

Spatial ecology of the multi-kingdom human intestinal microbiome

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Abstract

The human gut microbiome is typically characterized from stool samples, yet microbial communities vary across intestinal regions and microenvironments. Here, we performed spatially resolved deep whole-genome shotgun sequencing of paired luminal and mucosal samples collected across six intestinal segments from organ donors to characterize the organization of bacterial, archaeal, fungal, viral, and resistome components within the human intestine. Distinct microbial domains exhibited different spatial organization patterns: bacterial communities showed moderate longitudinal partitioning, methanogenic archaea displayed strong distal enrichment and mucosal association, viral distributions largely mirrored predicted bacterial hosts, and fungal communities showed comparatively weak spatial structure and strong donor dependence. Mucosa-associated communities showed a trend toward enrichment in enzymatic functions associated with host substrates. In contrast, resistome profiles were shaped predominantly by donor identity rather than anatomical location, highlighting the individualized nature of antimicrobial resistance reservoirs. Spatially distinct microbial association structures suggested niche-dependent ecological organization. Together, these findings support a model of the human intestine as a spatially structured, individualized microbial ecosystem and highlight the limitations of stool as a universal proxy for gut microbiome organization.

Introduction

The human intestinal tract harbors a dense, diverse community of microorganisms, collectively referred to as the gut microbiome. This ecosystem includes bacteria, archaea, fungi, protists, and viruses, whose combined abundance is estimated to exceed that of human cells¹. The gut microbiome contributes to host physiology through immune modulation, nutrient metabolism, and the production of bioactive metabolites such as vitamins and short-chain fatty acids². Microbial composition and activity are shaped by numerous host and environmental factors, including genetics, age, health status, diet, antibiotic exposure, and early-life influences such as birth mode and breastfeeding³.

Microbial communities vary along the intestinal tract, exhibiting longitudinal gradients in composition and function, as well as radial differences between the lumen and the mucosal interface^{4–7}. Despite this spatial heterogeneity, most current knowledge of the human gut microbiome derives from stool samples, which represent only the distal luminal environment. Comparisons with mucosal and colonic biopsy samples demonstrate substantial compositional differences, indicating that stool provides an incomplete view of intestinal microbial communities^{7,8}.

Physiological gradients along the intestine impose strong environmental filtering, shaping microbial community structure. The small intestine is characterized by higher oxygen levels, rapid transit, and elevated bile salt concentrations, conditions that limit microbial density and favor adaptable taxa⁹. In contrast, the large intestine supports dense anaerobic communities specialized for polysaccharide fermentation¹. Radial differences between luminal and mucosal compartments further create distinct

ecological niches, with mucosal communities linked to host barrier function and immune regulation, and luminal communities more associated with dietary metabolism^{10,11}.

These microenvironmental gradients drive microbial variation at both species and strain levels¹², complicating efforts to define a universal “healthy” microbiome. However, comprehensive multi-segment analyses of the human gut remain limited due to technical challenges in intestinal sampling. Previous approaches have relied on biopsies¹³, postmortem specimens¹⁴, or ingestible sampling capsules¹⁵, each with methodological constraints that can influence microbial characterization. These studies have characterized regional intestinal environments and microbial distributions using ingestible devices and spatial sampling approaches. Yet, comprehensive characterization of paired luminal and mucosal communities across multiple intestinal segments, integrating bacteria, archaea, fungi, viruses, resistomes, and strain-resolved genomes, remains limited.

Most microbiome research has focused on bacteria, driven in part by the widespread use of 16S rRNA gene sequencing and the relative scarcity of genomic resources for non-bacterial taxa¹⁶. Yet the gut microbiome also includes fungi, archaea, viruses, and parasites, which contribute to ecological stability and host health¹⁷. Viral communities, for example, may remain relatively stable in healthy individuals but exhibit marked alterations in disease states¹⁸. A multi-kingdom perspective is therefore essential for understanding the structure and function of gut ecosystems.

Here, we performed a spatially resolved, multi-kingdom analysis of the human intestinal microbiome using deep whole-genome shotgun sequencing of luminal and mucosal samples from six intestinal segments in organ donors. We characterized taxonomic composition, functional potential, resistome profiles, and microbial associations across intestinal niches and identified genomic divergence among strains recovered from different intestinal sites. By integrating spatial resolution with multi-domain profiling, this study provides a framework for understanding the human intestine as a structured and individualized ecosystem, with implications for precision microbiome diagnostics and therapeutics.

Results

Demographic information and clinical profiles

This study included eight organ donors, comprising five males and three females, aged 10 to 65 years. Donors represented adolescent, young adult, and middle-aged/older adult groups, and BMI values ranged from 22.7 to 52.5 among donors with available measurements, reflecting substantial variation in metabolic and physiologic status. Donors were predominantly White among those with recorded race information. Causes of death included anoxia or cardiovascular events, cerebrovascular events or stroke, cardiac arrest, gunshot wound, and drug overdose. Several donors had clinically relevant conditions or peri-donation features that may influence the intestinal microbiome, including prolonged CPR downtime, pancreatic transplantation affecting duodenal tissue availability, morbid obesity, fatty liver, hyperlipidemia, alcohol abuse, coronary artery disease, former smoking, hypertension, pre-diabetes,

methamphetamine exposure, and smoking history. One donor had no active or chronic infections noted, whereas another donor died from drug intoxication and underwent prolonged CPR and downtime. Together, these clinical and demographic features provide important context for interpreting individual donor variability in microbiome composition. However, donor-specific antibiotic exposure was not consistently recorded; although antibiotic use is an important factor shaping the microbiome, this limitation should be considered when interpreting interindividual differences. Demographic and clinically relevant donor information is summarized in **Table S1** and the **graphical abstract**.

Relative and absolute microbial abundance across the intestinal tract

Line plots of deep whole-genome shotgun (WGS)-derived relative abundance profiles revealed substantial interindividual variation in the distribution of major microbial kingdoms, including bacteriome, archaeome, virome, mycobiome, and other microbial groups, across intestinal segments from the duodenum to the descending colon (**Figure S1**). Absolute abundance analysis by qPCR demonstrated a progressive increase in bacterial load from the small intestine to the ascending colon ($P < 0.0001$), with luminal samples generally exhibiting higher bacterial abundance than mucosal samples ($P = 0.0115$). In contrast, fungal abundance measured by ITS copy number remained relatively stable across intestinal regions and sampling milieus. Neither bacterial nor fungal loads were significantly associated with donor identity, age, or sex (**Figure S2**).

Overview of the intestinal microbiome throughout the intestinal tract

The overall intestinal microbiome of the cohort comprised 3,508 taxa meeting domain-specific inclusion thresholds. This broad taxonomic representation highlights substantial interindividual variability while preserving consistent spatial structuring along the intestinal axis (Fig. 1, **Figure S3**, **Table S2**).

