Microcosm cultures of a complex synthetic community reveal ecology and genetics of gut microbial organization

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Running title: Mucin microcosm culture of complex synthetic gut community

Keywords: mucosa lumen biogeography, microcosm, biofilm, gut microbiome, adhesion, colonization
Abstract

The behavior of microbial communities depends on both taxonomic composition and physical structure. Metagenomic sequencing of fecal samples has revealed the composition of human gut microbiomes, but we remain less familiar with the spatial organization of microbes between regions such as lumen and mucosa, as well as the microbial genes that regulate this organization. To discover the determinants of spatial organization in the gut, we simulate mucosal colonization over time using an in vitro culture approach incorporating mucin hydrogel microcosms with a complex yet defined community of 123 human strains for which we generated high-quality genome assemblies. Tracking strain abundance longitudinally using shotgun metagenomic measurements, we observe distinct and strain-specific spatial organization in our cultures with strains enriched on mucin microcosms versus in supernatant, reminiscent of mucosa versus lumen enrichment in vivo. Our high taxonomic resolution data enables a comprehensive search for microbial genes that underlie this spatial organization. We identify gene families positively associated with microcosm-enrichment, including several known for biofilm and adhesion functions such as efflux pumps, gene expression regulation, and membrane proteases, as well as a novel link between a coenzyme F420 hydrogenase gene family and lipo/exopolysaccharide biosynthesis. Our strain-resolved abundance measurements also demonstrate that incorporation of microcosms yields a more diverse community than liquid-only culture by allowing co-existence of closely related strains. Altogether these findings demonstrate that microcosm culture with synthetic communities can effectively simulate lumen versus mucosal regions in the gut, providing measurements of microbial organization with high taxonomic resolution to enable identification of specific bacterial genes and functions associated with spatial structure.

Main

Human gut microbiomes consist of diverse microbial taxa [1, 2], with typical complexity ranging on the order of over a hundred species in a single individual [3]. Spatial organization of gut microbes is linked to community function and host health [4–10] – in particular, different taxa are enriched between mucosa and lumen [11–16], and mucosal colonizing bacteria may be particularly able to regulate host-microbiome interactions and immunomodulation [17–21]. However, we still lack a high-taxonomic-resolution view of ecological differences between lumen and mucosa, and accordingly possess a limited understanding of genetic factors underlying this spatial structure. As within-species dynamics exist within gut microbiomes [22–25], we hypothesize that distinct spatial organization may (i) occur at the
level of individual strains, and (ii) be associated with specific gene families and pathways that regulate mucosa versus lumen colonization.

To test our hypotheses, we develop an integrated experimental-computational workflow that compares lumen- and mucosal-like niches within a complex gut community. By using metagenomic sequencing, we are able to profile microbes with high taxonomic resolution, enabling strain- and gene-level analysis. We use a synthetic 123 strain community modeled closely after the recently published hCom2 community [26,27], cultured *in vitro* with added mucin microcosms to provide a mucosal-like substrate for bacterial attachment distinct from the surrounding liquid supernatant [28,29]. To identify genetic correlates of microcosm colonization, we develop a computational workflow that uses a comprehensive search across KEGG Orthology (KO) gene families [30] to identify associations between gut spatial organization and underlying microbial genotypes, using phylogenetic regression to account for evolutionary relationships between taxa [31–33].

Our approach provides key advantages over existing alternatives: first, by using an in vitro approach that allows mucin microcosm and supernatant subpopulations to be independently sampled [29] – analogous to mucosa and lumen *in vivo* – we obtain information on spatial structure missing from stool sampling and traditional liquid culture. Independent sampling of lumen and mucosal subpopulations is also possible using *in vivo* human gut biopsy, but the invasiveness of this approach limits sample sizes and longitudinal measurements [34]. In contrast, our *in vitro* platform enables us to sample mucosal- and lumen-like community subpopulations across multiple passage timepoints, with statistical replicates. Second, using our defined 123-strain community – which we generate high quality genomes for each member therein – allows us to emulate the bacterial complexity found in human guts, yet still accurately quantify abundance using metagenomic sequencing even between closely related strains. Strain-level measurements are critical for enabling gene-level analysis, as they allow genetic comparisons between closely related taxa. By comparison, earlier work with microcosms used 16S sequencing of undefined communities to produce measurements with more limited taxonomic resolution and did not examine genes associated with microcosm colonization [29].

We demonstrate that this approach yields detailed strain-level measurements of differential spatial organization, revealing taxa which are reproducibly enriched or depleted on mucin microcosms relative to supernatant. Then, we identify numerous genes and biosynthetic gene clusters that distinguish micro-
cosm versus supernatant genomes consistently across phylogenetic lineages, including genes related to cell adhesion and biofilm formation whose presence differs between closely related strains with distinct microcosm enrichment profiles.

## Results

**Closed genomes enable strain-level metagenomic profiling of complex defined microbial communities**

Starting from isolate cultures of 123 bacterial strains that are prevalent in the human gut microbiome (Fig. 1A, Table S1), we first generate high-quality, contiguous genomes for all strains other than five with closed genomes already. For the other 118 strains, we perform hybrid assembly of long Nanopore (median $3.9 \times 10^4$ reads/strain) and short Illumina reads (median $1.7 \times 10^6$ reads/strain) (Fig. S1, Methods), successfully generating closed assemblies with no more than 10 contigs. By contrast, the closest available NCBI genome (Fig. 1A) is more fragmented (78/123 comprise more than 10 contigs) and less closely related to the strain in our defined community; 20/123 have $>0.1\%$ ANI difference to our strain, and 33/123 contain 100 or more differential KEGG Orthology (KO) gene families (see Methods, Fig. S2). Thus, our reference database of closed genomes that are exact strain matches is critical for accurate strain and gene-level characterization of metagenomic data. Next, isolate strains are combined into a single community using anaerobic automated liquid handling (See Methods, Fig. S3), and inoculated into cultures containing 0.5% mucin 1% agar microcosms and MEGA media with 6 3-day passages (Fig. 1B, Methods). As a control, we also culture in parallel the same inoculum with MEGA media only, i.e., liquid-only culture. We use metagenomic sequencing of microcosms and supernatant sampled independently ($1.2 \times 10^7$ read pairs per sample) at each passage to quantify strain relative abundances (see Methods, Fig. S4). To analyze read libraries with high taxonomic resolution, we use NinjaMap [27] with our custom genome database to generate strain-level abundances (Fig. 1C, Table S2) – we successfully validate NinjaMap results against lower taxonomic resolution species-level abundances generated using Kraken2 [35] with the UHGG database [2] (median $R^2 = 0.978070$ across samples, see Fig. S5).
Mucin microcosms increase community richness and promote strain-coexistence within *in vitro* cultures

Next, we characterize differences that result from spatial structure introduced by the incorporation of mucin microcosms. In cultures without microcosms, community richness drops from a median of 113.5/123 detected strains in the inoculum (detection cutoff 0.01% relative abundance, 1% horizontal coverage – some strains with non-viable glycerol stocks / isolates did not grow to sufficient ODs, see Fig. S7), stabilizing down to a median of 38.5/123 detected strains by the 4 late passages (passages P2-6, i.e., days 9-18). By contrast, microcosm cultures seeded with the same inoculum stabilize to a median of 62.5/123 and 65.5/123 detectable strains on microcosms and supernatant respectively (Fig. 1D). This significantly elevated richness (\( p < 1^{-5} \)), see Fig. S6) parallels results from hCom2 inoculated in mice (median 56/119 detected strains across 19 mice) [26], suggesting cultures with microcosms provide a closer analog to *in vivo* conditions than do liquid-only cultures. Increased richness is particularly noticeable in Firmicutes, Firmicutes_A, and Bacteroidota (see Fig. S6), while total abundance is higher for Firmicutes and Firmicutes_A, but lower in Bacteroidota (Fig. 1E).

