zoo-housed gorilla. Relative abundances of DNA and RNA reads assigned to eukaryotic classes, obtained through shotgun metagenomics and RNA-seq on the same fecal samples.

Nearly all eukaryotic transcripts (Figure 4) were assigned to Neocallimastigomycetes, a class of anerobic gut fungi that has been identified before in the rumen and gut of other herbivores (95% of eukaryotic RNA reads, 19.0% of the total metatranscriptome, 0.07% of the total metagenome). The remaining 5% of eukaryotic transcripts (1.1% of the total metatranscriptome) are known to be found in soil or on plants, like the pathogenic genus *Melamspora*, which infects the *Salix* species (willow tree) that are consumed by the ZHG's. These are thus not considered true members of the gut microbiome, but most likely originated from the passage of food through the gastrointestinal tract.

A total of 10.9% of MT reads originated from Thermoplasmata and 8.3% of all MT reads originated from Methanobacteria. Relative to their genetic abundance in the gut metagenome (0.4%), Archaea were overrepresented in the transcriptome (19.3%). Methanogenic archaea are known symbionts of Neocallimastigomycetes, which produce hydrogen utilized by methanogens (Bauchop & Mountfort, 1981; Li et al., 2021), or live in symbiosis with hydrogen forming gut bacteria (Ma et al., 2020). The metatranscriptome analysis elucidated the substantial contribution of anaerobic fungi and archaea to the activity of the gut microbiome, together accounting for 38.3% of the assignable metatranscriptome.

The gorilla microbiome harbors an extensive repertoire of carbohydrate active enzymes

Shifting our perspective from a taxonomic to a functional one, we characterized the carbohydrate active enzyme repertoire of the ZHG gut microbiome. We utilized the classification of the Carbohydrate Active enzyme database (CAZyDB), which provides 'a sequence-based family classification linking the sequence to the specificity and 3D structure of the enzymes that assemble, modify and breakdown oligo- and polysaccharides' [30]. We identified genes and transcripts belonging to a total of 268 different CAZyme families. The CAZyDB classifies enzymes in six superfamilies (Supplemental Table S2). Most CAZymes identified in the gorilla gut metagenome (MG) are glycoside hydrolases (GH, 53.7%). The percentual contribution of each GH family to the total of GH families in the CAZy metagenome and metatranscriptome has been depicted in Supplemental Figure S3. Percentages mentioned in text below represent the percentual contribution of each family to the total CAZyome. Cellulases and hemicellulases were not found among abundant GH families in the metagenome that make up more than 2% of the metagenome. GH13 (4.6%) is involved in the breakdown of starch. GH2 (3.2%), GH3 (3.2%), GH20 (1.9%), GH1 (1.8%), GH29 (1.6%), GH43 (1.5%), GH92 (1.5%) and GH97 (1.3%) all contain oligosaccharide-degrading enzymes. GH78 (1.7%) and GH23 (1.7%) are debranching enzymes. In the metatranscriptome however, cellulase and hemicellulases are observed within the abundant GH families (Figure 5). We refer to 'abundant' in case of more than 2% of all GH families in the metatranscriptome. Families GH5 (3.5% MT, 0.98% MG), GH9 (2.1% MT, 0.3% MG), GH48 (2.1% MG, 0.01% MT) and GH6 (1.7% MT, not detected in MG) contain cellulases. GH11 (2.6% MG, 0.01% MT) and GH10 (1.4% MT, 0.3% MG) contain endo-hemicellulases. Abundant GH families in the metatranscriptome which are involved in the degradation of oligosaccharides are GH1 (4.6% MT, 1.8% MG), GH18 (1.9% MT, 0.5% MG). GH57 (1.1% MT, 0.5% MG). GH77 (3.1% MT, 0.9% MG) contains debranching enzymes.