At the phylum level, the bacteriome was dominated by Firmicutes (57.8%) and Bacteroidota (29.4%), followed by Proteobacteria (7.6%) and Actinobacteriota (3.2%), with all other phyla accounting for 1.1% of the total relative abundance. Segment-level patterns were consistent with intestinal redox and substrate gradients. Bacteroidota and obligate anaerobic Firmicutes were enriched in colonic segments, whereas Proteobacteria were relatively more abundant in proximal small intestinal sites, reflecting adaptation to higher oxygen availability and rapid transit. Segment-level analysis revealed distal enrichment among several anaerobic taxa. *Bacteroides thetaiotaomicron* had low mean relative abundance in the duodenum and jejunum, at 0.33% and 0.15%, respectively, but increased in the colon, reaching 2.5% in the ascending colon, 2.3% in the transverse colon, and 4.5% in the descending colon. *Bifidobacterium longum* showed a mean relative abundance of approximately 1%-1.5% from the duodenum to jejunum but increased to approximately 8% in the ileum and ascending colon. In contrast, facultative aerobic bacteria such as *Escherichia coli* were more abundant in the upper small intestine, with a mean relative abundance of 10% in the duodenum and 7% in the jejunum. Lumen-mucosa differences were modest overall. Most taxa exhibited less than 1.2-fold differences between

compartments; however, several taxa showed milieu-specific enrichment. For example, *Enterocloster bolteae* and *E. coli* were enriched in the mucosa by 3.4-fold and 2.9-fold, respectively, whereas *Limosilactobacillus fermentum* showed 3.5-fold enrichment in the lumen.

Fungal diversity was limited relative to bacterial diversity. The mycobiome was dominated by Ascomycota and Basidiomycota and showed strong donor dependence, with limited spatial structuring. *Saccharomyces cerevisiae* had the highest abundance among fungal taxa and showed mild enrichment in colonic segments in individual donors, but these patterns were not consistent across the cohort. *Candida albicans* was enriched in the colon, with a mean relative abundance of 2%-5%, compared with 0.3%-0.6% in the small intestine. Fungal taxa were detected in both lumen and mucosal samples without a clear compartmental preference, suggesting weak niche specialization and a largely transient ecological role.

The archaeome diversity remained markedly lower than bacterial diversity and was dominated by methanogens within the phylum Euryarchaeota. Methanogenic archaea exhibited the strongest spatial signal. *Methanobrevibacter smithii* was nearly absent in proximal segments (< 0.001%) but reached 0.0542% in the ascending colon, consistent with anaerobic specialization. Other methanogens displayed similar distal enrichment patterns. In contrast to bacteria, methanogens demonstrated marked mucosal enrichment. *M. smithii* averaged 0.047% in mucosa compared with 0.0013% in lumen, indicating preferential enrichment within mucosal-associated communities.

The gut virome consisted exclusively of bacteriophages. Viral distributions mirrored those of their bacterial hosts rather than exhibiting independent spatial gradients. This host-dependent pattern suggests that viral biogeography is constrained primarily by bacterial community structure rather than by direct environmental filtering along the intestinal axis.

The non-fungal eukaryotic microbiome included the taxon *Toxoplasma gondii*, which showed consistently high relative abundance across donors and intestinal segments. Further evaluation of reads classified as *T. gondii* showed that the high classification rate was largely driven by unplaced scaffolds in the NCBI RefSeq reference genome (GCF_000006565.2). Approximately 90% of reads classified as *T. gondii* aligned to the full reference genome, whereas only 0.005% aligned after removal of unplaced scaffolds. Alignment to alternative *T. gondii* assemblies was also low, ranging from 0.01% to 0.24%, except for one GenBank assembly (GCA_009761385.1), which showed an average alignment rate of approximately 53%. To assess potential host-related alignment, we examined human sequencing reads with no expected *T. gondii* contamination, including reads from the 1000 Genomes Project. More than 200,000 reads aligning to human chromosome 1 also aligned to four unplaced scaffolds in the *T. gondii* reference: KE140923.1, KE140740.1, KE140442.1, and KE140584.1. Additional shared alignments were observed for human chromosomes 3, 4, and 5. These regions were not annotated as genes in either the human or *T. gondii* reference. Together, these results indicate that *T. gondii* was detected in the metagenomic data, but its estimated abundance was likely inflated by reads mapping to unplaced scaffolds that are highly similar to human genomic regions.

Overall, these findings indicate a hierarchical pattern of ecological structuring across microbial domains. Bacteria exhibit moderate spatial niche partitioning along the intestinal axis (PERMANOVA $R^2 = 0.053$), archaea display distal enrichment consistent with anaerobic specialization, fungi show weak spatial structure and strong donor dependence (PERMANOVA $R^2 = 0.125$, $P = 0.005$), and viruses largely track bacterial host distributions (**Table S3**). This hierarchy underscores the intestine as a spatially heterogeneous ecosystem in which environmental filtering, host factors, and trophic dependencies operate at different strengths across microbial domains.

Core microbiome structure and continuity across the intestinal axis

A cohort-level intestinal core microbiome was defined using relative abundance and prevalence thresholds of 0.0001 and 50%, respectively (Fig. 2a, **Table S4**). The small intestine core comprised 15 taxa, and the large intestine core comprised 21 taxa, with 14 taxa shared between the two regions. Shared taxa included common gut commensals such as *Escherichia coli* and *Bifidobacterium longum*. The large intestine additionally contained characteristic anaerobic commensals, including *Ruminococcus gnavus*, *Bacteroides thetaiotaomicron*, *Clostridium scindens*, and *Eubacterium rectale*, consistent with specialization in carbohydrate fermentation, short-chain fatty acid production, and mucin degradation. Adjacent intestinal sections showed substantial overlap in core taxa, supporting gradual ecological transitions along the intestinal axis rather than discrete microbial communities. Core communities also differed between luminal and mucosal niches. The luminal core comprised 22 taxa, whereas the mucosal core contained 13 taxa, all of which were also present in the lumen. No taxa were uniquely identified as mucosa-specific core members under the applied thresholds.

Despite a conserved core microbiome, interindividual variation remained substantial. Across donors, accessory taxa consistently outnumbered core taxa, with accessory-to-core ratios ranging from 2 to 15 (Fig. 2b), indicating that donor-specific variation was driven primarily by accessory microbiota rather than changes in core membership. Beta dispersion analysis further demonstrated substantial within-donor compositional variability (mean Bray–Curtis distance 0.43–0.60). Core taxa also varied in abundance stability, with coefficients of variation ranging from 0.24 to 1.84. Facultative and environmentally responsive taxa showed the greatest variability, whereas anaerobic core taxa remained comparatively stable across donors (Fig. 2c).

To determine whether this taxonomic variability was accompanied by functional differentiation, we next evaluated enzyme commission (EC) profiles across intestinal segments and niches. Bray–Curtis dissimilarity of EC profiles revealed limited functional separation across intestinal sections, tissue milieus, or donor identities (**Figure S4, Table S5**). EC richness and Shannon diversity showed a trend toward higher functional diversity in the large intestine than in the small intestine, with the jejunal mucosa exhibiting the lowest functional diversity and the ascending colon mucosa showing the highest richness (**Figure S5**). However, these differences were not statistically significant, indicating substantial overlap in metabolic capacity across intestinal regions.