Beyond phylum level effects, abundance shifts also occur at strain level. Addition of microcosms increases abundance for a diverse set of strains including *Bacteroides caccae* ATCC-43185, *Lactobacillus ruminis* ATCC-25644, *Coprococcus comes* ATCC-27758 (which displays extremely sticky / slime phenotype in pure culture), two strains in family Marinifilaceae (*Butyricimonas virosa* DSM-23226, *Odoribacter splanchnicus* DSM-20712), and both sulfur reducing bacteria (*Desulfovibrio piger* ATCC-29098 and *Bilophila wadsworthia* ATCC-49260 from phylum Desulfobacterota). Some taxa are largely unaffected by microcosms, such as three Bifidobacterium strains, while few taxa are negatively affected by microcosms, with three closely related Veillonella strains being notable exceptions (see Fig. S8). These strain-level abundance shifts do not always align with corresponding phylum-level shifts, emphasizing the value of our highly-resolved taxonomic measurements.

One of the most striking abundance shifts revealed by strain-level analysis is the co-existence of closely related strains with the addition of microcosms. In liquid-only culture, *Bacteroides dorei* DSM-17855 outcompetes two closely related (ANI > 99%) strains, *Bacteroides dorei* 5-1-36-D4 and *Bacteroides sp.* 9-1-42FAA (Fig. 1F). By contrast, these three strains coexist stably in culture when microcosms are present. Other examples can be found between two closely related (ANI ~ 80%) Firmicutes_A strains:
Subdoligranulum sp. 4-3-54A2FAA and Subdoligranulum variabile DSM-15176 (Fig. 1G), and between two closely related (ANI ∼ 80%) Firmicutes_C strains: Acidaminococcus fermentans DSM-20731 and Acidaminococcus intestini D21 (Fig. 1H). These observations of co-existence (see Fig. S8 for additional examples) concur with increased richness detected in microcosm cultures.

To better understand why community richness increases with mucin microcosms, we additionally grow our inoculum in cultures with 1% agar microcosms (plain-agar, i.e., no mucin). We observe that plain-agar microcosm culture also exhibits overall enhanced richness compared with liquid-only culture (Fig. S6). However, we do note some specific strain-level differences between mucin-agar and plain-agar microcosm cultures (See Fig. S7, S8). This suggests that increased richness result largely – but not entirely – from having a physical surface to colonize rather than the nutrients provided by mucin. These results are reminiscent of similar effects in bacterial biofilms, where increased diversity has been attributed to expanded spatial niches and reduced competition [36–38].
Figure 1: (Previous page) Microcosm cultures yield stable, diverse communities with coexistence of closely related strains. (A) We generate closed, high quality genomes for each strain in a 123-member microbial community, representative of taxa in the human gut. De novo generated genomes are more contiguous than closest previously available NCBI genomes, and represent exact matches to our strains. (B) We use this 123-member community to inoculate cultures incorporating mucin microcosms as well as non-microcosm controls. We passage (P) each culture 6 times (3 days between passages), independently sampling bacterial DNA from microcosm, supernatant, and no-microcosm control at each timepoint for downstream metagenomic sequencing. (C) We use NinjaMap to obtain community relative abundances from metagenomic sequencing data, here we plot median abundance of each strain at each passage timepoint, across experimental conditions. (D) Number of detected strains after culture stabilization (∼P3 and later) is higher in microcosm versus no microcosm cultures, indicating enhanced community richness. Grey dashed line indicates median number of strains detected (56) using same threshold with the 119-member hCom2 community in mice [26] (E) Addition of microcosms leads to broad taxonomic shifts in community composition relative to no-microcosm control, visualized here at phylum level. (F) Strain-resolved abundance patterns of 3 B. dorei strains (ANI > 99%) in our community demonstrates stable co-existence enabled by addition of microcosms, compared with dominance of a single B. dorei strain without microcosms. (G) Strain-resolved abundance patterns of 2 Subdoligranulum strains (ANI ∼ 80%) in our community demonstrates stable co-existence enabled by addition of microcosms. Subdoligranulum variabile DSM-15176 in particular also exhibits increasing abundance over passage timepoints. (H) Strain-resolved abundance patterns of 2 Acidaminococcus strains (ANI ∼ 80%) demonstrates more stable co-existence when cultured in the presence of microcosms.

Strains exhibit distinct enrichment profiles between microcosm and supernatant communities

We next characterize spatial organization within microcosm cultures by comparing subpopulations sampled from microcosm and supernatant, testing our hypothesis that strain-level spatial differences occur within gut communities. For the 86/123 prevalent strains that are detected in at least 10% of passaged samples (see Methods), we quantify a microcosm enrichment score – defined as the log fold change in abundance between paired microcosm and supernatant samples (i.e., derived from the same culture tube) – for each strain and each passage (Fig. 2A, Table S3). We also calculate a single aggregate, normalized log-microcosm-enrichment score for each strain based on late passage measurements (see Methods). These scores reflect the preference of each strain to grow on microcosms versus in the supernatant, with positive scores indicating microcosm preference.

Aggregating at phylum level, we observe enrichment toward mucin microcosms in Desulfo bacterota, Firmicutes (primarily Bacillus-like), and Firmicutes_A (primarily Clostridia-like), and enrichment toward supernatant in Actinobacteriota, Bacteroidota, and Firmicutes_C (primarily Negativicutes-like), with
no obvious time-dependent signal (Fig. 2B). These results are largely consistent between mucin-agar and plain-agar microcosms, with the exception of Desulfobacterota which is not enriched on plain-agar microcosms. Certain individual strains also exhibit similar trends, such as Eubacterium ventriosum ATCC-27560 which exhibits microcosm preference with mucin-agar microcosms but not plain-agar (Fig. S9).

At strain level, we find a diverse range of enrichment profiles over time (Fig 2A), including several strains with opposite enrichment relative to their phylum. For instance, *Bacteroides sp.* 2-1-22 prefers microcosms, while *Clostridiales bacterium* VE-202-14 from phylum Firmicutes_A prefers supernatant. Moreover, closely related strains can exhibit different enrichment phenotypes: *Bacteroides dorei* 5-1-36-D4 and DSM-17855 exhibit similar abundance in supernatant and microcosm (log enrichment scores \( \approx 0 \)), but *Bacteroides sp.* 9-1-42FAA displays consistent enrichment toward supernatant (log enrichment scores \( < 0 \), see Fig. 2C). *Subdoligranulum variabile* DSM-15176 and *Acidaminococcus fermentans* DSM-20731 prefer mucin microcosms more than their respective counterparts, *Subdoligranulum sp.* 4-3-54A2FAA (Fig. 2D) and *Acidaminococcus intestini* D21 (Fig. 2E). These findings support our hypothesis that distinct strain-level spatial organization occurs within gut communities.

