Monkey Microbiome Across Sites and Age Groups: Model for Investigating Inaccessible Human Niches

**INTRODUCTION**

The study of the human microbiome has witnessed substantial progress in recent years. With the understanding of microbial composition in various body regions and its association with disease, there is growing interest in investigating the interplay between them. Evidence has shown the extensive occurrence of oral microbes translocating to the gut(1). Studies also revealed the associations between oral-gut microbial translocation with various diseases. Dysbiosis and immune defense caused by oral microbes can result in dysregulation in the gut(2). This oral-gut translocation is also found to be closely linked with conditions such as inflammatory bowel disease (IBD) and colorectal cancer (CRC), where an abnormal enrichment of oral microbes in the gut has been found(3). Thus, a deeper understanding of this oral-gut translocation process could reveal its association with human health and bring therapeutic opportunities.

The problem of lacking spatial resolution exists. So far, human microbiome studies have relied on a limited region of samples, primarily saliva and stool samples. However, they offer incomplete representations of the oral and gut microbiota(4). The microbiota composition can vary significantly at different sites in the mouth and along the GI tract. Moreover, studies have found that the mucosa-associated microbes, because of their proximity to the epithelium, could directly impact gut barrier function and the immune system(5). As a result, the mucosal microbiome could have a great influence on immune function and gut homeostasis. Though the gut luminal and mucosal microbiome are very different, there are few studies focusing on the gut mucosal microbiome. Therefore, how microbes cross the acid barrier, interact with the mucosal surface, and acquire mutations to colonize in specific locations has not been well studied.

Due to inherent limitations posed by the non-invasive collection of samples from human participants, animal models can be utilized for such studies. Non-human primates, being more closely related to humans, could provide a good approach. Here we first validate Rhesus Monkeys as suitable animal models and subsequently adopt them to study microbial translocation more closely. We perform a meta-analysis on the microbial translocation patterns of humans, monkeys, and mice to determine ﻿if that of monkeys resembles humans more. Our studies take samples from different sites along the GI tract, which extensively profile the microbial composition and functionality throughout the whole oral-gut axis. By analyzing the microbial translocation events along the GI tract and their gene functions, our primary aim is to gain an in-depth understanding of the mechanism of microbial translocation and its potential implications for health.

Moreover, we want to further explore the relationship between oral-gut microbial translocation and aging. A noteworthy finding showed shared strains between oral and gut frequently included abundant *Streptococcus salivarius* and *Streptococcus parasanguinis*, whose total relative abundance in the gut was significantly higher in older subjects(3). In light of this, we hypothesized that older individuals may be more susceptible to microbial translocation due to age-related immune changes, potentially compromising the body's barriers against such translocation. To test this, we included both young monkeys and old monkeys in our sample collection, comprehensively profiling the within-host microbiome landscape of Rhesus Monkeys in youth and age.

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**MATERIALS AND METHOD**

**Study Design**

The study was approved by the Fujian Maternity and Children's Health Hospital institutional review board. A total of four Rhesus monkeys (two 8-year-old and two 20-year-old) were selected for the study. The animals were housed and cared for according to ethical guidelines for the treatment of non-human primates, with appropriate veterinary care provided at Fujian Maternity and Children's Health Hospital, Fujian, China. Samples along the GI tract were collected after sacrifice and stored at −80°C.

Human samples are from the study that uses capsules to collect the content of human intestinal samples(4). It collected content from six body sites (saliva, four parts of different pH in intestines, and stool) from 12 patients. Raw FASTQ files were downloaded from NCBI under BioProject PRJNA822660 for ﻿Shalon et al..

**Monkey sample collection**

Post-sacrifice, a series of tissue and content samples were collected. Swabs were taken from the tongue. Tissue and content samples were taken from two sites (4 cm apart) in the esophagus, two sites in the stomach, five sites in the small intestine, and seven sites in the large intestine, with sampling points spaced evenly along the organ's length. Content was collected first, followed by mucosal tissue sampling after washing with PBS. All samples were stored at −80°C immediately after collection to preserve their integrity for downstream analyses.

**DNA extraction and shotgun metagenomics sequencing**

DNA was extracted from fresh frozen luminal contents samples using ﻿MagPure Soil DNA KF Kit (Magen Biotech, Guangzhou, China), followed by library preparations. Inputs were sequenced on the Illumina (please specify model) platform, and paired-end reads were generated. All 70 metagenomic samples provided more than 7x10^7 raw reads (average read 7.5x10^7). The metagenomic raw data were quality-controlled using Kneaddata (v0.12.0, huttenhower.sph.harvard.edu/kneaddata/). Reads mapped to the rhesus monkey database (Feb. 2019, rheMac10, The Genome Institute at Washington University School of Medicine, NCBI Assembly ID: 2345051) were filtered. Among them, 32 samples had less than 5% of the reads left after filtering. The average reads passing filter of all samples are 5.4x10^7.