Together, these findings support a conserved functional backbone across intestinal regions and niches, with regional specialization arising primarily through differential enrichment of taxa adapted to local environmental conditions rather than broad shifts in metabolic potential.

Interindividual variation as the primary determinant of the resistome

A diverse array of antimicrobial resistance (AMR) genes was identified across bacterial species, including *E. coli*, *E. fergusonii*, *Ruminococcus torques*, and *Megasphaera stantonii*, which were the most frequently detected carriers of AMR genes among donors (Fig. 3a and **Table S6**). Individual donors were the primary drivers of the observed resistance patterns (PERMANOVA $R^2 = 0.1$, $P = 0.2$), rather than the sampling milieu or sections (PERMANOVA $R^2 = 0.01$, $P > 0.3$). Specifically, donor 2 (OD2) showed donor-specific enrichment of AMR-gene-carrying taxa. Most AMR gene carriers from the Proteobacteria phylum were detected only in OD2, and AMR gene-carrying *Staphylococcus* and *Bacillus* species were also exclusive to this donor. OD2 had previously undergone pancreatic transplantation, which may represent an important clinical context for interpreting this distinct AMR profile. However, because antibiotic exposure records were not available, the specific factors contributing to the OD2-associated AMR pattern could not be directly determined.

Overall, total AMR burden, that is, the relative abundance (proportion of each gene) and richness (number of unique genes) of AMR genes, revealed differences in AMR distribution between segments, milieu, and sections regarding 32 drug classes and 36 gene families (Fig. 3b, **Figure S6-7**). Mucosal samples had a significantly higher mean relative abundance of AMR genes by drug class than luminal content, with a gradient of increasing mean relative abundance from the jejunum to the descending colon. Tetracycline, Fluoroquinolone, Macrolide, Aminoglycoside, and β -lactam were among the highest AMR gene richness recorded.

Moreover, analysis of the resistome profiles by AMR gene families revealed significant differences in the overall mean relative abundance and richness of AMR genes between the large and small intestines and between lumen and mucosa samples. The most abundant gene families were the major facilitator superfamily (MFS) antibiotic efflux pump, the resistance-nodulation-cell division (RND) antibiotic efflux pump, the mosaic tetracycline resistance gene, and the ribosomal protection protein. No differences were observed by individual donor gender or age.

Fifty-six AMR genes were present in all segments (Fig. 3c), while additional resistance genes exhibited segment-specific distributions, suggesting distinct regional resistome profiles along the intestinal tract. Antibiotic efflux-related AMR genes were predominant in all segments, followed by antibiotic inactivation and antibiotic target protection (Fig. 3d).

Species clustering analysis

Microbial influences on host health arise not only from individual taxa, but also from interactions within microbial communities¹⁹. A study by Pascual-Garcia et al.²⁰ postulated the existence of an intermediate organizational module, termed the Metabolically Cohesive Consortium (MeCoCo), a type of consortium that exhibits a positive feedback loop, in which the consortium modifies the environment by both creating and using resources, resulting in stable and reproducible community dynamics. The assembly of these intermediate consortia is not random and serves as an intermediate level of organization between species and communities. To identify such biological patterns of organization within the complex communities of the gut microbiome, we applied the Compositionality Corrected by Permutation and Renormalization (CCrepe) correlation structure to the relative abundances of species observed in the small intestine compared with those in the large intestine. In the colon, we identified nine distinct niche-associated co-abundance structures, which we designated persistent microbial community states (PMCs). A subsequent correlation analysis, considering only the top 10 most abundant taxa in each cluster, identified smaller units, which we termed units of correlation structure (UCS). Several UCS were identified in the small and large intestines as components of environment-specific PMCs (Fig. 4, **Figure S8, Table S7-8**).

Our analysis revealed that several species had opposite correlations in the small intestine compared to the large intestine. For example, *B. adolescentis* and *L. fermentum* were strongly correlated (CCrepe score = 0.9968, $q = 0.0022$) in the small intestine but almost entirely uncorrelated (CCrepe score = 0.05124, $q = 1.0$) in the large intestine. Similarly, *Dorea longicatena* and *Lactococcus lactis* were strongly correlated (CCrepe = 0.9598, $q = 6.084 \times 10^{-6}$) in the small intestine but largely uncorrelated (CCrepe score = 0.0687, $q = 0.9598$) in the large intestine, and to a lesser extent, *[Ruminococcus] gnavus* and *Christensenella minuta* were strongly correlated in the small intestine (CCrepe score = 0.8621, $q = 1.1425 \times 10^{-11}$), but only marginally correlated (CCrepe score = 0.3825, $q = 0.9011$) in the large intestine. In contrast, *Megasphaera massiliensis* and *Christensenella minuta* were largely uncorrelated in the small intestine (CCrepe score = 0.4571, $q = 0.0466$) but strongly correlated (CCrepe score = 0.8734, $q = 1.0$) in the large intestine. Similarly, *B. thetaiotaomicron* and both *Klebsiella pneumoniae* and *Parabacteroides merdae* were uncorrelated (CCrepe score = 0.0038, $q = 1.0$ and CCrepe score = 0.1002, $q = 1$, respectively) in the small intestine, but strongly correlated (CCrepe score = 0.8394, $q = 0.0059$ and CCrepe score = 0.8532, $q = 0.0097$, respectively) in the large intestine.

As in the small and large intestines, no single species showed differential abundance between the lumen and the mucosa. However, the correlation structure of species relative abundances in the lumen differed from the CCrepe correlation structure observed for species relative abundances in the mucosa. Moreover, UCSs formed substantial clusters specific to the lumen or mucosa. As in the small versus large intestines, several species showed opposite correlations in the mucosa compared to the lumen. For example, *L. fermentum* and *B. adolescentis* were highly correlated (CCrepe score = 0.9834, $q = 11.8566 \times 10^{-6}$) in the mucosa but uncorrelated (CCrepe score = -0.0303, $q = 0.9834$) in the lumen. *Listeria monocytogenes* and *Christensenella minuta* were highly correlated (CCrepe score = 0.9092, $q = 0.0004$) in the mucosa but uncorrelated (CCrepe score = -0.0627, $q = 1$) in the lumen, as are *Phocaeicola*

dorei and *Bacteroides salyersiae* with CCrepe score of 0.965 ($q = 6.766 \times 10^{-5}$) in the mucosa, but CCrepe score 0.292 ($q = 1$) in the lumen. In contrast, *L. fermentum* and *Listeria monocytogenes* were highly correlated (CCrepe score = 0.9999, $q = 0.022$) in the lumen but uncorrelated (CCrepe score = -0.0177, $q = 1$) in the mucosa. *Lactococcus lactis* was correlated with both *Blautia wexlerae* (CCrepe score = 0.7530, $q = 0.02837$) and *Anaerobutyricum hallii* (CCrepe score = 0.9674, $q = 4.2932 \times 10^{-5}$) in the lumen, but uncorrelated with both (CCrepe score = 0.0315, $q = 1$; CCrepe score = 0.0398, $q = 1$, respectively) in the mucosa.