Finally, as external validation we compare our in vitro microcosm enrichment results against an *in vivo* dataset [15] with paired mucosal and lumen samples (see Methods, Table S6). We find our *in vitro* microcosm-enrichment strain scores exhibit similarity to *in vivo* mucosal-enrichment species scores within inter-subject variability (Fig. S10, Table S7). We also observe general agreement at phylum level: Bacteroidota is enriched toward both supernatant *in vitro* and lumen *in vivo*, while Firmicutes_A and Firmicutes are enriched toward microcosm / mucosa. However, discrepancies also exist, as Actinobacteriota is enriched toward supernatant *in vitro* and mucosa *in vivo* (Fig. S10). These results suggest that our experimental platform provides a close – though not exact – approximation of *in vivo* structure.
Figure 2: Strain level differences exist between mucin microcosm and supernatant communities. (A) Strains exhibit different microcosm enrichment phenotypes, both within and between clades – positive (red) scores indicate higher relative abundance on microcosms versus supernatant. (B) Aggregated at phylum level, taxa exhibit evidence of distinct spatial structure: Desulfobacterota, Firmicutes and Firmicutes_A are enriched on microcosms, while Actinobacteriota, Bacteroidota and Firmicutes_C are enriched in supernatant. (C) One of the three Bacteroides dorei strains (sp. 9-1-42FAA) exhibits consistent microcosm depletion relative to the other two strains (5-1-36-D4 and DSM-17855). (D) Subdoligranulum variabile DSM-15176 exhibits consistent microcosm enrichment relative to the closely related strain Subdoligranulum sp. 4-3-54A2FAA. (E) Acidaminococcus fermentans DSM-20731 exhibits consistent microcosm enrichment relative to the closely related strain Acidaminococcus intestini D21. Additionally, microcosm enrichment of Acidaminococcus fermentans DSM-20731 increases with time towards later passages.
Phylogenetic regression predicts genes associated with mucosal colonization

We next test for statistical associations between microcosm enrichment and underlying microbial genotypes, evaluating our hypothesis that key microbial genes may regulate spatial organization in the gut. Using kofamscan [30] to comprehensively search all genomes against all defined KO families, we generate a genotype matrix consisting of 9857 KOs detected in the 86 prevalent strains. Each entry in this $86 \times 9857$ matrix corresponds to maximum kofamscan/hmmer bitscore hit for a particular KO in a particular genome (Fig. 3A) – higher scores reflect gene presence. We then test for each of the 9857 KOs whether its genotype pattern across the 86 strains is significantly associated with the corresponding pattern of microcosm enrichment scores (phenotype). We perform significance tests using phylogenetic regression with phylolm [32] to account for evolutionary relationships between strains (see Methods).

Our approach identifies 244 KO families significantly associated with increased enrichment on mucin microcosms relative to supernatant applying FDR correction at $p<0.01$ threshold (Fig. 3B, see also Methods, Table S8). Out of these KOs, we highlight several illustrative examples whose genotype patterns align with differential microcosm enrichment in the *B. dorei, Subdoligranulum* and *Acidaminococcus* strains featured in Fig. 2C-E. From the three *B. dorei* strains, we find two KO gene families in particular – K00441 (coenzyme F420 hydrogenase subunit beta [EC:1.12.98.1], Fig. 3C, 4A) and K08217 (MFS transporter, DHA3 family, macrolide efflux protein, Fig. 4B) – which have strong homology hits in *Bacteroides dorei* 5-1-36-D4 and DSM-17855, but not in *Bacteroides sp.* 9-1-42FAA. Mapping the K00441 coenzyme F420 hydrogenase hits to their genomic loci in 5-1-36-D4 and DSM-17855, we find the gene resides in the midst of lipo/exopolysaccharide (LPS/EPS) biosynthesis gene clusters (Fig. 3C). Performing gene neighborhood analysis across all 123 strain genomes to search for KOs enriched within 10 kilobases (kb) of K00441 annotated genes, we find 107 hits (see Methods, Table S5), which are dominated by KOs with LPS/EPS biosynthesis functions including numerous glycosyltransferase, epimerase, sugar-reductase, polysaccharide membrane transporter genes, suggesting a previously uncharacterized link between coenzyme F420 hydrogenase and microbial LPS/EPS production.

Beside K00441 and K08217 in *B. dorei*, we also note a strong hit to a DEAD box helicase gene family – K14440, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 [EC:3.6.4.12] – in *Subdoligranulum variabile* DSM-15176 (Fig. 4C) and a membrane...
protease gene family – K14743, membrane-anchored mycosin MYCP [EC:3.4.21.-] – in Acidaminococcus fermentans DSM-20731 (Fig. 4D) which are absent in their less microcosm-enriched relatives. Intriguingly, LPS/EPS biosynthesis [39–43], membrane transporters/efflux pumps [44–50], membrane proteases [51–56], and DEAD box helicase gene regulators [41,57–60] all have known links to biofilm formation and adhesion. Aggregating all 244 microcosm-associated KOs by KEGG BRITE gene categories, we identify several BRITE categories enriched for significant KOs, representing antibiotic resistance genes, glycosyltranferases (E.C. 2.4), phosphotransferases (E.C. 2.7), transcriptional regulators, and proteases (Table S11), further supporting the importance of these gene functions in mucosal colonization.

Testing for clade-specific effects using within-phylum phylogenetic regression, we find K14440 and K14743 to be among the most significant hits in Firmicutes/Firmicutes_A/Firmicutes_C, while K00441, K14743 and K08217 are among the most significant hits for Bacteroidota (Table S9). As an external validation, we repeat our workflow using the Suez et. al *in vivo* dataset [15] to identify a list of KOs associated with mucosal enrichment (Table S10), and find statistically significant overlap between genes associated with microcosm enrichment *in vitro* and genes associated with mucosal enrichment *in vivo*, \( \log \text{-} \text{odds} \text{-} \text{ratio} = 4.0, p = 9.7 \times 10^{-21}, \text{Fig. S11} \). Thus, we confirm that measurements from our *in vitro* synthetic community cultures are sufficiently detailed to inform a computational gene-level analysis of gut spatial organization, revealing that genes related to biofilm formation and adhesion likely play key roles in modulating gut microbial structure.
Figure 3: **Phylogenetic regression identifies genes associated with mucin microcosm enrichment**

(A) Phylogenetic regression identifies significant associations between log microcosm enrichment score (red/blue indicates positive/negative microcosm enrichment respectively) and gene presence absence patterns (lighter/darker shades of gray indicate gene presence/absence respectively) across the most prevalent 86 strains detected in passaged samples. We use this model to test a total of 9857 KEGG KO gene families determined using kofamscan [30], accounting for phylogenetic relatedness between strains assuming Brownian motion along evolutionary branches. (B) Volcano plot of phylogenetic regression test, where each dot represents one KEGG KO – horizontal line at FDR=0.01. Horizontal axis is clipped at 0.1 and 99.9 percentiles, highlighted gene families colored in red. (C) *Bacteroides dorei* 5-1-36-D4 and DSM-17855 both harbor a coenzyme F420 dehydrogenase gene (KEGG KO K00441, *hmmer.bitscore* = 190.7, 188.7) colocalized amongst LPS/EPS related gene clusters – these features are collectively missing from the corresponding region in the *Bacteroides sp.* 9-1-42FAA genome.
Figure 4: Phylolm-identified gene families have presence patterns that align with differential microcosm enrichment (A) Comparison of microcosm enrichment pattern (left) with gene presence pattern (right) of K00441 coenzyme F420 hydrogenase subunit beta [EC:1.12.98.1], across family Bacteroidaceae strains. (B) Comparison of microcosm enrichment pattern (left) with gene presence pattern (right) of K08217 MFS transporter, DHA3 family, macrolide efflux protein, across family Bacteroidaceae strains. (C) Comparison of microcosm enrichment pattern (left) with gene presence pattern (right) of K14743 membrane-anchored mycosin MYCP [EC:3.4.21.-], across phylum Firmicutes_A, Firmicutes_C, and Firmicutes strains. (D) Comparison of microcosm enrichment pattern (left) with gene presence pattern (right) of K14440 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 [EC:5.6.2.-], across phylum Firmicutes_A, Firmicutes_C, and Firmicutes strains.
**Strain enrichment on microcosms is associated with presence of lipo/exopolysaccharide biosynthesis gene clusters**