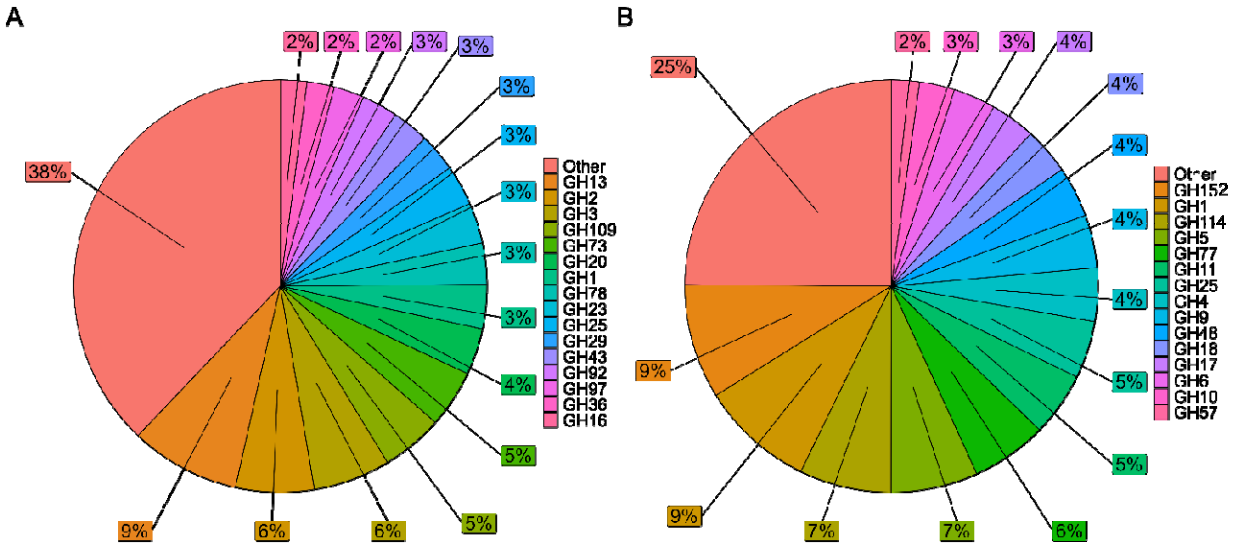


Figure 5 Glycoside hydrolyase families in the gut metagenome and metatranscriptome of a zoo-housed gorilla. Percentages represent the percentual contribution of each glycoside hydrolase (GH) family to the total number of GH families found by **A)** shotgun metagenomics (DNA) and **B)** metatranscriptomics or RNA-seq (mRNA) on fecal samples of a ZHG.

Mucin degradation by gut microbes requires the expression of GH33 (sialidases) to access mucin glycans (Glover et al., 2022). A total of 0.84% of the metagenomic CAZyome is accounted for by GH33, but no transcripts are observed. We thus observe no direct producer of mucin degrading activity in the ZHG gut microbiome at the moment of sampling. Other GH families known to be involved in mucin degradation are GH16, GH29, GH95, GH20, GH2, GH35, GH42, GH98, GH101, GH129, GH89, GH85, and GH84 (Glover et al., 2022), but these are involved in the general degradation of oligosaccharides, and their presence cannot be considered direct evidence for mucin degrading activity. Glycosyltransferases (GT) contribute to 32.0% of the MG CAZyome and 20.8% in the MT CAZYome, but are not considered further here, since they are involved in polysaccharide synthesis instead of their breakdown. Carbohydrate esterase (CE) families aid the deacetylation of plant polysaccharides (9.9% MT, 11.5% MG). Esterification of plant polysaccharides is a defense mechanism against breakdown by microorganisms, and without the aid of CE families, GH families cannot access the plant polysaccharides [44]. The most abundant and active CE family is CE4, which catalyzes the de- acylation of several polysaccharides (2.4% MG ,7.0% MT) (Supplemental Figure S4). Carbohydrate binding modules (CBM) target their substrate (mostly cellulose and hemicellulose) and promote prolonged interaction, thereby potentiating the enzymatic activities of other CAZymes. CBMs contribute 3.7% to the MG CAZyome and 10.3% to the MT CAZYome. Most activity is observed from CBM family 22, which mainly acts on xylan, but some members can also bind with lower affinities to β-1,3/β-1,4-glucans. CBM48 specifically binds to starch, and is appended to family GH13, the most abundant GH family in the metagenome (Supplemental Figure S5). The class of Auxiliary Activities enzymes (AA, 0.3% MG, 2.8% MT) contains catalytic enzymes potentially involved in plant cell degradation through an ability to aid GH, PL and CE enzymes gain access to the carbohydrates of the plant cell wall. PL families are responsible for cleavage of the glycosidic bonds of uronic acid-containing polysaccharides by a β-elimination mechanism (0.8% MG, 0.6% PL). S-layer homology (SLH) modules are part of the scaffolding subunit of a cellulosome (0.3% MG, 0.3% MT).

Notably, the MT CAZyome does not predict the activity of CAZymes in the MG samples. No (hemi)cellulases were found amongst the abundant GH families in MG samples, but the metatranscriptome indicated they are amongst the most active families of the CAZyome. The most represented GH family in the metagenome, GH13 (starch degrading), only contributed to 0.96% in the total MT CAZyome. Based on a metagenome analysis alone, we would have underestimated the (hemi)cellulase activity of the zoo-housed microbiome, just as we would have underestimated the contribution of anaerobic fungi and archaea. Presence and activity of debranching enzymes and oligosaccharide active enzymes allow for further consumption of degraded plant material by hemicellulases, or breakdown of oligosaccharides directly available in the diet. Interestingly, for families like GH6 or GH48, none or extremely little MG reads were found, but significant levels of MT reads were detected. We further investigated two of the cellulase families for which this is the case, GH6 and GH48, with ShortBRED, a system for profiling protein families of interest at very high specificity in shotgun metaomic sequencing data (Kaminski et al., 2015).