**2bRAD-M sequencing**

DNA was extracted from fresh frozen mucosal tissue samples using ﻿CretMag Multi Sample DNA Kit (Cret Biotech, Suzhou, China), followed by 2bRAD-M reduced metagenomics sequencing by Qingdao OE Biotech Co., Ltd. (Qingdao, China). Clean reads were generated by removing reads with more than 8% unknown bases and discarding low-quality reads in which over 20% of the bases had a quality score below Q30. Utilizing 2bRAD-M sequencing data, our computational pipeline (https://github.com/shihuang047/2bRAD-M), was employed to characterize species-level microbial communities. This bioinformatics pipeline depends on a pre-existing 2bRAD tag database (2bTagDB), containing species-specific markers derived from microbial genomes obtained from GTDB (R214). We collected air, scissor, forceps, and monkey skin swabs as negative controls. Taxa present at >5% relative abundance in any of these controls were identified as potential contaminants.

**Microbial Profiling**

MetaPhlAn 4 was used to perform species-level metagenomic phylogenetic analysis. A New MetaPhlAn database (v.4.1.0, Feb 20th, 2024) with non-human primates MAGs was used. HUMAnN 3 (v.3.8) was used to profile microbial gene family abundances and pathway abundance. Enriched gene sets (Log fold change > 2) were subjected to Gene Ontology (GO) enrichment analysis using the enricher function (default parameters) from R package clusterProfiler (v.4.16.0).

Kraken2 (v.2.1.2) was also used to perform taxonomic profiling, using “core\_nt” database (released in September 2024). After profiling with Kraken2, species-level abundances were refined using Bracken, which employs a Bayesian model to reassess read assignments (Supplementary table).

2bRAD-M pipeline (https://github.com/shihuang047/2bRAD-M) was also employed to characterize species-level microbial communities. This bioinformatics pipeline depends on a pre-existing 2bRAD tag database (2bTagDB), containing species-specific markers derived from a comprehensive dataset of microbial genomes obtained from GTDB v214. Sequencing reads were electronically digested to fragments containing BcgI recognizing cites. Microbial species were identified for each microbiota using the GTDB, and their relative abundance was estimated by normalizing the sequencing coverage of single-copy, species-specific markers. Specifically, the average read coverage of 2bRAD markers for each species was calculated to reflect the number of individuals from that species in the sample. The relative abundance was then determined by dividing this coverage by the total number of individuals from all identified species within the sample.

**Metagenomic assembly and functional profiling**

Metagenomic samples with reads > 3 million after filtering were assembled with MEGAHIT (v.1.2.9). Bowtie2 (v. 2.5.0) was used for alignment between the reads and the assembled contigs. Assembled contigs were binned with MetaBAT 2 (v.2.12.1) into 4,815 genome bins. CheckM (v.1.2.2) was used to assess quality. Bins with >50% completeness and <10% contamination were selected, resulting in 1,963 representative MAGs across all samples.

Taxonomic classification of each reconstructed genome was conducted using GTDB-Tk (v.2.4.0) with the R220 database release (24th April 2024) via the ‘classify\_wf’ command. ARG annotation for the MAGs was carried out using the DeepARG (v.1.0.2) deep learning algorithm. Identification of secondary metabolite BGCs was achieved through antiSMASH (v.7.0.1). All computational tools were executed with default settings.

**﻿Statistical analysis**

The statistical analysis was conducted with R (v.4.2.2). The profiling results of all monkey and human samples were imported to R in the form of feature tables. Phyloseq package (v.1.42.0) was used for conveniently analyzing the data(6). The feature table was first used for Alpha diversity estimation. We calculated Bray Curtis dissimilarity for beta diversity estimation.

We performed PERMANOVA test based on species-level beta diversity. PERMANOVA was performed in R using the adonis2 function in vegan (v.2.6-4) (7). Principal Coordinate Analysis (PCoA) and Uniform manifold approximation and projection (UMAP) based on the distance metrics were conducted to reduce the dimensionality of microbiome data for better visualization. The resulting first two principal coordinates (i.e., PC1 and PC2) were visualized via a scatter plot using the R package ggplot2(8).

Low-abundance (relative abundance < 0.1%) species were filtered using R package metagMisc (v.0.5.0). Differential abundance analysis was carried out through R package ANCOM (v.2.0.3)(10).

MetaCyc pathway abundance of each body location was compared using Kruskal‐Wallis test. Differential abundance pathways (BH adjusted p < 0.01) were presented on the heatmap using R package pheatmap (v.1.0.12).

**Microbial Load Measurement**

DNA extracted from 0.025 g luminal content sample with an elution volume of 10 ul was sent to qPCR for microbial load quantification. qPCR amplification was performed on a Light-Cycler Roche 480 instrument (Roche Molecular Systems, Switzerland). The reaction system had a total volume of 10μL, including 5μL 2×TB Green II (Takara, Beijing), 0.2μL forward and reverse primers (10μM), 0.2μL 50×Rox, and 1μL template DNA. The absolute abundances of bacteria were determined using the 16S rRNA gene V4-V5, using the primer pair 515F (GTGCCAGCMGCCGCGG) and 907R (CCGTCAATTCMTTTRAGTTT). The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, 72°C for 30 s. All standard curves were prepared by a 10-fold gradient dilution method for plasmid DNA, and the R2 values of the standard curves of bacteria were greater than 0.997. After qPCR, amplification and melting curves were examined to ensure quality, with a single melting peak confirming specificity. Cp values were obtained using LC480 software. The coefficient of variation (CV) was calculated for each set of triplicate reactions; data with CV <5% were considered valid. A standard curve was generated by plotting the log10 of known copy numbers against average Cp values, yielding a linear equation with slope between -3.9 and -3.3 and R² ≥ 0.99. Absolute copy numbers in samples were determined by substituting Cp values into this equation and reverse-transforming the result from log10. The average copy number of the triplicates was reported as the final absolute abundance (copies/g DNA) for each sample.