To explore mechanisms of community structure beyond individual genes, we next investigate microcosm enrichment of biosynthetic gene clusters (BGCs). We use deepBGC [61] to search for BGCs across our strain genomes, annotate BGCs based on their KEGG KO presence, and apply hierarchical clustering to categorize 1103 detected BGCs into 256 groups with similar KO co-occurrence patterns (Fig. 5A, Table S12). We then map presence/absence of each of these 256 BGC-groups against the 86 prevalent strains in our experiment (Fig. S12), and apply phylogenetic regression to test for associations between microcosm enrichment and BGC-groups.

Our approach yields a total of 7/256 significant BGC-groups positively associated with microcosm enrichment (Fig. 5B), the three largest of which consist of 18 or more BGC representatives (BGC-group 157 – see Fig. 5C, BGC-group 120, and BGC-group 69). Filtering for the most common KEGG KOs in each of these BGC-groups, we discover that BGC-group 157 and BGC-group 120 consist of likely EPS related gene clusters, typified by glycosyltransferase, epimerase and other EPS related KOs (Fig 5D, Table S13). BGC-group 69 consists largely of gene clusters populated by membrane transporter genes. KOs in other microcosm enriched BGC-groups include more polysaccharide related genes (BGC-groups 198, 186, 161) and AraC transcriptional regulator genes (BGC-group 34). These findings at the BGC-level further reinforce our KO-level results, showing that membrane-related functions such as LPS/EPS and transporters, as well as key gene regulators, likely regulate spatial organization in our *in vitro* model of the human gut.

**Discussion**

Applying mucin microcosm culture with our defined community of human gut strains, we present here the first strain-resolved measurements of spatial structure within the context of a complex gut microbial community. By *a priori* generating a database of high quality closed reference genomes, our approach enables high taxonomic resolution abundance measurements using metagenomic sequencing, while effectively recapitulating spatial structure in the gut microbiome. These measurements show with high taxonomic resolution how a complex gut microbial community is spatially organized upon introduction...
Figure 5: Strain enrichment on mucin microcosms is associated with exopolysaccharide gene clusters. (A) Schematic of approach used to generate and group BGCs across strains using deep-BGC and hierarchical clustering. (B) Volcano plot of phylogenetic regression test, each dot represents one BGC-group, horizontal line at FDR=0.01 cutoff. Top hit BGC-group 157 highlighted in green. (C) Comparison of microcosm enrichment pattern (left) with presence pattern (right) of BGC-group 157 across strains in family Bacteroidaceae. (D) Example of a representative gene cluster in BGC-group 157 from the Bacteroides strain with highest microcosm enrichment score (Bacteroides-sp.2-1-22_cluster_1_1984776-2023109.1). Gene label colors reflect frequency of KO family among all BGCs in group – in cases where a gene maps to multiple KOs, * marks mapped KO with highest frequency in BGC-group.
of microcosms, demonstrating that microcosms enhance community richness to a level similar to *in vivo* observations, including instances of co-existence between closely related strains. We find clear enrichment signals *within* microcosm cultures where certain strains prefer to grow on the microcosms versus in the supernatant, or vice versa. Microcosm enrichment phenotypes can differ significantly even between closely related strains, supporting our hypothesis that spatial organization in the gut occurs at strain-level and trends would be missed at coarser taxonomic resolution.

Another benefit of using strain-resolved metagenomics is that we can identify gene families that specifically occur in strains with microcosm enrichment (or depletion) phenotypes. We do so using phylogenetic regression, a rigorous statistical approach that adjusts for evolutionary relationships between strains. This analysis identifies several gene families related to microbial adhesion and biofilm formation, including efflux pumps (e.g., K08217) that are known to mediate collective biofilm phenotypes such as quorum sensing and antibiotic resistance [44–50], and membrane proteases (e.g., K14743) which can enhance motility / colonization on surfaces [51–56]. We also find genes involved in biosynthesis of LPS/EPS which are known to mediate bacterial adhesion [39–43], such as glycosyltransferase and epimerase genes, as well as a particular gene family K00441 (coenzyme F420 hydrogenase subunit beta [EC:1.12.98.1]) for which we report significant genomic colocalization with other known LPS/EPS genes, suggesting a previously uncharacterized functional link. We also find several groups of biosynthetic gene clusters containing membrane transporters and LPS/EPS genes associated with microcosm enrichment. Beyond membrane-associated functions, our analysis also highlights regulatory genes such as SWI/SNF DEAD box helicases (K14440). Intriguingly, such genes have not only been shown to be involved in biofilm formation [41,57–60], but also specifically drive expression of efflux pumps and LPS/EPS genes [57]. We speculate that in mucosa-associated taxa, key regulator genes act as master switches for a host of bacterial functions that alter outer membrane composition to enhance biophysical interactions with the mucosal surface and thus increase mucosal colonization fitness, leading to global spatial organization of these taxa towards the mucosa (Fig. 6).

We conclude by noting several limitations to our work and point to areas for further exploration. First, we only show statistical associations – not causal mechanisms – between genotypes and microcosm enrichment, meaning hits should be cautiously interpreted as potential genetic factors deserving of followup investigation. Synthetic biology in genetically tractable gut strains can be used to test our
predictions by altering the expression of identified gene families using gene knockout, knockdown or knockin experiments [62–64]. Second, while our in vitro results generally parallel those from earlier in vivo work [15, 26], we do find limited discrepancies (e.g., microcosm depletion of Actinobacteriota), meaning our current platform provides a close but still imperfect replica of the in vivo gut environment. More realistic culture conditions can be explored, potentially through modification of media conditions (e.g., addition of bile acids, different carbon sources). Third, our current approach based on metagenomic sequencing provides accurate quantification of strain and gene abundance, but it does not assay gene expression or spatial localization on microcosms. Future work using gut microbial metatranscriptomic analysis [65, 66] and multiplexed FISH imaging [67–69] can greatly complement current capabilities and mitigate these shortcomings. Fourth, it remains unclear how strain-strain interactions affect structure. Follow-on studies with our platform that incorporate strain dropout can address these questions. Finally, in addition to strains from healthy Western guts, future work should incorporate taxa found in dysbiotic and non-Western guts to explore how spatial structure varies between healthy and diseased states, and across global geographic regions. Ultimately we believe the platform presented here has the potential to transform the standard for in vitro investigation of gut microbiota, in a manner that recognizes the important interplay between spatial structure and strain-level ecology.