Genomic diversity among reconstructed bacterial genomes

From donor shotgun metagenomes, we reconstructed 12 near-complete bacterial genomes representing taxa with limited representation among close reference genomes: *E. lenta* ($n = 3$), *B. longum* ($n = 4$), *Collinsella aerofaciens* ($n = 2$), and single genomes of *L. fermentum*, *E. bolteae*, and *Escherichia coli* (**Table S9**). Comparative CAZyme profiling revealed species- and niche-specific functional patterns (Fig. 5a, b). *E. lenta* assemblies from the large intestine were enriched in GT126, GT32, and GT2 glycosyltransferases. *B. longum* assemblies from the small intestine showed increased CE20, CE8, GH172, and CBM13 families associated with carbohydrate degradation and binding. Across all assemblies, lumen-associated genomes were enriched in carbohydrate turnover genes, while mucosa-associated genomes contained more enzymes linked to glycan degradation and host-associated carbohydrate utilization.

Two *B. longum* genomes were recovered from distinct intestinal sites within Donor 1 (duodenum and ascending colon), enabling within-donor comparison of the strains across niches. The assemblies were highly complete and comparable in size (2.31 vs 2.28 Mb), yet whole-genome alignment revealed substantial divergence, with only 86.2% of the genome aligning at $\geq 95\%$ identity. Comparative analysis against the reference genome identified assembly-specific genes, many of which encoded hypothetical proteins (Fig. 5c). However, functional annotation revealed enrichment of carbohydrate metabolism-related genes in the duodenal assembly, whereas the ascending colon assembly contained more genes associated with transcriptional regulation, transport systems, and stress response pathways (Fig. 5d; **Table S10**), suggesting niche-specific genomic adaptation along the intestinal axis.

Bacteriophages of the intestinal tract

Using the viral-inclusive profiling tool Phanta, we characterized the intestinal virome and identified core bacteriophage populations across intestinal regions. Core vOTUs (prevalence $\geq 50\%$) were largely restricted to the large intestine, where two Caudoviricetes-associated vOTUs were consistently detected, including one predicted to infect *Parabacteroides* spp. In contrast, the small intestine exhibited minimal virome core structure, with only a single lumen-associated vOTU predicted to infect *Anaerostipes* spp.

Analysis of predicted bacterial hosts showed that bacteriophages in the large intestine primarily targeted common gut commensals, including *Alistipes*, *Bacteroides*, *Faecalibacterium*, *Parabacteroides*, and *Streptococcus*. In the small intestine, *Anaerostipes* was the predominant predicted phage host. No antimicrobial resistance genes were detected in any of the 218 assembled vOTUs screened against the CARD and ResFinder databases.

Discussion

Data from our study support a model of the human intestine as a spatially structured, multi-kingdom ecosystem in which microbial composition, function, and resistance profiles are shaped by interacting environmental gradients and host-specific constraints^{14,22,23}. By integrating multi-domain profiling with high spatial resolution across six intestinal segments and paired luminal and mucosal niches, we showed that microbial community assembly is governed by a hierarchy of ecological filters, with filter strength varying along both longitudinal and radial axes.

Along the intestinal axis, physicochemical gradients impose differential selection pressures that structure microbial communities¹⁰. The proximal small intestine, characterized by higher oxygen availability, exposure to bile, rapid transit, and fluctuating nutrient inputs, favors aerotolerant and metabolically flexible taxa adapted to dynamic conditions. In contrast, the distal colon supports dense anaerobic communities shaped by slow transit, stable redox conditions, and abundant fermentation substrates. Radially, the mucosal interface constitutes a distinct habitat defined by host-derived glycans, immune surveillance, and microaerophilic conditions generated by epithelial oxygen diffusion. Microorganisms occupying this niche must balance metabolic versatility with adhesion, immune evasion, and host interactions, thereby enriching enzymatic functions that process host-derived substrates. Together, these gradients define a continuum of ecological filtering strength, from strong abiotic constraints proximally to increased biotic interactions and metabolic interdependence distally. In our study, spatial structuring differed markedly across microbial domains, indicating that distinct ecological forces govern community assembly. In agreement with a previous study based on 16S rRNA amplicon sequencing⁹, bacterial communities exhibited moderate niche partitioning along intestinal oxygen and substrate gradients. Archaea showed strong distal enrichment dominated by methanogens²⁴, while viral distributions largely mirrored those of their bacterial hosts, suggesting host-dependent virome organization^{25–27}. In contrast, fungal communities displayed weak spatial structure and strong donor dependence, consistent with transient colonization and diet-responsive dynamics^{28,29}. Together, these findings highlight distinct ecological drivers shaping different microbial kingdoms across the intestinal tract.

A notable feature of this dataset is the predominance of donor identity as a determinant of community composition and resistome structure. Interindividual variation consistently exceeded differences attributable to intestinal segment or sampling milieu across multiple microbial domains, particularly fungi and antimicrobial resistance profiles. This persistence of donor-specific signatures across spatial

niches suggests that host history, including diet, immune tone, medication exposure, and early-life microbial acquisition, imposes a stable ecological template that constrains community assembly throughout the intestine. Importantly, these samples derive from organ donors, a context that may further influence microbiome structure through end-of-life factors such as critical illness, hospitalization, antibiotic administration, and physiological stress preceding death^{30,31}. While such factors may introduce additional variability or transient perturbations, they do not override the dominant signal of donor identity, underscoring the resilience of individualized microbiome configurations. Together, these findings argue against a universal "healthy" microbiome and instead support the concept of personalized microbial baselines shaped by host-specific ecological filters.

The resistome further highlighted strong donor-specific variation. Although regional differences in antimicrobial resistance gene abundance and mechanisms were observed, individualized profiles predominated, suggesting that the gut resistome reflects cumulative antimicrobial exposures and a history of horizontal gene transfer. Resistance determinants were relatively enriched in mucosal communities, indicating that host-associated niches may serve as reservoirs for persistent resistance genes. These findings are clinically relevant in organ donation, as donor-derived multidrug-resistant organisms can influence organ utilization, prophylactic strategies, and post-transplant infection risk³¹.

Correlation analyses showed that microbial relationships vary by intestinal location, with distinct groups of organisms co-occurring in specific niches. Species pairs that were strongly correlated in the small intestine were often uncorrelated or inversely correlated in the colon, and similar context-dependent relationships were observed between luminal and mucosal communities. These patterns suggest that microbial association structures are spatially contingent and vary across intestinal niches. Such consortia may stabilize ecosystem function through cooperative metabolism and resource partitioning, providing a mechanistic basis for resilience across intestinal niches²⁰. The spatial contingency of these associations suggests that successful microbiome-targeted interventions may require consideration of niche compatibility and community context rather than introducing individual strains in isolation.

Our findings confirm previously reported limitations of stool as a proxy for the intestinal microbiome. Stool primarily represents the distal luminal environment and therefore overrepresents high-biomass anaerobes and lytic phage activity while underrepresenting mucosa-associated communities and proximal niches^{7,32}. Functional enrichment at the mucosal interface and microbial populations adapted to the small intestine are largely invisible to fecal analyses. Rather than a comprehensive representation of the gut microbiome, stool should be viewed as a niche-specific sampling device. This distinction has important implications for interpreting disease associations and for the design of diagnostics and therapeutics based on fecal profiling.