**Metabolite Extraction and Sample Preparation**

Stock solutions of each bile acid standard were prepared at a concentration of 1 mg/mL in methanol (MeOH). A primary mixed standard stock solution (MSS) was generated by combining the individual standards and diluting them with MeOH–water (1:1, v/v) to the required concentrations. Calibration curves were constructed by serially diluting the MSS to final concentrations of 100, 40, 16, 6.40, 2.56, 1.02, 0.41, 0.16, 0.07, 0.03, and 0.01 ng/mL. A mixed standard at 12.8 ng/mL was used as the standard quality control (STD-QC). All standards and biological samples were stored at −80 °C prior to analysis.

Freeze-dried samples were extracted according to a protocol optimized for bile acid analysis. Ice-cold MeOH–water (1:1, v/v, containing internal standard) was added to each sample, followed by homogenization using two steel beads in a grinder (60 Hz, 2 min). Subsequently, acetonitrile containing 5% ammonia (v/v) was added. The mixture underwent ultrasonic extraction in an ice-water bath for 20 min and was then centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected, dried under a gentle nitrogen stream, and reconstituted in MeOH–water (1:1, v/v, containing internal standard). A secondary ultrasonic extraction (5 min, ice bath) was performed before filtration through a 0.22 μm organic phase membrane filter. A pooled quality control (QC) sample was prepared by combining aliquots from all biological samples.

**Targeted Bile Acid Quantification by UPLC–MS/MS**

Ultra-performance liquid chromatography (UPLC) was performed using a Nexera UHPLC LC-30A system (Shimadzu) equipped with a Phenomenex Kinetex C18 column (2.1 mm × 100 mm, 2.6 µm). The column temperature was maintained at 45 °C,and the injection volume was 5 μL. The mobile phase consisted of (A) water with 0.1% formic acid and (B) a mixture of methanol–acetonitrile–2-propanol (1:1:1, v/v/v) with 0.1% formic acid. The flow rate was set to 0.3 mL/min with the following gradient: 0–2 min, 30% B; 2–5 min, linear increase to 40% B; 5–13 min, 55% B; 13–17 min, 80% B; 17–19 min, 90% B; and 19–20 min, re-equilibration to 35% B. Samples were kept at 4 °C during analysis.

Mass spectrometric detection was carried out on an AB SCIEX SelexION™ Triple Quad™ 5500 system equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes. Nitrogen was used as the collision gas. The instrument parameters in negative ion mode were: curtain gas (CUR) 35 psi, ion spray voltage (IS) −4500 V, temperature (TEM) 450 °C, ion source gases 1 and 2 (Gas1 and Gas2) both at 55 psi.

Targeted bile acids were analyzed using multiple reaction monitoring (MRM) mode. MRM transitions, declustering potentials (DP), and collision energies (CE) were optimized for each compound. Quantification was performed based on external calibration curves, and the final metabolite concentrations were normalized to sample weight and expressed as absolute concentrations (ng/mg). Analyst software was used for data acquisition and SCIEX OS-MQ software for quantitation.

**Strain-level profiling**

The metagenomic raw data were quality-controlled using ﻿Kneaddata. Reads mapped to the human database are filtered. Then, MetaPhlAn 4 is used to perform species-level metagenomic phylogenetic analysis. ﻿Consensus markers (species strains) are generated for each sample to profile SNV. A total of 493 species-level genome bins (﻿SGBs) are included. Then the corresponding species marker genes are extracted from the database. Lastly, ﻿StrainPhlAn (﻿--phylophlan\_mode accurate --sample\_with\_n\_markers 10) is used to build the multiple sequence alignment and the phylogenetic tree.

**Translocation analysis**

﻿Sample pairwise distances are extracted from .tre files, and nGD is calculated from normalizing the phylogenetic distances by the total branch length. Then SGB-specific nGD threshold is determined to separate same-individual closely related strain from different individual nGD distributions. For SGBs detected in at least 30 pairs of same-individual samples, nGD thresholds were defined based on maximizing Youden’s index, and limiting at 5% the fraction of different individuals to share the same strain as a bound on a false discovery rate. For SGBs detected in less than 30 same-individual sample pairs, the nGD corresponding to the 3rd percentile of the different individual nGD distribution was used. Pairs of strains with pairwise nGD below the strain identity threshold were defined as strain-sharing events.

**﻿Keystone species identification**

SGB transmissibility between two samples: the number of strain-sharing events detected for an SGB divided by the total potential number of strain-sharing events based on the presence of a strain-level profile

For different body sites within one sample: weighted average of SGB transmissibility depending on sample distance

**RESULTS**

**Comparative Microbiome Profiling in Humans and Monkeys**

Our analysis was based on gastrointestinal (GI) tract samples from both rhesus monkeys and humans. Content and mucosal samples (N = 152) were collected along the GI tract, including the oral cavity, esophagus, stomach, small intestine, and large intestine in rhesus monkeys (see Methods section). To achieve high-resolution profiling, we performed shotgun metagenomics on high-biomass luminal content samples and reduced metagenomics 2bRAD-M(12) on low-biomass mucosal samples. We also include human intestinal samples for comparative analysis. Human intestinal shotgun sequencing data were obtained from BioProject PRJNA822660, which were collected using capsule-based sampling. However, as noted by the original authors, a limitation of this dataset is the uncertainty regarding the exact location of sample collection(4).