Methods

Hybrid assembly of microbial isolates

Strains are cultured in isolation until stationary phase, followed by DNA extraction using phenol chloroform. DNA is sequenced using both Oxford Nanopore long-read and Illumina short-read sequencing,
followed by hybrid assembly using custom bioinformatic workflow (Fig. S2) built using Unicycler [70],
RScaf [71] and TGS-GapCloser [72] – workflow is available as docker images, see Software availability
below.

**Community phylogeny**

Phylogenetic tree structure of the community is generated using GTDB-tk [73], using our genome
assemblies as input.

**Genome annotation and gene classification**

Genomes are annotated using NCBI PGAP [74]. Predicted protein sequences are then mapped using
kofamscan [30] to the to KEGG Orthology database.

**Mucin microcosm preparation**

Mucin microcosms are prepared similarly to previously described protocols [28,75], using boiled 0.5%
porcine mucin (Sigma M2378) and 1% agar (BD 214030) solution solidified onto K1 biofilm carriers (Evo-
lution Aqua MEDIAK1). Mucin free agar-only microcosms are prepared using 1% agar solution.

**In vitro culture of synthetic community with mucin microcosms**

To construct the full *in vitro* synthetic community, we first culture each strain in isolation in 1.8 mL of its
preferred media in a 96 well deep well plate (Table S1). Because of the large range of growth rates and
stationary phase cell densities, strains are inoculated in a staggered fashion with slow growers inoculated 3
days prior and fast growers inoculated 1 day before community assembly. Fastidious growers are cultured
in 10 mL and concentrated to increase final cell density. Individual isolate cultures are sequenced to
verify purity. On the day of community assembly, cell density for each strain is estimated using OD
measured on a plate reader (BioTek Epoch). Using this measurement, each strain is normalized to a
maximum OD of 0.3 using liquid handling robotics. Cultures are pelleted and washed with PBS, and
then combined to form a mixture of 123 strains (epMotion 5073). Strains are combined in an anaerobic
environment equipped with automated liquid handling in order to reduce potential cross contamination
and other human errors when concurrently handling many strains (Fig. S3).
Following assembly of our bacterial community, the mixture is used to inoculate cultures in 15 mL tubes comprising MEGA media and 5 microcosms each. Cultures are left to grow at 37°C in anaerobic conditions for 3 days without agitation, at which point they are passaged. Passaging consists of transferring a single microcosm from the old culture tube to a new culture tube. This process is repeated 5 times for a total of 6 passages - for each subsequent passage, the previously transferred-in microcosm is discarded prior to transferring of a microcosm to the next culture. For liquid-only cultures, inoculating loops (Fisherbrand 01-189-165) are used for passaging. Supernatant pellets and microcosm samples are saved and frozen at each passage point.

For each condition, we culture the community in biological triplicate cultures (i.e. 3 separate culture tubes). Each culture tube is sampled with technical triplicates – for microcosm samples, we pick 3 microcosms out of each culture tube to store at −80°C prior to DNA extraction, while for supernatant and liquid-only cultures, we take 3 separate 1mL aliquots from each tube, pellet, then store at −80°C prior to DNA extraction. This yields a total of 9 read libraries for each passage and experimental condition. The initial inoculum communities are sampled in duplicate, each sample sequenced 3 times each.

**DNA extraction, Library prep and sequencing**

DNA is extracted from pellets and microcosms using ZymoBIOMICS 96 DNA Kit and bead beating with 0.1mm glass beads (Benchmark Scientific D1031-01). Extracted DNA from each sample is quantified in 384 well plates on a fluorescent plate reader (BioTek Neo2) using the Quant-iT PicoGreen assay (ThermoFisher). To generate input DNA for our high-throughput and low-volume Nextera XT library preparation process, DNA samples are normalized to at maximum of 0.2 ng/μL in a 384 well plate using a low volume cherry picking liquid handler (SPT). Library preparation is done in 384 well plates using a low-volume 16 channel liquid handler (SPT) and follows the chemistry of the Nextera XT process but in a total volume of 4 μL in order to reduce library preparation cost. Libraries are quantified again using the Quant-iT PicoGreen assay and normalized. After pooling and cleaning using Ampure XP beads (Beckman), libraries are sequenced on a Novaseq 6000 (Illumina) to a mean depth of $1.2 \times 10^7$ read pairs per sample. In addition to DNA derived from microbial communities, we also sequenced all input strains used to construct the community to ensure strain purity and identity.
Read mapping and abundance estimation

Read mapping is performed with NinjaMap as previously described [27], using our de novo generated genomes as reference database. Briefly, reads are aligned to genome sequences, with only perfect unique matches considered in the first round. Ambiguous reads are held in escrow for the first round, and subsequently assigned in a statistically weighted manner determined by initial abundance estimates from the first round of alignment. This generates relative abundance and horizontal genome coverage estimates for each strain in each sample's read library. We consider a strain present in a sample if it exceeds a 1% horizontal coverage and 0.01% relative abundance cutoff. Out of all 270 passage samples (6 passages × 5 experimental conditions – mucin-agar microcosms, mucin-agar supernatant, plain-agar microcosms, plain-agar supernatant, no-microcosms – × 9 replicates), we use a prevalence cutoff of 10% presence (i.e. present in 27 or more samples) to focus on the most prevalent strains. For downstream abundance-related analysis, we collapse technical (i.e., within tube) triplicates to their median abundance measurements, while considering biological (different culture tubes) triplicate measurements separately. Table S2 lists relative abundance and horizontal coverage across strains, passages, replicates and experimental conditions.

Mucosal enrichment calculations

For each strain, and passage, microcosm enrichment scores are calculated as log ratio of microcosm to supernatant abundance, for 3 biological replicates, replacing zeros with half-minimum non-zero value prior to taking log. For each strain, a single aggregate microcosm is generated by taking the mean of over mean over standard deviation of 12 log ratio scores in the late passages (P3-6, 4 passages × 3 biological replicates). Table S3 lists enrichment scores per strain.

Gene neighborhood enrichment test

Based on results from kofamscan for each gene in each genome, a gene is annotated with a KO-label if it exhibits overlap greater than $0.5 \times$ coverage with the KO’s pHMM model, as well as a bitscore greater than $0.5 \times$ the KO’s bitscore threshold. We count the frequency of all annotated KOs within 10 kb of K00441-labeled genes across the full community genome database. To generate p-value estimate of this measured frequency, we compare it against a null distribution generated by 1000 random gene
order permutations. In each of these 1000 permutations, we randomly reassign gene labels within each of the 123 genomes prior to conducting frequency counts. \( p < 0.01 \) indicates 990 or more times out of 1000, the actual co-occurrence of a particular KO within 10 kb of K00441 is greater than random.

**Phylogenetic regression**

For each KO family, and each strain, we determine the maximum hmmer bitscore hit to the KO’s pHMM out of all the strain’s proteins. Aggregating across KOs and strains, this yields a strain-by-KO genotype matrix, where each entry is the highest bitscore value – higher bitscores indicate gene presence. We then test for association between this genotype and microcosm enrichment score (phenotype). While such genotype-phenotype tests are in many ways similar to those conducted in genome association studies (GWAS), the application of ordinary least squares (OLS) regression, often used in GWAS, is not appropriate here due to phylogenetic relationships between strains. These relationships mean that assumptions of independence between measurements inherent to OLS are violated. We confirm the presence of a non-star phylogeny between strains by generating a phylogenetic tree based on strain genomes, using bac120 multiple sequence alignment with GTDB-tk [73] (Fig. 1A). Therefore, to account for this phylogenetic relatedness, for each KO we apply phylogenetic regression to test for significant association between mucosal enrichment scores and maximum hmmer bitscore (standard scaled) across strains. We implement this test using the R package phylolm [32], assuming a Brownian motion model along evolutionary branches, using the bac120 phylogenetic tree as input. This generates effect size estimates and \( p \)-values for every KO. We filter KOs for significance applying a \( p < 0.01 \) cutoff with Benjamini-Hochberg FDR correction. In addition to running phylogenetic regression across all 86 prevalent strains, we also run these models across subsets of these strains grouped by phylum to search for clade-specific hits. For this analysis, we group Firmicutes, Firmicutes_A, Firmicutes_C phyla into a single clade.