The recovery of reference-divergent yet highly similar *Bifidobacterium longum* genomes across donors further indicates that human intestinal microbiomes harbor cohort-associated lineages that remain underrepresented in reference databases. Niche-specific differences in gene content between duodenal and colonic genomes within a single donor suggest niche-associated genomic divergence potentially

related to substrate availability, reinforcing the role of spatial selection in shaping strain-level diversity. These findings emphasize the need for expanded reference genomes and spatially resolved sampling to improve functional annotation and ecological inference.

Several limitations should be considered. The number of donors was constrained by logistical challenges associated with multi-segment intestinal sampling; however, the high spatial resolution of paired luminal and mucosal samples across six anatomical regions enabled robust within-host comparisons and donor-aware analyses. The reproducibility of spatial patterns across donors supports the biological relevance of observed gradients despite interindividual variability. Although samples were processed under anaerobic conditions within a defined postmortem interval, postmortem effects cannot be entirely excluded. Future studies incorporating larger cohorts, longitudinal sampling, and integrated host profiling will be essential to further elucidate mechanisms of host–microbiome interaction and to refine spatial models of gut ecology.

Collectively, spatially resolved organ-donor sampling revealed domain-specific ecological organization of the human intestinal microbiome across longitudinal and mucosal-luminal niches. Microbial domains respond differently to spatial constraints, functional metabolic profiles remained comparatively conserved despite taxonomic variability, and donor-specific signatures persist across niches. Recognizing the gut as a heterogeneous, individualized ecosystem challenges reliance on stool as a universal proxy and provides a framework for spatially informed, personalized microbiome diagnostics and therapeutics.

Conclusion

Spatially resolved organ-donor sampling reveals domain-specific ecological organization of the human intestinal microbiome across longitudinal and mucosal-luminal niches. Our findings indicate that the human gut microbiome is a spatially structured, multi-kingdom ecosystem shaped by environmental gradients, host interactions, and individual history. Microbial domains responded differently to intestinal niches, with pronounced bacterial and archaeal partitioning, host-associated functional enrichment at the mucosal interface, and virome distributions constrained by bacterial hosts. The predominance of donor-specific signatures in community composition and the resistome underscores the personalized nature of gut microbial ecology. The identification of novel strains and niche-dependent associations further indicates that local ecological conditions are associated with distinct community structures throughout the intestinal tract. Recognizing the gut as a heterogeneous and individualized ecosystem has important implications for microbiome research, challenging reliance on stool as a universal proxy and guiding the development of precision microbiome-based diagnostics and therapeutics.

Methods

Ethical approval and donor selection criteria

Organ donor samples were obtained from Honor Bridge (Formerly Carolina Donor Services, Durham, NC) between April 2017 and February 2021. The inclusion criteria for the donors were as follows: individuals 65 years old or younger, declared brain-dead, and tested negative for HIV, hepatitis, syphilis, tuberculosis, and COVID-19. Additionally, donors had no history of undergoing bowel surgery, did not experience severe abdominal trauma, nor had a history of cancer or chemotherapy treatment. Samples from pancreas donors were collected from the distal duodenum, whereas samples from non-pancreas donors were collected from the mid-section of the duodenum. The University of North Carolina's Institutional Review Board established that this investigation did not fall under the human subjects research category.

Organ donor sample processing

To preserve microbial community integrity, intestinal segments were transported intact on ice to suppress microbial metabolism immediately following procurement, and dissection commenced within 8 hours after cross-clamping, with comparable procurement intervals across donors. Segments remained unopened during transport to minimize oxygen exposure prior to anaerobic processing and were not opened or flushed prior to anaerobic processing. Upon arrival, all samples were transferred directly into an anaerobic chamber (Coy Laboratory Products Inc., MI) maintained at 37°C with a gas mixture of 85% N₂, 10% H₂, and 5% CO₂. All instruments, buffers, and collection materials were pre-reduced in the chamber overnight to limit oxidative stress during processing.

Cold-chain transport and rapid transfer to anaerobic conditions were employed to suppress microbial growth and metabolic activity during the postmortem interval. Luminal contents and mucosal scrapings were collected immediately after chamber entry using sterile instruments, with care taken to minimize handling time and environmental exposure.

The intestinal samples were obtained from six segments (duodenum, jejunum, ileum, ascending colon, transverse colon, and descending colon), and each fragment was subdivided into luminal contents and mucosal scrapings, yielding 12 samples per donor (**Figure S9**). Mucosal scrapings were collected using a sterile surgical blade, and luminal contents were obtained using sterile disposable culture loops. Mucosal samples were obtained from intact tissue surfaces, which are less susceptible to rapid postmortem compositional shifts than luminal contents. All procedures were designed to minimize oxygen-driven shifts and postmortem bloom.

Bacterial DNA extraction and host depletion

The QIAamp DNA Microbiome Kit was utilized to extract microbial DNA from complex samples while concurrently depleting host DNA. This approach combines mechanical and enzymatic lysis with Benzonase® nuclease treatment and specialized magnetic beads to enhance the representation of

microbial DNA in the final extract, thereby improving the sensitivity and accuracy of downstream analyses.

Whole Genome Shotgun (WGS) sequencing

A total of 5 ng of genomic DNA was prepared using the Nextera XT Sample Preparation Kit (Illumina, San Diego, CA) as described³³. Briefly, the target DNA underwent simultaneous fragmentation and tagging using the Nextera Enzyme Mix. This enzyme mix fragments the input DNA and appends the bridge PCR (bPCR)-compatible adaptors. During this process, index 1 (i7) and index 2 (i5) were incorporated between the downstream bPCR adaptor, the core sequencing library adaptor, and the primer sequences needed for cluster formation. The thermal profile for the amplification process consisted of an initial extension step at 72°C for 3 minutes and an initial denaturing step at 95°C for 30 seconds. Subsequently, there were 15 cycles of denaturing at 95°C for 10 seconds, annealing at 55°C for 30 seconds, a 30-second extension at 72°C, and a final extension for 5 minutes at 72°C. After amplification, the DNA library was purified using Agencourt® AMPure® XP Reagent. Each sample was then quantified and normalized before pooling. Finally, the DNA library pool was loaded onto the Illumina platform reagent cartridge and analyzed using the Illumina instrument³⁴.

Data analysis

Sequencing output from the Illumina Nova Seq 6000 platform was converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.20.0³⁵. The quality of the demultiplexed sequencing reads was verified using FastQC³⁶. The adapters were trimmed using Trim Galore³⁷. The median was 58,298,622 reads per sample, and the mean was 76,974,408 reads per sample. Six samples had fewer than 1 million reads, 77 had more than 10 million, and 35 had more than 100 million. The percentage of unclassified reads was relatively low, with a median of 4.81% and a mean of 6.71% across samples, ranging from 0.47% to 22.01%. The resulting paired-end reads were classified with Kraken2³⁸ and Bracken 2.5³⁹, and all reads identified as hosts were eliminated. Paired-end reads were joined with vsearch 1.10.2⁴⁰. The resulting single-end reads were again trimmed of any remaining adapters using Trim Galore. Estimates of taxonomic composition, gene family, path abundance, and path coverage were produced from the remaining reads using HUMAnN3⁴¹. Core taxa were identified from Kraken 2.1.2⁴² estimates species occurring with relative abundance greater than 0.0005 and occurring in at least 50% of the samples for each milieu and tissue type.