The cellulase families GH48 and GH6 originate from anaerobic gut fungi

The family of GH48 contains cellulases and its transcripts were detected, but at very low quantity in the metagenome. GH6 was found in MT samples but was not detected at all in MG samples. From the 1405 known GH48 DNA sequences 110 corresponding protein sequences are deposited in UniProt, of which we detected eight using SHORTBRED in our MT data (Supplemental Table S3), and none in the MG data. Within the GH48 family we detected two transcripts of presumed fungal origin. H2BPU6, a putative cellulase of *Neocallimastix patriciarum*, has been detected previously in the rumen and functionally described by transcriptomic analysis (Li et al., 2014). The latter, Q8J1E3, encodes a Cellulase Cel48A protein and originates from *Piromyces* sp. strain E2, isolated from the gut of an Indian elephant. Cellulase 48 is the major cellulosome component of *Piromyces* (Steenbakkers et al., 2002). Both these species are members of the class Neocallimagistomycotes which was shown earlier to account for 19.0% of taxonomically assignable transcripts. The other GH48 transcripts found with ShortBRED originated from an uncultured actinobacterium from soil and uncultured bacteria from the rumen. From GH6, a cellobiohydrolase first found in *Piromyces rhizinflatus* was detected.

Hydrogenosomal activity and symbiosis with methanogens

Characteristic for the Neocallimagistomycotes is that they possess hydrogenosomes instead of mitochondria (Czajkowski et al., 2020). Malic enzyme (ME) is considered a marker for hydrogenosomal activity [48]. From the sixteen malic enzyme proteins attributed to Neocallimagistomycotes in UniProt, transcripts from ME of *Neocallimastix californiae* and *Piromyces sp.* (strain E2) were detected (A0A1Y2CXE5 in all samples and A0A1Y3NKG2 in three out of four samples). Other proteins involved in hydrogenosomal metabolism are succinate—CoA ligase subunit alpha (Q7Z941, originating from *Neocallimastix patriciarum*, with the beta subunit being detected in two out of four samples from another genome (P53587, *Neocallimastix frontalis*). Also expected to be in the hydrogenosome are homoacotinase, Aconitate hydratase, NADH dehydrogenase [ubiquinone] flavoprotein 1, Arg5,6 arginine biosynthetic enzyme, Alanine-tRNA ligase, Dynamin-type G domain-containing protein and MSF1-domain-containing protein. An overview of these results is given in Supplemental Table S4.

From the scientific literature it is known that the hydrogen produced in the hydrogenosome of anaerobic gut fungi is converted to methane by methanogenic archaea (Li et al., 2021). Methyl-coenzyme M reductase (MCR) catalyzes the final step in methanogenesis and is considered a marker gene for methanogenesis (Chen et al., 2020). The alpha, beta, gamma, C and D subunit of the MCR gene annotated as COG ids (COG4058, COG4054, COG4057, COG4056 and COG4055, respectively). CPM values of the collective of these COG IDs

were 14 times to 145 times as high in the metatranscriptome as in the corresponding metagenome samples, indicating an overrepresentation of methanogenic activity in MT samples, in line with the Kraken2/Bracken taxonomy assignment. For methanogenesis, abundance in the metagenome thus does not reflect contribution in activity. Our data indicates that anaerobic fungi and their symbiotic methanogens contribute substantially to enzymatic activity in the ZHG gut microbiome (Figure 6).