**Comparison with in vivo dataset**

We analyze from the Suez 2018 [15] dataset all read libraries from untreated (i.e., naive) individuals, for whom lumen and mucosal reads were available from cecum, descending colon, and terminal ileum, i.e., a total of 6 read libraries per individual. We first use Kneaddata (part of the Biobakery suite [76]) to
perform host (i.e. human) filtering of read sequences, resulting in 13 individuals for which all 6 libraries exceed a read depth of $10^4$ reads (Table S6). For these 13 individuals, we obtain abundance estimates at all 6 sites by mapping reads to UHGG database using Kraken2 [2, 35]. We then calculate normalized mucosal enrichment scores for each species defined as log ratio of mucosal to lumen abundance. Scores are normalized by taking mean-over-standard-deviation across all individuals and sites (13 individuals × 3 sites – cecum, descending colon and terminal ileum – for 39 total measurements). We determine gene presence absence for these species, across KOs, by using kofamsn scan to search the UHGG pangenome database [2], and then apply phylogenetic regression as described above to test for associations between gene presence absence and mucosal enrichment score across UHGG species. The regression uses enrichment scores from 676 species detected with greater than 0.01% relative abundance in at least 10% of in vivo read libraries, which contain a total of 12,822 detected KEGG KO gene families (Table S7, S10).

**Extraction and grouping of biosynthetic gene clusters using DeepBGC and hierarchical clustering**

DeepBGC [61] is used to extract BGCs from our de novo genomes. For each identified BGC, we generate a list of present KOs based on if contained genes map to KO’s pHMM with overlap greater than $0.5 \times$ coverage, as well as a bitscore greater than $0.5 \times$ the KO’s bitscore threshold. We filter out BGCs with fewer than 3 present KOs, and then use hierarchical clustering to cluster all remaining BGCs based on their binary KO presence/absence profile into 256 BGC-groups, applying a Jaccard distance metric. We then map presence of each BGC-group within community strains, and use this presence/absence matrix to test for associations with microcosm-enrichment applying phylogenetic regression as described above.

**Data availability**

All sequencing data of this study is deposited in the Sequence Read Archive (SRA), accession codes pending. Genomes are deposited in Genbank (NCBI), also pending.
**Code availability**

Code used for analysis and visualization available at:

https://github.com/xiaofanjin/gut-community-microcosms

Software used for nanopore basecalling and hybrid assembly available at:

https://github.com/czbiohub/microbiome-data-analysis/

https://github.com/FischbachLab/nf-hybridassembly

**Ethics declarations**

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgements**

We thank A. Lind, P. Bradley, A. Bustion, and I.H. Riedel-Kruse for helpful discussions regarding data analysis and feedback on the manuscript, A. Cheng and M. Fischbach for sharing bacterial strains, and S. Jain and X. Meng for help building the genome assembly framework. This work is supported by funding from Chan Zuckerberg Biohub, Gladstone Institutes, NSF grant #1563159.

**Author’s contributions**

XJ, BY and KP contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. JY and AW contributed to the implementation of the research.
References

[1] Wei-Lin Wang, Shao-Yan Xu, Zhi-Gang Ren, Liang Tao, Jian-Wen Jiang, and Shu-Sen Zheng. Application of metagenomics in the human gut microbiome. *World journal of gastroenterology*, 21(3):803–814, Jan 2015.

[2] Alexandre Almeida, Stephen Nayfach, Miguel Boland, Francesco Strozzi, Martin Beracochea, Zhou Jason Shi, Katherine S Pollard, Ekaterina Sakharova, Donovan H Parks, Philip Hugenholtz, Nicola Segata, Nikos C Kyripides, and Robert D Finn. A unified catalog of 204,938 reference genomes from the human gut microbiome. *Nature Biotechnology*, 39(1):105–114, 2021.

[3] Petar Scepanovic, Flavia Hodel, Stanislas Mondot, Valentin Partula, Allyson Byrd, Christian Hammer, Cécile Alanio, Jacob Bergstedt, Etienne Patin, Mathilde Touvier, Olivier Lantz, Matthew L Albert, Darragh Duffy, Lluis Quintana-Murci, Jacques Fellay, Laurent Abel, Andres Alcover, Hugues Aschard, Kalla Astrom, Philippe Bousso, Pierre Bruhns, Ana Cumano, Caroline Demangel, Ludovic Deriano, James Di Santo, Françoise Dromer, Darragh Duffy, Gérard Eberl, Jost Enninga, Jacques Fellay, Odile Gelpi, Ivo Gomperts-Boneca, Milena Hasan, Serge Hercberg, Olivier Lantz, Claude Leclerc, Hugo Mouquet, Sandra Pellegrini, Stanislas Pol, Antonio Rausell, Lars Rogge, Anavaj Sakuntabhai, Olivier Schwartz, Benno Schwikowski, Spencer Shorte, Vassili Soumelis, Frédéric Tangy, Eric Tartour, Antoine Toubert, Mathilde Touvier, Marie-Noëlle Ungeheuer, Matthew L Albert, Lluis Quintana-Murci, and The Milieu Intérieur Consortium. A comprehensive assessment of demographic, environmental, and host genetic associations with gut microbiome diversity in healthy individuals. *Microbiome*, 7(1):130, 2019.

[4] Carey D. Nadell, Knut Drescher, and Kevin R. Foster. Spatial structure, cooperation and competition in biofilms. *Nature Reviews Microbiology*, 14(9):589–600, 2016.

[5] Carolina Tropini, Kristen A Earle, Kerwyn Casey Huang, and Justin L Sonnenburg. The Gut Microbiome: Connecting Spatial Organization to Function. *Cell Host and Microbe*, 21(4):433–442, Apr 2017.

[6] Kudelka Matthew R., Hinrichs Benjamin H., Darby Trevor, Moreno Carlos S., Nishio Hikaru, Cutler Christopher E., Wang Jianmei, Wu Huixia, Zeng Junwei, Wang Yingchun, Ju Tongzhong, Stowell Sean R., Nusrat Asma, Jones Rheinallt M., Neish Andrew S., and Cummings Richard
D. Cosmc is an X-linked inflammatory bowel disease risk gene that spatially regulates gut microbiota and contributes to sex-specific risk. *Proceedings of the National Academy of Sciences*, 113(51):14787–14792, dec 2016.

[7] Weiguang Chen, Fanlong Liu, Zongxin Ling, Xiaojuan Tong, and Charlie Xiang. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. *PLOS ONE*, 7(6):e39743, jun 2012.

[8] Ian M Carroll, Young-Hyo Chang, Jiwon Park, R Balfour Sartor, and Yehuda Ringel. Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Gut Pathogens*, 2(1):19, 2010.