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| --- | --- | --- | --- | --- | --- | --- |
| **Group** | **Subgroup** | **Df** | **SumOfSqs** | **R2** | **F** | **Pr(>F)** |
| all | age | 1 | 1.41 | 0.030 | 4.49 | 0.001 |
| all | species | 1 | 8.64 | 0.184 | 32.80 | 0.001 |
| all | location | 6 | 11.17 | 0.238 | 7.31 | 0.001 |
| large intestine | species | 1 | 2.79 | 0.392 | 20.65 | 0.001 |
| small intestine | species | 1 | 3.95 | 0.261 | 16.26 | 0.001 |
| stomach | species | 1 | 2.60 | 0.397 | 11.85 | 0.001 |
| oral | species | 1 | 0.90 | 0.437 | 7.75 | 0.003 |

**Table 1** | Bray Curtis dissimilarity statistics of all samples with different age, species and location. Results were generated using PERMANOVA adnois test.

We combined intestinal content samples from both humans (N = 77) and monkeys (N = 70) to have a holistic comparison. Host differences emerged as the most significant factor influencing microbial variation, followed by location-based differences (Table 1). In contrast, age had a minimal effect on microbial community structure, indicating that host species and location are the primary drivers of gut microbiome composition.

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**Figure 1** | **a**, PCoA plot based on Bray-Curtis dissimilarity between microbial composition determined by Kraken2 of both human and monkey luminal content samples **b**, PCoA plot based on Bray-Curtis dissimilarity between pathway abundance of both human and monkey luminal content samples **c**, Keystone species with differential abundance between human and monkey luminal content samples. **d**, Dot plot showing the Gene Ontology (GO) enrichment analysis of S. copri genes from monkey samples using hypergeometric testing.

We performed Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarities of species-level microbiome data (Fig. 1a). PC1 (22.6% of variance) mainly separates samples by host species (human and monkey), whereas PC2 (17.1% of variance) captures GI site-based variation. Oral samples from humans and monkeys cluster closely, suggesting conserved oral microbiomes across host species. Location-wise, oral, esophagus and stomach samples cluster together, indicating related upper GI communities. In contrast, small intestine and large intestine samples form distinct clusters, particularly in monkeys, where site-based stratification is clearer. The human data, collected via capsule sampling, display more overlap across different GI locations, suggesting higher variability by site. As for pathway abundance profiles, human and monkey samples are largely segregated along PC1, similar to that of species abundance (Fig. 1b). Along PC2, only that of monkey samples are stratified by GI site, showing that the functions of microbiome in different GI locations have much smaller variations in human than in monkey.

We next identified differentially abundant species between humans and monkeys across various body locations using ANCOM (ALR-transformed, cutoff = 0.6). A total of 42 species exhibited significant differences in abundance between the two hosts between different locations (Supplementary Table 1). Notably, six out of seven differentially abundant species in the upper GI tract were recognized oral pathogens or opppotunistic pathogens—*Fusobacterium nucleatum, Fusobacterium polymorphum, Fusobacterium animalis, Streptococcus mitis, Streptococcus oralis,* and *Streptococcus pneumoniae*—all of which were enriched in monkey samples. Interestingly, *Fusobacterium polymorphum* was not only enriched in the oral cavity but also in the stomach subgroups, suggesting potential translocation of oral pathogens to the stomach.

In the intestinal tract, many species enriched in humans were probiotics or potential probiotics, including *Bifidobacterium adolescentis, Bifidobacterium longum, Anaerobutyricum hallii,* and *Anaerostipes hadrus.* These species are associated with gut health, particularly in sugar catabolism and short-chain fatty acid production(13–15). In contrast, monkeys consume diets rich in plant fibers and polysaccharides. Accordingly, species such as *Segatella copri* and *Treponema succinifaciens*, which specialize in breaking down these complex carbohydrates, were enriched in monkey samples (Fig. 1c).

Then we have a deeper investigation into *Segatella copri*, which exhibits substantial genomic and functional diversity, extending into multiple distinct clades(16). Kraken2 results showed that monkey intestines harbor a significantly higher proportion of *S. copri* compared to human intestines, and further strain-level identification was carried out through MetaPhlAn4. *S. copri* strains identified from monkey samples were classified into clade K, initially identified in non-human primates(16).

Previous genomic analyses revealed substantial variability in polysaccharide utilization loci (PULs) and carbohydrate-active enzyme (CAZYme) profiles among *S. copri* strains, affecting their ability to metabolize plant-derived polysaccharides(16, 17). Specifically, substantial variability has been noted in genes encoding the SusC/SusD transporters, essential components involved in polysaccharide binding and uptake, and enzymes crucial for glycan degradation(17, 18).