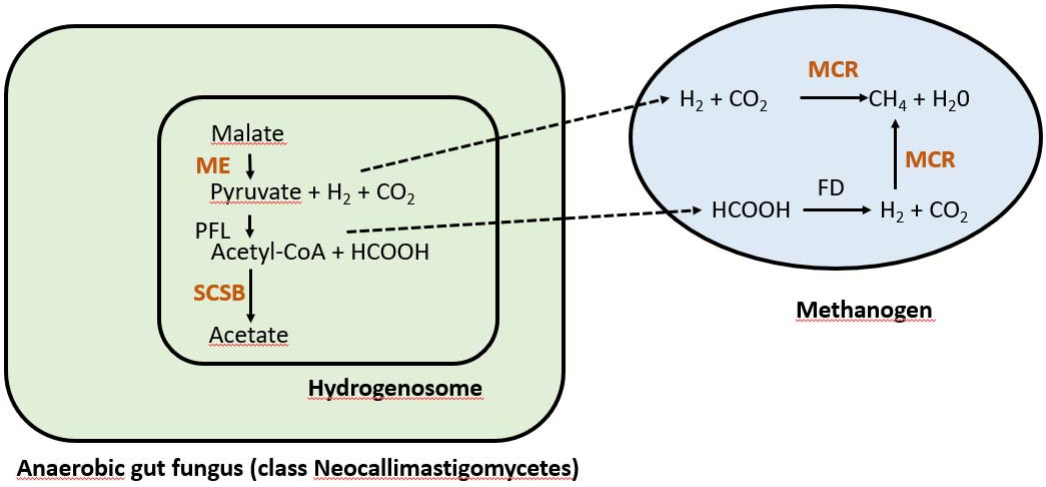


Figure 6. Hydrogenosome-methanogenesis pathway interaction between anaerobic fungi and methanogens. The hydrogen and formate produced by anaerobic fungi when degrading plant polysaccharides is used by methanogens to produce methane. Enzymes for which transcripts were detected are indicated in red: ME, Malic Enzyme; SCSB, Subunit B of Succinyl-CoA Synthetase; MCR, Methyl-coenzyme M reductase. Figure adapted from Li et al. (2016).

Discussion

In this study, we determined the microbiome composition of a population of zoo-housed Western lowland gorillas through 16S rRNA V3-V4 amplicon sequencing. Their gut microbiome composition shows a decreased alpha diversity in terms of species richness compared to previously obtained data from wild western lowland gorillas, as indicated by rarefaction and a significantly lower Chao1 estimator, ACE, and Fisher diversity index. Additionally, we observed a decreased beta diversity for the ARTIS gorilla population compared to groups of WG's. As observed in previous studies on the primate zoo-housed microbiome, we found characteristic members of the native gorilla microbiome were lost. The phylum Chloroflexi and the genus *Olsenella* are reduced to a relative abundance around the methodological detection limit in the ZHG microbiome, whilst they are the third most abundant phylum and most abundant genus in the WG microbiome. Correlation of these species to consumption of high-fiber foods may explain this difference (Davenport et al., 2014; Hicks et al., 2018). A striking observation is the emergence of high numbers of *Lactobacillus* species in the microbiome of the ZHG's. Although *Lactobacillus* is known to colonize the mammalian gut, they are highly abundant compared to WG data. An anatomical explanation may be given here: gorillas share a similar gut anatomy to other monogastric herbivores. Typical for this gut anatomy type is a simple stomach of which the proximal part consists of non-glandular squamous cell epithelium. In horses, it has been shown that *Lactobacillus* species can colonize this area of the stomach and form biofilm-like structures (Yuki et al., 2000). Although this phenomenon has not been studied or observed before in gorillas, based on the similarity in anatomy of the gorilla and monogastric herbivore stomach, we hypothesize that the stomach of gorillas may be susceptible to colonization by *Lactobacillus* species as well. Colonization of the non-glandular part of the stomach is believed to be beneficial to the host by preventing colonization of pathogenic microorganisms (Yuki et al., 2000). This hypothesis of *Lactobacillus* colonization of the gorilla stomach would clarify the presence of these *Lactobacillus* species in our compositional data but does not explain why relative abundances are so high compared to WG compositional data. Again, diet most likely explains this observation. Whilst the diet of WG's almost exclusively consists of high fiber fruits and plant material, the diet of ZHG's inevitably contains higher levels of oligosaccharides (Gänzle et al., 2012). These are mainly available in the upper part of the mammalian intestinal tract (Gänzle et al., 2012). The summed effect of easy adhesion to the upper intestinal epithelium and a large availability of oligosaccharides in this part of the gastrointestinal tract may explain why *Lactobacillus* species can flourish in the intestinal tract of ZHG's. However, based on relative abundances alone we cannot draw confident conclusions on the absolute abundances and distribution of microbes over the gastrointestinal tract.

An important limitation in our comparison of zoo-housed compositional data with that of WG's is the large methodological differences between the datasets. Datasets are obtained with different sequencing platforms, sequenced a different amplicon, and are mapped against the taxonomic data at different moments in time. We therefore did not perform any differential abundance analysis by statistical testing and aimed to interpret the composition of zoo-housed and gorilla microbiomes as independent datasets. The acquisition of 16S rRNA data or shotgun metagenomics and RNA-seq data for both ZHG's and WG's would greatly strengthen the comparisons made in this study but is outside the scope of this project and challenging in general.