Core Enzyme Commission (EC) numbers were identified from HUMAnN3⁴³ and estimates occurring with relative abundance greater than 0.0001 and occurring in at least 50% of the samples. Tree representation of Kraken classified species and EC reactions was constructed from NCBI Taxonomy Common Tree⁴⁴ and the ENZYME - Enzyme nomenclature database, respectively⁴⁵. The pathways of the core enzyme were identified using the KEGGREST function of the R software package⁴⁶. Thirteen

metabolism pathways were used in the annotation. The seven functional classes were based on the type of reaction in which they are used to catalyze. EC tree annotations were built with the Interactive Tree of Life⁴⁷. Correlations between species relative abundance were calculated with CCREPE for Kraken estimated species occurring with more than 5000 reads and in at least 20% of the samples for the given intestinal section or milieu. Principal coordinate analysis and visualization were performed using QIIME 2⁴⁸. Clustering was calculated using the Ward D method⁴⁹ implemented in R 4.2.2⁵⁰ and Leiden⁵¹.

To identify viral sequences from whole-metagenome data, both assembly- and reference-based approaches were applied. Raw reads were pre-processed using fastp⁵² (v0.23.4; parameters: ‘--length_required 50 --detect_adapter_for_pe’) for adapter trimming and quality filtering. Reads corresponding to the human genome were then identified and removed by mapping reads to Homo sapiens genome (GCF_009914755.1_T2T-CHM13v2.0) using Kraken (v2.1.3). Finally, sequences corresponding to the common Illumina spike ΦX174 were removed using the ‘bbduk.sh’ script from the bbmap suite (v39.06; <https://sourceforge.net/projects/bbmap/>; parameters: ‘k = 31 hdist = 1’). Throughout these steps, read quality was assessed using FastQC9 (v0.12.1) and MultiQC10 (v1.24.1).

For the assembly-based method, reads were assembled per sample and per donor using MEGAHIT⁵³ (v1.2.9), retaining contigs ≥ 5 kbp. Redundant sequences were removed using Vclust⁵⁴ (v1.2.8; ANI $\geq 95\%$, qcov $\geq 85\%$). Viral contigs were identified with geNomad⁵⁵ (v1.8.1), Jaeger⁵⁶ (v1.1.26), and circular contigs with VRCA. Viral predictions were quality-assessed with CheckV⁵⁷ (v1.0.3); prophage sequences were trimmed and re-evaluated. Only contigs meeting quality criteria (e.g., $> 50\%$ completeness and $< 5\%$ contamination, or novel sequences $> 10\text{kb}$) were retained. Final viral operational taxonomic units (vOTUs) were clustered using Vclust (Leiden algorithm, ANI 95%).

Taxonomy was assigned via geNomad. Host predictions were made using iPhoP⁵⁸ (v1.3.3), and viral lifestyle (virulent vs temperate) was inferred using BACPHLIP⁵⁹ (v0.9.6; confidence ≥ 0.95). Abricate (v1.0.1, <https://github.com/tseemann/abicate>) with the CARD⁶⁰ and Resfinder⁶¹ databases was used to screen for AMR genes.

Quantification of vOTUs was performed by mapping reads to assembled contigs using bowtie2⁶² (v2.5.4, end-to-end mode). Read counts were processed using SAMtools⁶² (v1.21), normalized by contig length and library size, and filtered to exclude spurious mappings ($< 75\%$ 1x coverage).

For the reference-based approach, filtered reads were mapped using Phanta⁶³ (v1.1.0) with the “uhggv2_uhgv_mqplus” database. Host and lifestyle predictions followed Phanta’s documentation, with virulence scores > 0.5 considered virulent.

All downstream virome analyses were performed in R (v4.4.1). α -diversity was computed using the vegan package (v2.6.8). β -diversity was based on Aitchison distance using CLR-transformed data, with vOTUs present in $< 5\%$ of samples excluded. Statistical tests included PERMANOVA, ANOSIM, and PERMDISP (1,000 permutations). Visualizations were generated using ggplot2 and patchwork.

Determination of community-level differences

Community-level differences in microbial composition were evaluated using a multifactor, donor-aware framework. The OTU table was treated as compositional count data and transformed using a centered log-ratio (CLR) transformation following the addition of a pseudocount of 1 to address zero values. Community dissimilarities were calculated in Aitchison space (Euclidean distance on CLR-transformed data), and permutational multivariate analysis of variance (PERMANOVA) was performed to test for differences in composition. To account for the non-independence of samples from the same donor, permutations were constrained within donor using the Freedman–Lane approach. The model included tissue (intestinal segment), sample type (milieu: lumen versus mucosa), and their interaction (tissue × sample type) as fixed effects, with donor included to control for host-specific variation.

Core microbiome definition

Shotgun metagenomic reads were taxonomically classified using Kraken/Bracken against a comprehensive microbial reference database. Relative abundances were calculated for each taxon by normalizing read counts to the total reads per sample. To minimize host-derived artifacts, taxa corresponding to mammalian genomes (e.g., *Mus musculus* misclassifications) were excluded prior to analysis. A cohort core microbiome was defined separately for each intestinal region and sampling milieu by applying abundance and prevalence thresholds. Taxa were first filtered to retain those with a per-sample relative abundance $\geq 0.01\%$ (0.0001 as a fraction). Prevalence was calculated as the proportion of samples within each subset (e.g., small intestine, colon, lumen, mucosa) in which a taxon exceeded this abundance threshold. Taxa present in $\geq 50\%$ of samples within a given subset were designated as core members. Core microbiomes were computed independently for bacteria, fungi, archaea, viruses, and protists to account for domain-specific ecological dynamics and to prevent cross-domain differences in abundance distributions from biasing core definitions.

Genome assembly and comparative genome analysis

Metagenome-assembled genomes (MAGs) were reconstructed from donor shotgun metagenomic sequencing data and filtered for high completeness and low contamination prior to downstream analysis. Genome assembly quality metrics, including genome size, contig number, N50, completeness, and contamination, were assessed using QUAST⁶⁴.

Comparative genome analyses were performed against closely related reference genomes downloaded from NCBI. Pairwise genome alignment and sequence similarity were assessed using minimap2⁶⁵, and alignment statistics including genome coverage and sequence identity were calculated from the resulting alignments. Whole-genome structural comparisons and identification of shared and assembly-

specific genomic regions were performed using MUMmer4⁶⁶. Gene content differences between assemblies were subsequently characterized through functional annotation and CAZyme profiling.