These genetic traits align closely with the dietary adaptations of the host, notably toward fiber-rich diets. Strains derived from individuals adhering to plant-rich diets consistently exhibit enhanced genetic capabilities for complex fiber degradation, such as arabinan, arabinoxylan, xylan, and pectin, reflected in their expanded repertoires of glycoside hydrolases and polysaccharide lyases(19). We performed differential gene and Gene Ontology (GO) enrichment analysis by extracting gene family profiles assigned to *S. copri* from the HUMAnN3 output (Fig. 1D). The monkey-derived strains identified in this study highlighted a significant over-representation of hydrolase activity, acting on glycosyl bonds (GO:0016798). Genes encoding phosphoribosyltransferases and glycosyltransferases were the top contributors. In addition, glycosyltransferase activity (GO:0016757) was also enriched, with key contributors including genes encoding glycoside hydrolase xylanase and glycosyl hydrolase family 97 proteins. These enzymes are associated with the degradation of plant-derived polysaccharides such as xylans and beta-glucans, indicative of robust polysaccharide metabolism suited to a fibrous diet.

**Spatial Stratification of the Microbiome Along the GI Tract**

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**Figure 2** | **a**, Bar plots showing microbial load measured by qPCR quantification of 16S rRNA gene copy number per g sample collected from various regions of the GI tract. Upper panel: oral, esophagus (E), stomach (S), small intestine (SI). Lower panel: large intestine (LI). **b**, Species-level microbiome profiles: Alpha diversity (Shannon index) across luminal sites, and Bray-Curtis dissimilarity between oral samples and all other sites, with comparisons also stratified by age group. **c**, Functional pathway profiles: Alpha diversity (Shannon index) of MetaCyc pathway abundance across luminal sites, and Bray-Curtis dissimilarity between oral samples and other sites, also stratified by age group. **d**, Heatmap of MetaCyc pathways showing groups of pathways with significantly different abundances across oral cavity, esophagus, stomach, small intestine, and large intestine samples (Kruskal-Wallis test, adjusted p < 0.01).

We next examined the spatial microbial distribution patterns in the whole GI tract through the monkey samples we collected. We quantified microbial load across body sites using 16S rRNA gene-targeted qPCR (Fig. 2a) and calculated the 16S copy number per g sample. The oral cavity harbored a relatively high number of microbes, while the microbial load was markedly depleted in the esophagus and stomach. In the small intestine—particularly the ileum—the microbial load began to rise, increasing dramatically in the large intestine. By the rectum, microbial load was approximately 2000-fold higher compared to the small intestine, representing the highest density along the GI tract.

We then compared alpha diversity across different body sites in monkeys using Shannon indices (Fig. 2b). Age was not a significant factor as samples of the same region from young monkeys did not show any statistical significance (Fig. S2). Clear trends were observed in the microbiome in different locations. Microbial diversity was relatively high in the oral cavity, esophagus, and stomach, with a noticeable decline in the small intestine, then increased again.

This U-shaped trend in species diversity across the GI tract reflects the distinct ecological environments of each region. The upper GI tract is exposed to a wide range of transient microbes introduced through food and the environment, contributing to higher diversity. In contrast, the small intestine, with its faster transit time, stronger host immune surveillance, and exposure to bile acids, likely imposes selective pressure that limits microbial richness. The large intestine and stool harbor more stable and dense microbial communities, supporting the rebound in diversity observed in these sites. Interestingly, microbial richness in the stomach remained high despite its acidic environment. A Venn diagram analysis revealed substantial overlap in species composition between the stomach and the upper GI tract (Fig. S3), suggesting that many microbes introduced from the oral cavity are not eliminated by gastric acid. Furthermore, this suggests the stomach may harbor a more complex and resilient microbial community than traditionally assumed.

We also analyze the functional composition of microbial communities in different body sites by HUMAnN 3. Interestingly, despite a reduced microbial load, pathway diversity reached saturation across most sites, including the stomach and small intestine (Fig. 2c). The heatmap presents the MetaCyc pathways significantly different between different body locations (Fig. 2d).

Functional pathways enriched in most body sites primarily include amino acid biosynthesis, nucleotide biosynthesis, and coenzyme production—core metabolic processes essential for microbial growth (Supplementary Table 2). The large intestine exhibits a broader metabolic repertoire, with enrichment of pathways involved in the TCA cycle, as well as sugar and vitamin degradation. Notably, the stomach shows surprising functional diversity. In addition to sharing some metabolic capabilities with the large intestine, it displays unique pathway enrichments. These include aerobic respiration (PWY-3781, PWY-7279) and terminal O-glycan modification (PWY-7434), which may support immune evasion. Pathways for ceramide degradation (PWY66-388) and folate metabolism (PWY-7200) are also enriched, suggesting microbial adaptation to the stomach’s nutrient-limited and physiologically challenging environment.

We then investigated the microbial distance between different body sites by Bray Curtis dissimilarity. The microbial composition exhibited a gradient of dissimilarity along the digestive tract, progressing from the esophagus and stomach to the small and large intestines relative to the oral samples (Fig. 2b). Notably, distance based on species profiles revealed that older monkeys exhibited significantly greater divergence between oral and intestinal microbiota, particularly in the small and large intestine (Fig. 2a). This suggests an age-related increase in regional microbial differentiation along the gastrointestinal tract. Specifically, small intestine and large intestine samples showed greater divergence from oral samples in older monkeys, indicating an age-related increase in microbial community divergence. In contrast, dissimilarity between locations calculated by pathway abundance remained uniformly low across body sites, suggesting that microbial functional profiles are more conserved than taxonomic profiles. In contrast, in terms of functional dissimilarity, young monkeys exhibited greater functional dissimilarity between oral and intestinal sites (Fig. 2c). These results highlight a decoupling between taxonomic and functional divergence with aging, where microbial species composition becomes more heterogeneous, but their metabolic capabilities converge.