By annotation of the metagenomic and metatranscriptomics CAZyome the, we identified 268 distinct Carbohydrate Active enzyme (CAZyme) families. None of the abundant GH families in the metagenome contain cellulases or hemicellulases. Instead, starch or other oligosaccharide degrading enzymes and debranching enzymes are the most represented GH families in the metagenome. The metatranscriptome however reveals the activity of multiple cellulases and hemicellulases, next to these oligosaccharide-degrading enzymes and debranching enzymes. From a methodological point of view, it is important to note that, had we only resorted to metagenomics for functional profiling, we would have concluded that the ZHG microbiome is hardly displaying potential for cellulose and hemicellulose degradation. However, metatranscriptomics reveals the activity of cellulases and hemicellulases, and that of Neocallimastigomycetes and methanogens in the gorilla microbiome, which we would not have identified or largely underestimated by metagenomics alone. A first possible explanation for this difference is that due to the short half-lives of mRNA, transcripts active in the upper intestinal tract may be less represented in the metatranscriptome than those in the hindgut. Since oligosaccharides are mainly digested in the upper intestinal tract, this may explain the overrepresentation of oligosaccharide-degrading enzymes in the metagenome compared to the metatranscriptome. A second explanation could be that part of the (hemi)cellulases are of eukaryotic origin. Interestingly, activity of at least two families of cellulases (GH6 and GH48) can be attributed to members of the Neocallimastigomycetes, who are underrepresented (0.07%) in the metagenome, but who contribute 19.0% to the total metatranscriptome. Splicing effects may explain the lack of detection of eukaryotic genes in the metagenome, since methods for metagenomics analysis are tailored towards the bacterial microbiome, and thus do not account for introns and exons in assembly and contig annotation or lack reference genomes from eukaryotes in their databases(Lind & Pollard, 2021). We presume this is the reason no shotgun metagenomics studies find or mention the presence of Neocallimastigomycetes in the gorilla microbiome (Campbell et al., 2020; Hicks et al., 2018). In fact, only one previous study utilizing ITS1 sequencing discusses the presence of Neocallimastigomycetes in the gorilla gut (Schulz et al., 2018). The authors of this study recognized that anaerobic fungi are well known members of the ruminant in the intestinal tract and known to be essential in the breakdown of plant material (Czajkowski et al., 2020; Gruninger et al., 2014). ITS1 sequencing results subsequently suggested that these fungi were indeed members of the gorilla microbiome (Schulz et al., 2018). Later studies all utilize 16s rRNA amplicon sequencing (which does not detect eukaryotes) or shotgun metagenomics and do not mention these anaerobic gut fungi again, probably due to the earlier mentioned methodological limitations. Similarly, in our shotgun metagenomics data, all analyses except for the taxonomy assignment with Kraken2/Bracken did not detect these anaerobic fungi at all. Our RNA-seq results however show that they are highly active in the gorilla gut, and that they contribute to cellulase activity. Fungal isolates from the rumen microbiome were found to degrade about 70% of dried leaf blades and stems in *in vitro* digestion studies (Akin et al., 1990). Neocallimastigomycetes may thus be major players in the degradation of plant cell wall material by the gorilla microbiome and are preserved in the zoo-housed microbiome. Isolation of Neocallimastigomycetes and novel cellulases from the gorilla gut microbiome could be of great biotechnological interest for biomass degradation. Their polysaccharide-degrading efficiency is highest when in symbiosis with methanogenic archaea (Bauchop & Mountfort, 1981), which are also overrepresented in the metatranscriptome compared to the metagenome. In human microbiome data it has been found as well that metagenome analysis vastly underestimates methanogenesis, when compared to metatranscriptome data (Franzosa et al., 2014, 2018). It should be noted that not all methanogenic activity found in our data originates from methanogens that are symbionts to