Quantitative PCR (qPCR) analysis

qPCR assays were conducted on a QuantStudio Q6 instrument (Applied Biosystems) as described^{23,67}. Primer sets used in the study are shown in **Table S11**. The standard curves were generated using the components of a microbial community standard (Zymo Research Corporation, Irvine, CA, USA).

Declarations

Conflict of interest statement

The authors declare no conflicts of interest regarding the research.

Supplementary information

Supplementary data to this article are attached as “supplementary information.”

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Data availability statement

The whole-genome shotgun metagenomic sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1470551. Processed metadata tables, computational workflows, analysis scripts, and visualization resources are publicly available through https://github.com/Yunan0525/Organ_Donor_2026.

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Figures

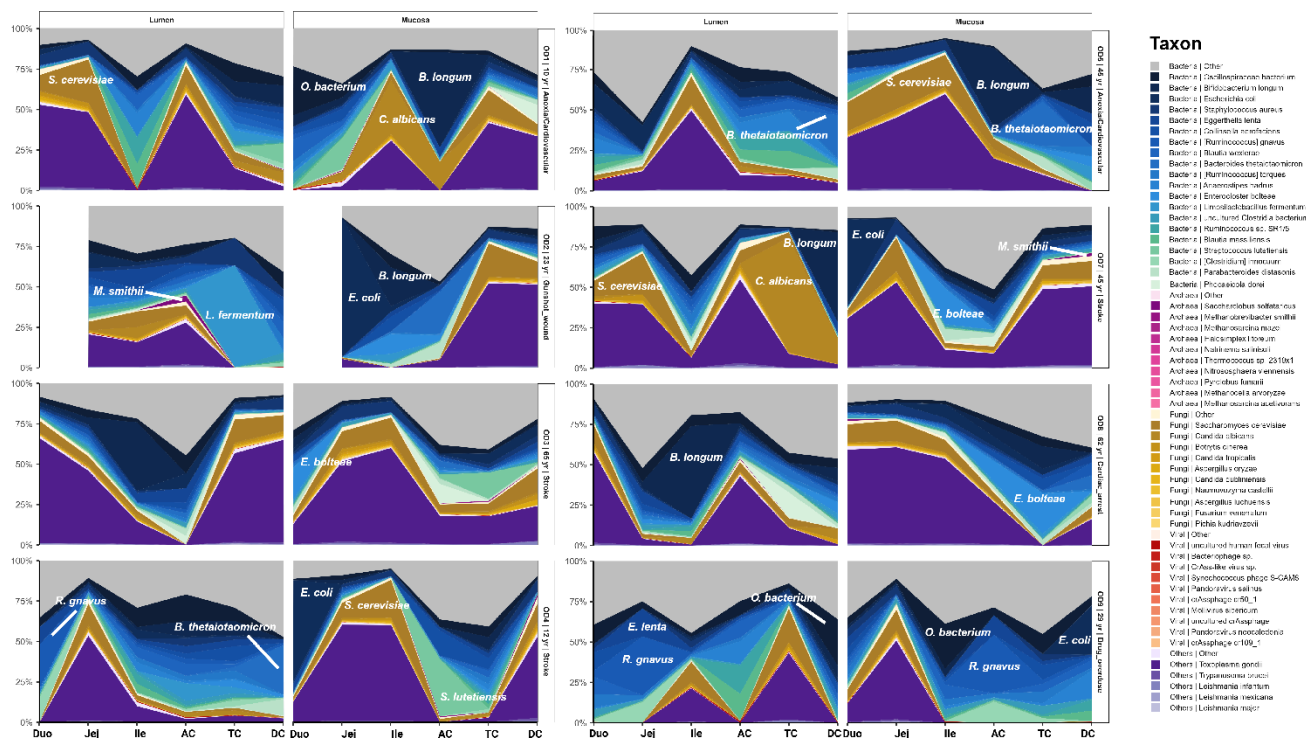


Figure 1

Multi-kingdom microbial composition across intestinal segments in human organ donors. Stacked area plots show the relative abundance of microbial taxa across six intestinal segments, including the duodenum (Duo), jejunum (Jej), ileum (Ile), ascending colon (AC), transverse colon (TC), and descending colon (DC), for all eight organ donors. Lumen and mucosa samples are shown separately for each donor. The dominant taxa within each microbial group are displayed individually, including the top 20 bacterial taxa, top 10 fungal, archaeal, viral taxa, and top 5 other taxa. Remaining lower-abundance taxa within each group were collapsed into group-specific “Other” categories. Distinct color schemes represent bacteriome, fungi, archaea, viral, and other microbial groups, with darker shades indicating higher-ranked taxa by overall abundance within each group.

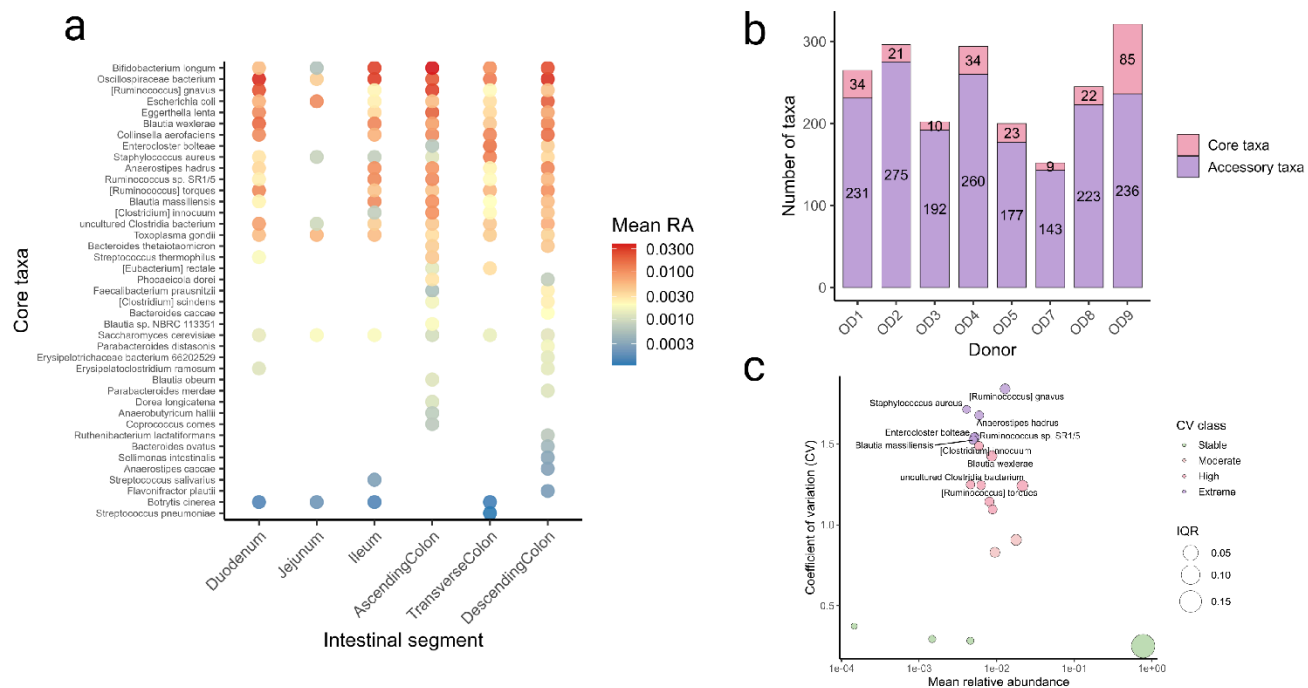


Figure 2

Spatial distribution and interindividual variability of core microbiome taxa. (a) Dot plot showing segment-specific core taxa across intestinal regions. Each point represents a taxon identified as core within a given segment, and color indicates its mean relative abundance. (b) Stacked bar plot showing the number of core and accessory taxa for each donor, illustrating interindividual differences in microbiome composition. (c) Bubble plot showing interindividual variability of global core taxa. Each point represents a taxon, with the x-axis indicating mean relative abundance and the y-axis indicating the coefficient of variation (CV). Point size reflects the interquartile range (IQR), highlighting differences in the dispersion of abundance across donors.