**Distinct Mucosal and Luminal Communities**图形用户界面, 图表

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**Figure 3** | **a**, PCoA plot based on Bray-Curtis dissimilarity between microbial composition determined by 2bRAD-M of monkey mucosal samples (PERMANOVA, p = 0.001, F = 12.88) **b**, PCoA plot based on Bray-Curtis dissimilarity between microbial composition determined by 2bRAD-M of monkey blood samples (PERMANOVA, p = 0.907, F = 0.49) **c**, Stacked bar plot showing the relative abundance of microbial phylum across different mucosal and luminal content samples. Samples are grouped based on sub-location: Proximal Jejunum (PJ), Duodenojejunal Flexure (DJ), Ileum (IL), Cecum (CE), Proximal Colon (PC) and Descending Colon (DC). d, Heatmap showing species with significantly different abundances between mucosal and luminal samples (Kruskal-Wallis test, adjusted p < 0.01).

The high-resolution profiling of low-biomass mucosal tissue or blood samples has always been hindered by the contamination of human DNA. Here, we employed 2bRAD-M sequencing method(12) which is designed for tackling this issue, enabling accurate species-level profiling of mucosal microbiota. We performed PCoA based on Bray-Curtis dissimilarity to cluster the species-level microbial profiles from stomach tissue, small and large intestinal tissue, and blood samples (Fig. 3a). Microbial communities in the mucosa were clearly clustered by body sites, while age-related differences were still minimal. Stomach and large intestine samples formed relatively tight and overlapping clusters, whereas small intestine samples were more broadly distributed, spanning the space between stomach and large intestine clusters. This dispersion suggests that microbial communities infiltrating the small intestine are more heterogeneous in composition, probably due to the functional complexity of the small intestine.

Blood samples formed a distinct cluster, separate from all GI tract tissue. We collected blood samples from arm, leg and neck. To explore whether blood collected from these sites had a difference, we performed PCoA on blood samples alone (Fig. 3b). Most samples clustered tightly together, indicating that the microbial composition of peripheral blood is consistent across collection sites. A few outliers were observed, likely due to contamination events. A conserved core microbial signal in blood was observed with minor fluctuations.

We then compared the microbial communities between mucosal and luminal content samples. In order to make it comparable, we performed 2bRAD-M pipeline analysis on shotgun metagenomics luminal sample data. A stacked bar plot of phylum-level relative abundances revealed distinct microbial signatures across GI tract locations and between luminal content and mucosal samples (Fig. 3c). Firmicutes and Bacteroidota were dominant phyla in both mucosa and luminal content. Proteobacteria were consistently enriched in luminal content, likely reflecting their ability to exploit oxygenated and nutrient-rich environments in the gut lumen(20). In contrast, Pseudomonadota were more prevalent in mucosal samples, particularly in the small intestine, suggesting a stronger adaptation to host-associated niches. Additionally, Campylobacterota exhibited increased abundance in the mucosa of the large intestine, likely benefiting from the microaerophilic environment and its genes for hydrogenotrophic metabolism(21).

We then dive deeper to compare the species-level differential abundance between the microbiome in mucosa and luminal content (Fig. 3d, Supplementary Table). Luminal content was enriched with strict anaerobes such as *Faecalibacterium duncaniae*, *Prevotella* *copri*, and *Butyricicoccus* *intestinisimiae*, which are specialized in fermenting dietary polysaccharides to produce SCFAs like butyrate and propionate(19, 22). In contrast, mucosal samples were dominated by aerotolerant or microaerophilic species including *Lactobacillus johnsonii*, *Faecalibacterium* *prausnitzii*, and *Treponema succinifaciens*, many of which exhibit strong adaptation to the host interface via mucus adhesion, motility, and even exhibit immune modulation activity(23–25). These results underscore the spatial structuring of the gut microbiome, where luminal and mucosal compartments harbor functionally distinct microbial communities adapted to differences in oxygen availability, substrate type, and host interactions.

**Distinct Bile Acids Regional Patterns and Isoallo-LCA Enrichment**

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**Figure 4** | **a**, Box plots showing total bile acid concentrations across GI tract in monkeys (left) and humans (right). **b**, Stacked bar plot showing relative bile acid composition by chemical class across GI tract in monkeys (left) and humans (right). **c**, Isoallo-LCA concentrations across monkey GI compartments. **d**, Scatter plot of Odoribacteraceae bacterium absolute abundance (multiplied by microbial load) with Isoallo-LCA concentration. (Pearson r = 0.44, *p* = 0.002).

To further understand the functional implications of regional microbial variation across the gastrointestinal tract, we next investigated the composition and spatial distribution of bile acids. We first quantified the total bile acid concentration across GI compartments in monkeys (Fig. 1a). In contrast to human data, which exhibit a progressive decline in total bile acid levels from the proximal small intestine to stool, monkeys showed a clear increase in bile acid concentrations from the small to the large intestine.

We then examined the compositional distribution of bile acid classes by calculating the average relative abundance of four bile acid types (Fig. 1b). Human samples displayed a gradual decline in primary conjugated bile acids and an increase in secondary deconjugated bile acids in stool. In contrast, the monkey samples showed a sharp compositional shift between the small and large intestines. Secondary deconjugated bile acids dominated all large intestinal regions, whereas the small intestine exhibited a more mixed profile, with some presence of primary and secondary conjugated forms.