anaerobic fungi, since these methanogens can also be symbionts to methane producing bacteria of the gut microbiome. Based on our analyses, we cannot quantify the bacterial and fungal contributions of hydrogen production. It has however been found that anaerobic fungi and methanogens together degrade polysaccharides at a higher rate than a consortium of bacteria and methanogens (Ma et al., 2020), again underlining the biotechnological potential of Neocallimastigomycetes. Our study shows how metatranscriptomics can uncover the contribution of eukaryotes and archaea in the herbivore gut microbiome. Ideally, future research would include metatranscriptomics data of WG microbiomes, although we recognize that this is challenging, since fecal samples have to be collected as soon as possible after defecation due to the short lifetime of RNA. Alternatively, shotgun metagenomics analysis workflows should be further improved to detect eukaryotic material as well as prokaryotic genes. In conclusion, the ZHG microbiome is altered with regards to diversity, composition, and expectedly function, but hemi(cellulases), other carbohydrate active enzymes targeting polysaccharides and enzymes originating from Neocallimastigomycetes are still active in the zoo-housed microbiome. Key species belonging to Chloroflexi and *Olsenella* are decreased in the ZHG microbiome but persist at low abundances, and herbivore-characteristic activity of the microbiome is conserved. These results are promising considering conservation efforts and ask for further investigations to 'rewild' the zoo- housed gorilla microbiome. Our study highlights the contribution of eukaryotes and archaea to the gorilla gut microbiome, thereby showing the added value of RNA-seq and the importance of further improvements of metagenomics analysis pipelines for detection of eukaryotic genes in shotgun metagenomics data.

Funding

This research project was carried with internal funding from the ARTIS Heritage, Education and Academy team.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank all gorilla care takers at ARTIS Amsterdam Royal Zoo for their cooperation and collection of fecal samples. The authors gratefully acknowledge Yaro Laenen and Jasper Buikx from ARTIS-Micropia, and Eline Klaassens and Will Harley from BaseClear for their support to this research project.

Author contributions

Conceptualization, I.H. and R.K.; Methodology and data analysis, I.H., M.v.Z.L., M.B., W.P., R.K; Experiments: M.v.Z.L., R.K.; data curation, I.H., Writing -original draft preparation, I.H.; Writing - review and editing R.K.; Supervision, R.K. All authors have read and agreed to the published version of the manuscript.

References

Akin, D. E., Borneman, W. S., & Lyon, C. E. (1990). Degradation of leaf blades and stems by monocentric and polycentric isolates of ruminal fungi. *Animal Feed Science and Technology, 31*(3–4), 205–221. https://doi.org/10.1016/0377-8401(90)90125-r

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. *URL http://www.bioinformatics.babraham.ac.uk/projects/fastqc*

Bauchop, T., & Mountfort, D. O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Applied and Environmental Microbiology, 42*(6), 1103–1110. https://doi.org/10.1128/aem.42.6.1103-1110.1981

Campbell, T. P., Sun, X., Patel, V. H., Sanz, C., Morgan, D., & Dantas, G. (2020). The microbiome and resistome of chimpanzees, gorillas, and humans across host lifestyle and geography. *The ISME Journal, 14*(6), 1584. https://doi.org/10.1038/S41396-020-0634-2

Chen, H., Gan, Q., & Fan, C. (2020). Methyl-coenzyme M reductase and its post-translational modifications. *Frontiers in Microbiology, 11*, 578356. https://doi.org/10.3389/fmicb.2020.578356

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics, 34*(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Clayton, J. B., Gomez, A., Amato, K., Knights, D., Travis, D. A., Blekhman, R., Knight, R., Leigh, S., Stumpf, R., Wolf, T., Glander, K. E., Cabana, F., & Johnson, T. J. (2018). The gut microbiome of nonhuman primates: Lessons in ecology and evolution. *American Journal of Primatology, 80*(6). https://doi.org/10.1002/ajp.22867

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., Travis, D. A., Long, H. T., van Tuan, B., van Minh, V., Cabana, F., Nadler, T., Toddes, B., Murphy, T., Glander, K. E., Johnson, T. J., & Knights, D. (2016). Captivity humanizes the primate microbiome. *Proceedings of the National Academy of Sciences of the United States of America, 113*(37), 10376–10381. https://doi.org/10.1073/pnas.1521835113

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., & Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research, 42*(Database issue). https://doi.org/10.1093/nar/gkt1244

Czajkowski, R., Elshahed, M. S., Singh, B., Hess, M., Edwards, J. E., Paul, S. S., Puniya, A. K., van der Giezen, M., Shaw, C., & Fliegerová, K. (2020). *Anaerobic Fungi: Past, Present, and Future*. https://doi.org/10.3389/fmicb.2020.584893

Davenport, E. R., Mizrahi-Man, O., Michelini, K., Barreiro, L. B., Ober, C., & Gilad, Y. (2014). Seasonal Variation in Human Gut Microbiome Composition. *PLOS ONE, 9*(3), e90731. https://doi.org/10.1371/journal.pone.0090731

di Tommaso, P., Chatzou, M., Floden, E. W., Barja, P. P., Palumbo, E., & Notredame, C. (2017). Nextflow enables reproducible computational workflows. *Nature Biotechnology, 35*(4), 316–319. https://doi.org/10.1038/nbt.3820