[9] Maximilian Baumgartner, Michaela Lang, Hunter Holley, Daniel Crepaz, Bela Hausmann, Petra Pjevac, Doris Moser, Felix Haller, Fabian Hof, Andrea Beer, Elisabeth Orgler, Adrian Frick, Vineeta Khare, Rayko Evstatiev, Susanne Strohmaier, Christian Primas, Werner Dolak, Thomas Köcher, Kristaps Klavins, Timo Rath, Markus F Neurath, David Berry, Athanasios Makristathis, Markus Muttenthaler, and Christoph Gasche. Mucosal Biofilms Are an Endoscopic Feature of Irritable Bowel Syndrome and Ulcerative Colitis. *Gastroenterology*, 161(4):1245–1256.e20, oct 2021.

[10] Gopanandan Parthasarathy, Jun Chen, Xianfeng Chen, Nicholas Chia, Helen M O’Connor, Patricia G Wolf, H Rex Gaskins, and Adil E Bharucha. Relationship Between Microbiota of the Colonic Mucosa vs Feces and Symptoms, Colonic Transit, and Methane Production in Female Patients With Chronic Constipation. *Gastroenterology*, 150(2):367–379.e1, feb 2016.

[11] Sandra Macfarlane, Bahram Bahrami, and George T Macfarlane. *Mucosal biofilm communities in the human intestinal tract.*, volume 75. Elsevier Inc., 1 edition, jan 2011.

[12] Erwin G Zoetendal, Atte von Wright, Terttu Vilpponen-Salmela, Kaouther Ben-Amor, Antoon D L Akkermans, and Willem M de Vos. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and environmental microbiology*, 68(7):3401–3407, jul 2002.

[13] Gerardo M Nava, Hans J Friedrichsen, and Thaddeus S Stappenbeck. Spatial organization of intestinal microbiota in the mouse ascending colon. *The ISME Journal*, 5(4):627–638, 2011.
[14] Koji Yasuda, Keunyoung Oh, Boyu Ren, Timothy L Tickle, Eric A Franzosa, Lynn M Wachtman, Andrew D Miller, Susan V Westmoreland, Keith G Mansfield, Eric J Vallender, Gregory M Miller, James K Rowlett, Dirk Gevers, Curtis Huttenhower, and Xochitl C Morgan. Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. *Cell Host and Microbe*, 17(3):385–391, mar 2015.

[15] Jotham Suez, Zamir Halpern, Eran Segal, and Eran Elinav. Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features Article Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Feat. *Cell*, 174(6):1388–1405.e21, 2018.

[16] Stefania Vaga, Sunjae Lee, Boyang Ji, Anna Andreasson, Nicholas J Talley, Lars Agréus, Gholamreza Bidkhori, Petia Kovatcheva-Datchary, Junseok Park, Doheon Lee, Gordon Proctor, Stanislav Dusko Ehrlich, Jens Nielsen, Lars Engstrand, and Saeed Shoai. Compositional and functional differences of the mucosal microbiota along the intestine of healthy individuals. *Scientific Reports*, 10(1):14977, 2020.

[17] Ivaylo I Ivanov, Rosa de Llanos Frutos, Nicolas Manel, Keiji Yoshinaga, Daniel B Rifkin, R Balfour Sartor, B Brett Finlay, and Dan R Littman. Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host and Microbe*, 4(4):337–349, oct 2008.

[18] Koji Atarashi, Takeshi Tanoue, Minoru Ando, Nobuhiko Kamada, Yuji Nagano, Seiko Narushima, Wataru Suda, Akemi Imaoka, Hiromi Setoyama, Takashi Nagamori, Eiji Ishikawa, Tatsuihiro Shima, Taeko Hara, Shoichi Kado, Toshi Jinnohara, Hiroshi Ohno, Takashi Kondo, Kiminori Toyooka, Eiichiro Watanabe, Shin-ichiro Yokoyama, Shunji Tokoro, Hiroshi Mori, Yurika Noguchi, Hidetoshi Morita, Ivaylo I. Ivanov, Tsuyoshi Sugiyama, Gabriel Nuñez, J. Gray Camp, Masahira Hattori, Yoshinori Umesaki, and Kenya Honda. Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell*, 163(2):367–380, oct 2015.

[19] Round June L. and Mazmanian Sarkis K. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences*, 107(27):12204–12209, jul 2010.
[20] Gregory P Donaldson, S Melanie Lee, and Sarkis K Mazmanian. Gut biogeography of the bacterial microbiota. *Nature reviews. Microbiology*, 14(1):20–32, jan 2016.

[21] G P Donaldson, M S Ladinsky, K B Yu, J G Sanders, B B Yoo, W-C Chou, M E Conner, A M Earl, R Knight, P J Bjorkman, and S K Mazmanian. Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science (New York, N.Y.)*, 360(6390):795–800, may 2018.

[22] Siegfried Schloissnig, Manimozhiyan Arumugam, Shinichi Sunagawa, Makedonka Mitreva, Julien Tap, Ana Zhu, Alison Waller, Daniel R Mende, Jens Roat Kultima, John Martin, Karthik Kota, Shamil R Sunyaev, George M Weinstock, and Peer Bork. Genomic variation landscape of the human gut microbiome. *Nature*, 493(7430):45–50, 2013.

[23] Paul I Costea, Luis Pedro Coelho, Shinichi Sunagawa, Robin Munch, Jaime Huerta-Cepas, Kristoffer Forslund, Falk Hildebrand, Almagul Kushugulova, Georg Zeller, and Peer Bork. Subspecies in the global human gut microbiome. *Molecular Systems Biology*, 13(12):960, dec 2017.

[24] Duy T Truong, Adrian Tett, Edoardo Pasolli, Curtis Huttenhower, and Nicola Segata. Microbial strain-level population structure and genetic diversity from metagenomes. *Genome Research*, 27(4):gr.216242.116—-216242.116, feb 2017.

[25] Nandita R Garud, Benjamin H Good, Oskar Hallatschek, and Katherine S Pollard. Evolutionary dynamics of bacteria in the gut microbiome within and across hosts. *PLoS biology*, 17(1):e3000102, 2019.

[26] Alice G Cheng, Andrés Aranda-Díaz, Sunit Jain, Feiqiao Yu, Mikhail Iakiviak, Xiandong Meng, Allison Weakley, Advait Patil, Anthony L Shiver, Adam Deutschbauer, Norma Neff, Kerwyn Casey Huang, and Michael A Fischbach. Systematic dissection of a complex gut bacterial community. *bioRxiv*, page 2021.06.15.448618, jan 2021.

[27] Alice G Cheng, Po-Yi Ho, Sunit Jain, Xiandong Meng, Min Wang, Feiqiao Brian Yu, Mikhail Iakiviak, Ariel R Brumbaugh, Kazuki Nagashima, Aishan Zhao, Advait Patil, Katayoon Atabakhsh, Allison Weakley, Jia Yan, Steven Higginbottom, Norma Neff, Justin L Sonnenburg, Kerwyn Casey Huang, and Michael A Fischbach. In vivo augmentation of a complex gut bacterial community. *bioRxiv*, page 2021.06.15.448620, jan 2021.
[28] Pieter Van den Abbeele, Stefan Roos, Venessa Eeckhaut, Donald A MacKenzie, Melanie Derde, Willy Verstraete, Massimo Marzorati, Sam Possemiers, Barbara Vanhoecke, Filip Van Immerseel, and Tom Van de Wiele. Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. *Microbial biotechnology*, 5(1):106–115, jan 2012.