Strikingly, the most abundant bile acid species in the monkey large intestine was isoallo-lithocholic acid (Isoallo-LCA) (Fig. 4c), which was undetectable in both the monkey small intestine and all human samples. Isoallo-LCA was initially identified in the feces of healthy human centenarians, where it was found to be enriched compared to younger individuals(26). It has been shown to exhibit antimicrobial activity against gram-positive bacteria and immunomodulatory effects, including the promotion of regulatory T cell differentiation, suggesting a potential role in maintaining intestinal immune homeostasis(27).

To explore potential microbial contributors to isoallo-LCA production, we performed Pearson correlation analyses between isoallo-LCA concentrations and the absolute abundance (i.e., relative abundance multiplied by microbial load) of individual microbial taxa (Table ). Consistent with previous findings in humans, we found that the abundance of Odoribacteraceae bacterium strongly correlates with isoallo-LCA concentrations in our sample (Fig. 4d).

Our correlation analysis found many novel candiate species significantly associated with isoallo-LCA abundance. Notably, the three species with the lowest adjusted p-values were Segatella copri, Segatella oris, and Segatella bryantii. These species belong to the Bacteroidales order, which is known to harbor enzymes such as 5α-reductase (5AR) and 3β-hydroxysteroid dehydrogenase (3β-HSDH) implicated in isoallo-LCA biosynthesis pathways.

**DISCUSSION**

The analysis of gut microbiomes has previously been constrained to oral and fecal samples, primarily due to challenges in sample collection. To address this limitation, our study utilized rhesus monkeys as a non-human primate model, enabling a comprehensive assessment of both mucosal and luminal microbiome communities across multiple GI compartments. For comparative purposes, we aimed to leverage human GI tract samples. However, only three previous studies have successfully performed holistic sampling (4, 28, 29). Among these, we selected the dataset provided by Shalon et al., as Zmora et al. lacked oral samples, and She et al. relied exclusively on 16S rRNA sequencing rather than shotgun metagenomics.

Our analyses reveal conserved as well as distinct microbial patterns between rhesus monkeys and humans. Species-level profiles of different body sites were separated clearly on PC2. Functional pathway analysis revealed significantly greater spatial stratification in monkeys compared to humans. These observations underscore the utility of rhesus monkeys as a model for capturing fine-scale spatial microbiome variations, surpassing the resolution typically achievable in human studies.

Upon examining differential microbial compositions between humans and rhesus monkeys, we identified enrichment of oral pathogens in the upper GI tract of monkeys, likely attributable to inadequate oral hygiene conditions typical of captive environments. Probiotic species enrichment in human intestines probably reflects greater investigative efforts of human gut microbiota. Notably, the monkey gut microbiome consistently showed enrichment of species adept at dietary fiber metabolism, particularly Segatella copri and Treponema succinifaciens. This aligns closely with our hypothesis that distinct dietary habits impose evolutionary selection pressures, driving adaptations in microbial genetic and metabolic capacities.

Genomic analysis of monkey-derived S. copri strains further revealed an enrichment of glycoside hydrolase and glycosyltransferase genes, indicative of specialized adaptations for plant-derived polysaccharide metabolism. Additionally, the enrichment of T. succinifaciens observed in monkeys corroborates previous reports from traditional human societies, where similar Treponema species are abundant due to fiber-rich diets(30). Collectively, these findings suggest that such fiber-specialist microbial taxa represent prolonged dietary selection pressures, potentially lost in industrialized human populations due to dietary shifts away from fiber-rich foods. Consequently, these genetically distinct strains may hold promise as next-generation probiotic candidates.

Detailed analysis of spatial microbiome stratification in monkeys revealed an intriguing divergence from previous expectations regarding microbial diversity gradients. Contrary to the general assumption of low microbial load and diversity in the stomach (29) , our results revealed notably high diversity in this organ, which then decreased to low levels in the small intestine before increasing in the large intestine. This is possibly due to the pathological conditions in post-mortem human sampling in She et al. study.

In addition to taxonomic diversity, functional patterns further highlight key differences across GI tract regions. Moreover, the observed saturation of microbial pathway diversity across most GI compartments, coupled with low Bray–Curtis dissimilarity among pathway profiles, emphasizes the existence of high functional redundancy. Such redundancy, characterized by taxonomically diverse microbes performing similar metabolic roles, likely contributes to microbiome resilience, ensuring functional stability against perturbations(31).

Interestingly, the small intestine deviates from this trend, displaying relatively lower functional diversity and redundancy, indicative of its highly selective and dynamic environment. Consequently, reduced redundancy in this compartment could render it vulnerable to functional instability under stress or perturbation. In contrast, the stomach exhibited high functional diversity and apparent redundancy despite its acidic conditions. Differential pathway abundance analyses identified enrichments in aerobic respiration, ceramide degradation, and folate metabolism, challenging conventional views of the stomach as a microbiologically simple and functionally limited niche.

An intriguing aspect of our findings involves age-associated decoupling between microbial taxonomy and function. Specifically, oral and intestinal microbial communities showed increasing functional convergence with age, potentially reflecting diminished barriers between oral and gut environments, independent of taxonomic identity. Conversely, higher functional divergence observed in younger monkeys suggests a dynamic, transitional microbiome ecosystem characterized by specialized, compartment-specific functional roles that evolve over time.