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science, 14*(6), 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England), 29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Edgar, R. C., & Bateman, A. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Foster, Z. S. L., Sharpton, T. J., & Grünwald, N. J. (2017). Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. *PLoS Computational Biology*, *13*(2). https://doi.org/10.1371/journal.pcbi.1005404

Franzosa, E. A., McIver, L. J., Rahnavard, G., Thompson, L. R., Schirmer, M., Weingart, G., Lipson, K. S., Knight, R., Caporaso, J. G., Segata, N., & Huttenhower, C. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nature Methods*, *15*(11), 962–968. https://doi.org/10.1038/s41592-018-0176-y

Franzosa, E. A., Morgan, X. C., Segata, N., Waldron, L., Reyes, J., Earl, A. M., Giannoukos, G., Boylan, M. R., Ciulla, D., Gevers, D., Izard, J., Garrett, W. S., Chan, A. T., & Huttenhower, C. (2014). Relating the metatranscriptome and metagenome of the human gut. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(22). https://doi.org/10.1073/pnas.1319284111

Fu, L., Niu, B., Zhu, Z., Wu, S., & Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*, *28*(23), 3150. https://doi.org/10.1093/bioinformatics/bts565

Gänzle, M. G., Follador, R., & van Derlinden, E. (2012). Metabolism of oligosaccharides and starch in lactobacilli: a review. *Frontiers in microbiology*, 3, 340.https://doi.org/10.3389/fmicb.2012.00340

Glover, J. S., Ticer, T. D., & Engevik, M. A. (2022). Characterizing the mucin-degrading capacity of the human gut microbiota. *Scientific Reports 2022 12:1*, *12*(1), 1–14. https://doi.org/10.1038/s41598-022-11819-z

Gomez, A. (2014). *The gut microbiome of the western lowland gorilla (Gorilla gorilla gorilla): implications for overall ecology.* PhD-thesis. University of Illinois at Urbana-Champaign, Champaign, IL, USA.

Gomez, A., Petrzelkova, K., Yeoman, C. J., Vlckova, K., Mrázek, J., Koppova, I., Carbonero, F., Ulanov, A., Modry, D., Todd, A., Torralba, M., Nelson, K. E., Gaskins, H. R., Wilson, B., Stumpf, R. M., White, B. A., & Leigh, S. R. (2015). Gut microbiome composition and metabolomic profiles of wild western lowland gorillas (*Gorilla gorilla gorilla*) reflect host ecology. *Molecular Ecology*, *24*(10), 2551–2565. https://doi.org/10.1111/ mec.13181

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S. S., Fliegerova, K., Griffith, G. W., Forster, R., Tsang, A., Mcallister, T., & Elshahed, M. S. (2014). Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiology Ecology*, *90*(1), 1–17. https://doi.org/10.1111/1574-6941.12383

Hicks, A. L., Lee, K. J., Couto-Rodriguez, M., Patel, J., Sinha, R., Guo, C., Olson, S. H., Seimon, A., Seimon, T. A., Ondzie, A. U., Karesh, W. B., Reed, P., Cameron, K. N., Lipkin, W. I., & Williams, B. L. (2018). Gut microbiomes of wild great apes fluctuate seasonally in response to diet. *Nature Communications 2018 9:1*, *9*(1), 1–18. https://doi.org/10.1038/s41467-018-04204-w

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., & Huerta-Cepas, J. (2021). eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Molecular biology and evolution*, *38*(12), 5825-5829.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, L. J., von Mering, C., & Bork, P. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research*, *47*(D1), D309–D314. https://doi.org/10.1093/nar/gky1085

Houtkamp, I. (2022). Gorilla, Github repository, https://github.com/imhoutkamp/Gorilla

Kaminski, J., Gibson, M. K., Franzosa, E. A., Segata, N., Dantas, G., & Huttenhower, C. (2015). High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. *PLOS Computational Biology*, *11*(12), e1004557. https://doi.org/10.1371/journal.pcbi.1004557

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1). https://doi.org/10.1093/nar/gks808

Kopylova, E., Noé, L., & Touzet, H. (2012). SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*, *28*(24), 3211–3217. https://doi.org/10.1093/bioinformatics/bts611

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Li, D., Liu, C. M., Luo, R., Sadakane, K., & Lam, T. W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, *31*(10), 1674-1676.https://doi.org/10.1093/bioinformatics/btv033

Li, J., Su, X., Tian, Y., Dong, Z., Hu, S., Huang, L., & Dai, X. (2014). Gene diversity of the bacterial 48 family glycoside hydrolase (GH48) in rumen environment. *Wei Sheng Wu Xue Bao = Acta Microbiologica Sinica*, *54*(1), 53–61. https://europepmc.org/article/med/24783854