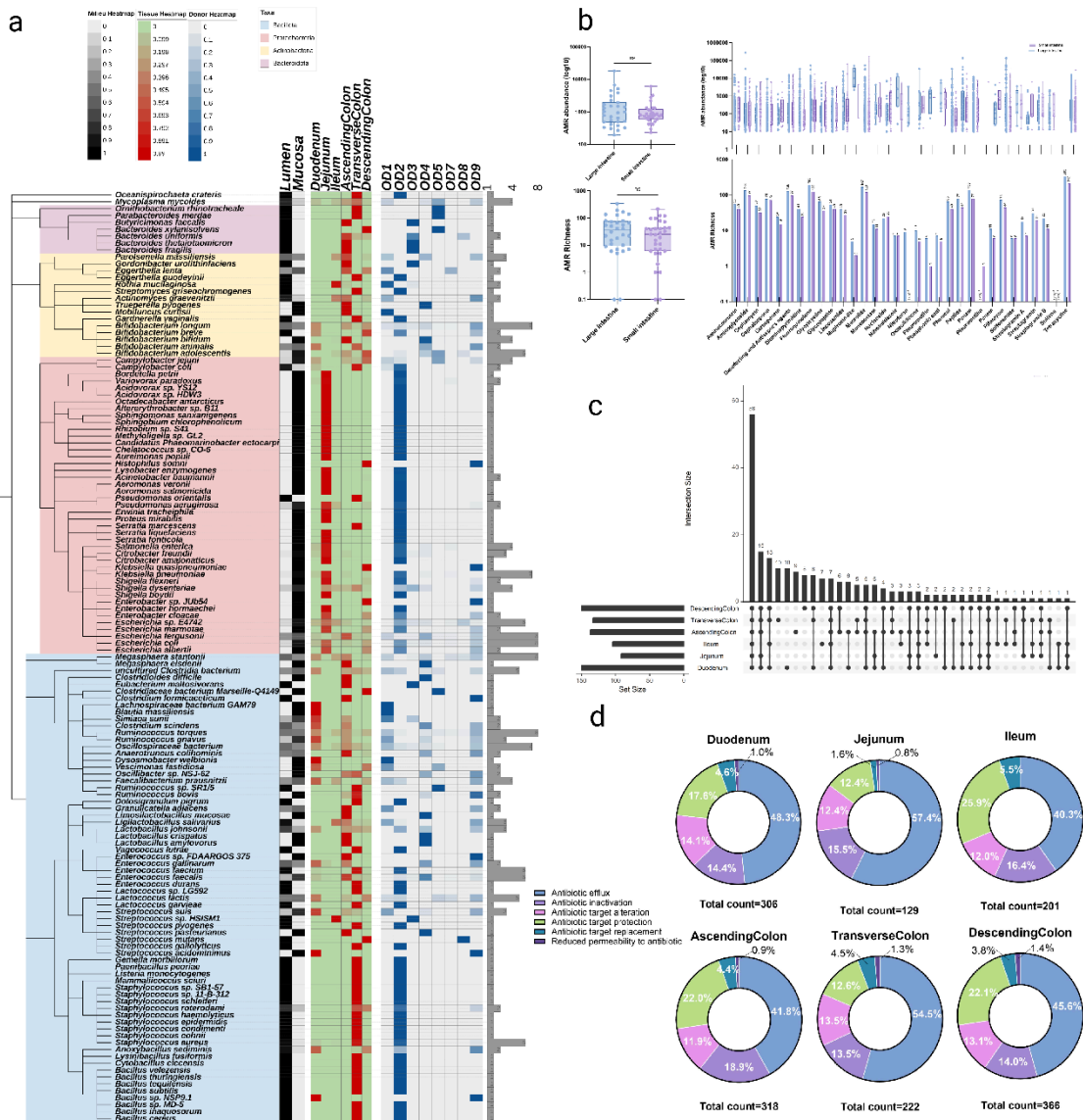


Figure 3

Distribution of antimicrobial resistance (AMR) genes across intestinal segments and donors. (a) Phylogenetic distribution of AMR gene-carrying species. The left panel shows a phylogenetic tree of bacterial species harboring antimicrobial resistance (AMR) genes. The right panels display associated heatmaps and summary metrics. The first heatmap shows the proportional distribution across sampling milieus (lumen vs. mucosa), with values indicating the fraction of observations in each milieu for a given species. The second heatmap shows the proportional distribution across sampling sections (Duodenum, Jejunum, Ileum, Ascending colon, Transverse colon, and Descending colon), with values indicating the

fraction of observations in each section for a given species. The third heatmap shows the proportional distribution across donors, with values indicating the fraction of observations from each donor for a given species. The bar plot indicates the number of donors in which each AMR gene–carrying species was detected. (b) Summary of AMR gene richness and relative abundance at drug class stratified by section.[YH1] (c) Distribution of AMR genes across intestinal segments. An UpSet plot illustrates the overlap of AMR genes among the six intestinal segments. (d) Composition of AMR drug classes across gastrointestinal segments. Donut plots display the relative composition of AMR mechanisms within each segment, showing both total gene counts and percentages for each resistance mechanism.

CAZyme families are shown based on mean abundance differences.

(c) Circular genome comparison between the reference genome and two *Bifidobacterium longum* genomes reconstructed from the same donor but different intestinal segments (duodenum, pink; ascending colon, purple), illustrating shared and strain-specific genomic regions. (d) Comparison of major metabolic pathway gene counts between the two *B. longum* strains, highlighting segment-associated functional differences in metabolic potential.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS10Bifidobacteriumlongumassemblies.xlsx](#)
- [TableS5FunctionCoreEC.xlsx](#)
- [TableS4Coretaxa.xlsx](#)
- [TableS3SummaryofPERMANOVAanalysis.xlsx](#)
- [TableS7Clusteranalysis.xlsx](#)
- [TableS8CCrepescore.xlsx](#)
- [TableS1Donorinformation.xlsx](#)
- [TableS2Alltaxaabundancetable.xlsx](#)
- [TableS6AMRgenes.xlsx](#)
- [TableS11qPCRprimers.xlsx](#)
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