Moreover, we addressed the mucosal microbiome, an under-investigated niche. Our mucosal profiling using the high-resolution 2bRAD-M method demonstrated distinct community structures compared to luminal counterparts. Specifically, luminal microbiomes were dominated by strict anaerobes specialized in dietary fiber fermentation and short-chain fatty acid production, whereas mucosal microbiomes exhibited enrichment in aerotolerant, host-adapted taxa associated with mucus adhesion, motility, and immune modulation. These results further highlight the importance of mucosal microbiome profiling for comprehensive microbiome characterization and functional understanding.

Lastly, looking at bile acid profiles, we found a sharp increase in isoall-LCA concentration in monkey large intestines. Isoallo-LCA has gained attention for its antimicrobial activity and its immunoregulatory properties. Our finding that isoallo-LCA accumulates in the monkey colon suggests that its role may not be limited to centenarians, but may represent a more widespread host–microbe co-metabolite with functional relevance in the primate gut.

While the enzymatic steps to convert LCA to isoallo-LCA have been mapped in selected human isolates, our findings suggest that additional taxa in the primate gut may contribute. Correlation analysis revealed previously found and novel species that associated with isoallo-LCA. While these associations are intriguing, they are hypothesis-generating and require experimental validation. Future studies involving in vitro culturing or metagenomic functional annotation will be needed to confirm their roles in isoallo-LCA production. Nonetheless, the identification of these species provides a promising direction for expanding our understanding of bile acid–microbiota interactions beyond previously established taxa.

A major strength of this study lies in the comprehensive sampling strategy employed across the gastrointestinal tract, encompassing both luminal and mucosal compartments. The ability to assign precise anatomical locations to each sample site enables a more accurate spatial mapping of the microbiome, a level of resolution that is often unattainable in human studies. Our study also leveraged high-resolution mucosal profiling using the 2bRAD-M method, which has proven effective in characterizing low-biomass microbial communities. This approach allowed us to access previously underexplored mucosal niches, providing deeper insight into host-associated microbial ecosystems.

One notable limitation of the study is the small number of individuals due to the scarcity of dissected rhesus monkey samples. Additionally, while rhesus monkeys offer valuable physiological similarities to humans, direct extrapolation of findings must be approached with caution due to inherent interspecies differences in immune function, diet, and gut physiology.

Our findings have several important implications for microbiome research and potential translational applications. The identification of microbial species in monkeys with genetic enrichment for plant fiber metabolism, such as S. copri, highlights promising candidates for next-generation probiotics tailored to dietary habits. Notably, the presence of microbial genes involved in the production of beneficial allo-LCA further demonstrates their functional relevance in maintaining host metabolic and immune homeostasis.

The observed patterns of functional redundancy, particularly in the distal gut, shed light on microbial ecosystem stability, suggesting that taxonomically diverse communities may perform overlapping functions to buffer against environmental perturbations. These insights may inform strategies aimed at preserving or restoring microbial resilience in microbiome interventions. Finally, our findings challenge prevailing assumptions about the stomach and other traditionally understudied gastrointestinal niches. The unexpected diversity and functional activity in these regions highlight the need for more nuanced investigations, which may ultimately revise our understanding of host–microbe interactions across the entire gut.

Supplementary Figure

**图表, 条形图

描述已自动生成**

Figure S1. Stacked bar plot showing the relative abundance (only showing those > 1%) of microbial taxa determined by Kraken2 across different samples. Samples are grouped and labeled based on the monkey's age and the sample location site.

图表, 瀑布图, 箱线图

AI 生成的内容可能不正确。

Figure S2. Alpha diversities (Shannon diversity, Simpson diversity) of young and old monkey lumenal microbiome. No significant differences were observed.

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Figure S3. Venn diagram showing the number of microbial species shared between the upper gastrointestinal (GI) tract (oral cavity and esophagus) and the stomach in monkeys. A total of 6,137 species were shared, while 577 and 4,003 species were unique to the upper GI tract and stomach, respectively.

**(Strain sharing)**

图表, 瀑布图

描述已自动生成

Strain-sharing rates between different locations were calculated as the number of strains shared between two sites divided by the number of shared SGBs profiled by StrainPhlAn (number of shared strains/ number of shared SGBs). Our hypothesis is the transmission rate is higher in old people than in young people.

**(Gene functions of monkey MAG)**

**图表

描述已自动生成**

**Figure x** | **a**, Phylogenetic tree of genera detected from monkey digestive tract using whole-genome sequencing (WGS) data annotated by age (inner ring) and location (outer ring) found. The phylum information is shown as colored with names annotated.

We further characterized these communities by employing a metagenomic assembly-based approach. The phylogenetic relationships among microbial genomes were analyzed using a phylogenetic tree generated from metagenome-assembled genomes (MAGs) and annotated with GTDB-tk. The tree demonstrates the taxonomic diversity of microbial communities at genus level across different gastrointestinal locations and age groups. Distinct microbial clades are observed, with dominant taxa including Bacteroidota, Bacillota, and Pseudomonadota. Distinct phylogenetic lineages were associated with each gastrointestinal location, suggesting niche-specific adaptations. Samples from young individuals and old individuals did not exhibit much difference, suggesting that age might have less influence on the taxonomic diversity at this resolution compared to location.

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