Li, Y., Jin, W., Cheng, Y., & Zhu, W. (2016). Effect of the Associated Methanogen Methanobrevibacter thaueri on the Dynamic Profile of End and Intermediate Metabolites of Anaerobic Fungus Piromyces sp. F1. *Current Microbiology*, *73*(3), 434–441. https://doi.org/10.1007/S00284-016-1078-9

Li, Y., Meng, Z., Xu, Y., Shi, Q., Ma, Y., Aung, M., Cheng, Y., & Zhu, W. (2021). Interactions between Anaerobic Fungi and Methanogens in the Rumen and Their Biotechnological Potential in Biogas Production from Lignocellulosic Materials. *Microorganisms 2021, Vol. 9, Page 190*, *9*(1), 190. https://doi.org/10.3390/microorganisms9010190

Lind, A. L., & Pollard, K. S. (2021). Accurate and sensitive detection of microbial eukaryotes from whole metagenome shotgun sequencing. *Microbiome*, *9*(1). https://doi.org/10.1186/s40168-021-01015-y

Ma, Y., Li, Y., Li, Y., Cheng, Y., & Zhu, W. (2020). The enrichment of anaerobic fungi and methanogens showed higher lignocellulose degrading and methane producing ability than that of bacteria and methanogens. *World Journal of Microbiology and Biotechnology*, *36*(9). https://doi.org/10.1007/S11274-020-02894-3

Masi, S. (2011). Differences in gorilla nettle-feeding between captivity and the wild: Local traditions, species typical behaviors or merely the result of nutritional deficiencies? *Animal Cognition*, *14*(6), 921–925. https://doi.org/10.1007/s10071-011-0457-7/figures/1

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. v., Lonsdorf, E. v., Muller, M. N., Pusey, A. E., Peeters, M., Hahn, B. H., & Ochman, H. (2016). Cospeciation of gut microbiota with hominids. *Science*, *353*(6297), 380–382. https://doi.org/10.1126/science.aaf3951

Moeller, A. H., & Sanders, J. G. (2020). Roles of the gut microbiota in the adaptive evolution of mammalian species. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1808), 20190597. https://doi.org/10.1098/rstb.2019.0597

Nishida, A. H., & Ochman, H. (2021). Captivity and the co-diversification of great ape microbiomes. *Nature Communications 2021 12:1*, *12*(1), 1–10. https://doi.org/10.1038/s41467-021-25732-y

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Schulz, D., Qablan, M. A., Profousova-Psenkova, I., Vallo, P., Fuh, T., Modry, D., Piel, A. K., Stewart, F., Petrzelkova, K. J., & Fliegerová, K. (2018). Anaerobic Fungi in Gorilla (Gorilla gorilla gorilla) Feces: an Adaptation to a High-Fiber Diet? *International Journal of Primatology*, *39*(4), 567–580. https://doi.org/10.1007/S10764-018-0052-8

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sonnenburg, E. D., & Sonnenburg, J. L. (2019a). The ancestral and industrialized gut microbiota and implications for human health. *Nature Reviews Microbiology 2019 17:6*, *17*(6), 383–390. https://doi.org/10.1038/s41579-019-0191-8

Sonnenburg, J. L., & Sonnenburg, E. D. (2019b). Vulnerability of the industrialized microbiota. *Science*, *366*(6464). https://doi.org/10.1126/science.aaw9255

Steenbakkers, P. J. M., Freelove, A., van Cranenbroek, B., Sweegers, B. M. C., Harhangi, H. R., Vogels, G. D., Hazlewood, G. P., Gilbert, H. J., & Op Den Camp, H. J. M. (2002). The major component of the cellulosomes of anaerobic fungi from the genus piromyces is a family 48 glycoside hydrolase. *Mitochondrial DNA*, *13*(6), 313–320. https://doi.org/10.1080/1042517021000024191

Yuki, N., Shimazaki, T., Kushiro, A., Watanabe, K., Uchida, K., Yuyama, T., & Morotomi, M. (2000). Colonization of the Stratified Squamous Epithelium of the Nonsecreting Area of Horse Stomach by Lactobacilli. *Applied and Environmental Microbiology*, *66*(11), 5030. https://doi.org/10.1128/AEM.66.11.5030-5034.2000

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y., & Yin, Y. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research*, *46*(W1), W95–W101. https://doi.org/10.1093/nar/gky418

Zhang, X., Li, L., Butcher, J., Stintzi, A., & Figeys, D. (2019). Advancing functional and translational microbiome research using meta-omics approaches. *Microbiome 2019 7:1*, *7*(1), 1–12. https://doi.org/10.1186/S40168-019-0767-6