[29] LinShu Liu, Jenni Firrman, Ceylan Tanes, Kyle Bittinger, Audrey Thomas-Gahring, Gary D Wu, Pieter Van den Abbeele, and Peggy M Tomasula. Establishing a mucosal gut microbial community in vitro using an artificial simulator. *PLOS ONE*, 13(7):e0197692, jul 2018.

[30] Takuya Aramaki, Romain Blanc-Mathieu, Hisashi Endo, Koichi Ohkubo, Minoru Kanehisa, Susumu Goto, and Hiroyuki Ogata. KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics*, 36(7):2251–2252, apr 2020.

[31] Alan Grafen and William Donald Hamilton. The phylogenetic regression. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 326(1233):119–157, dec 1989.

[32] Lam Si Tung Ho and Cecile Ane. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Systematic Biology*, 63:397–408, 2014.

[33] Patrick H Bradley, Stephen Nayfach, and Katherine S Pollard. Phylogeny-corrected identification of microbial gene families relevant to human gut colonization. *PLOS Computational Biology*, 14(8):e1006242+, aug 2018.

[34] Qiang Tang, Ge Jin, Gang Wang, Tianyu Liu, Xiang Liu, Bangmao Wang, and Hailong Cao. Current Sampling Methods for Gut Microbiota: A Call for More Precise Devices. *Frontiers in cellular and infection microbiology*, 10:151, apr 2020.

[35] Derrick E Wood, Jennifer Lu, and Ben Langmead. Improved metagenomic analysis with Kraken 2. *Genome Biology*, 20(1):257, 2019.

[36] Bjarke B Christensen, Janus A J Haagensen, Arne Heydorn, and Søren Molin. Metabolic commensalism and competition in a two-species microbial consortium. *Applied and environmental microbiology*, 68(5):2495–2502, 2002.

[37] Jan-Ulrich Kreft. Biofilms promote altruism. *Microbiology (Reading, England)*, 150(Pt 8):2751–60, aug 2004.
[38] Sivan Elias and Ehud Banin. Multi-species biofilms: living with friendly neighbors. *FEMS microbiology reviews*, jan 2012.

[39] Ian W Sutherland. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, 147(1):3–9, 2001.

[40] Barbara Vu, Miao Chen, Russell J Crawford, and Elena P Ivanova. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules*, 14(7):2535–2554, 2009.

[41] Tu Quoc Patrick H., Genevaxx Pierre, Pajunen Maria, Savilahti Harri, Georgopoulos Costa, Schrenzel Jacques, and Kelley William L. Isolation and Characterization of Biofilm Formation-Defective Mutants of Staphylococcus aureus. *Infection and Immunity*, 75(3):1079–1088, mar 2007.

[42] X. Wang, J. F. Preston, and T. Romeo. The pgaABCD Locus of Escherichia coli Promotes the Synthesis of a Polysaccharide Adhesin Required for Biofilm Formation. *Journal of Bacteriology*, 186(9):2724–2734, apr 2004.

[43] Dominique H Limoli, Christopher J Jones, and Daniel J Wozniak. Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiology spectrum*, 3(3):3, 2015.

[44] Kvist Malin, Hancock Viktoria, and Klemm Per. Inactivation of Efflux Pumps Abolishes Bacterial Biofilm Formation. *Applied and Environmental Microbiology*, 74(23):7376–7382, dec 2008.

[45] Thithiwat May, Akinobu Ito, and Satoshi Okabe. Induction of multidrug resistance mechanism in Escherichia coli biofilms by interplay between tetracycline and ampicillin resistance genes. *Antimicrobial agents and chemotherapy*, 53(11):4628–4639, nov 2009.

[46] Kayo Matsumura, Soichi Furuikawa, Hirokazu Ogihara, and Yasushi Morinaga. Roles of multidrug efflux pumps on the biofilm formation of Escherichia coli K-12. *Biocontrol science*, 16(2):69–72, 2011.

[47] Stephanie Baugh, Aruna S Ekanayaka, Laura J V Piddock, and Mark A Webber. Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm. *Journal of Antimicrobial Chemotherapy*, 67(10):2409–2417, oct 2012.
[48] Ilyas Alav, J Mark Sutton, and Khondaker Miraz Rahman. Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy*, 73(8):2003–2020, aug 2018.

[49] Stefano Sabatini, Miranda Piccioni, Tommaso Felicetti, Stefania De Marco, Giuseppe Manfroni, Rita Pagiotti, Morena Nocchetti, Violetta Cecchetti, and Donatella Pietrella. Investigation on the effect of known potent S. aureus NorA efflux pump inhibitors on the staphylococcal biofilm formation. *RSC Advances*, 7(59):37007–37014, 2017.

[50] Martina Pasqua, Milena Grossi, Alessandro Zennaro, Giulia Fanelli, Gioacchino Micheli, Frederic Barras, Bianca Colonna, and Gianni Prosseda. The Varied Role of Efflux Pumps of the MFS Family in the Interplay of Bacteria with Animal and Plant Cells. *Microorganisms*, 7(9):285, aug 2019.

[51] Kazuo Kobayashi and Yukako Ikemoto. Biofilm-associated toxin and extracellular protease cooperatively suppress competitors in Bacillus subtilis biofilms. *PLOS Genetics*, 15(10):e1008232, oct 2019.

[52] Jennifer M Pang, Emilie Layre, Lindsay Sweet, Ashley Sherrid, D Branch Moody, Anil Ojha, and David R Sherman. The polyketide Pks1 contributes to biofilm formation in Mycobacterium tuberculosis. *Journal of bacteriology*, 194(3):715–721, feb 2012.

[53] Mariah Bindel Connelly, Glenn M Young, and Alan Sloma. Extracellular proteolytic activity plays a central role in swarming motility in Bacillus subtilis. *Journal of bacteriology*, 186(13):4159–4167, jul 2004.

[54] Ana Yepes, Johannes Schneider, Benjamin Mielich, Gudrun Koch, Juan-Carlos García-Betancur, Kumaran S Ramamurthi, Hera Vlamakis, and Daniel López. The biofilm formation defect of a Bacillus subtilis flotillin-defective mutant involves the protease FtsH. *Molecular Microbiology*, 86(2):457–471, oct 2012.

[55] Shubhada R Hegde. Computational Identification of the Proteins Associated With Quorum Sensing and Biofilm Formation in Mycobacterium tuberculosis, 2020.

[56] Holger Rohde, Christoph Burdelski, Katrin Bartscht, Muzaffar Hussain, Friedrich Buck, Matthias A Horstkotte, Johannes K.-M. Knobloch, Christine Heilmann, Mathias Herrmann, and Dietrich Mack. Induction of Staphylococcus epidermidis biofilm formation via proteolytic processing of
the accumulation-associated protein by staphylococcal and host proteases. *Molecular Microbiology*, 55(6):1883–1895, mar 2005.

[57] S V Lynch, L Dixon, M R Benoit, E L Brodie, M Keyhan, P Hu, D F Ackerley, G L Andersen, and A Matin. Role of the rapA gene in controlling antibiotic resistance of Escherichia coli biofilms. *Antimicrobial agents and chemotherapy*, 51(10):3650–3658, oct 2007.

[58] Laís Moreira Granato, Simone Cristina Picchi, Maxuel de Oliveira Andrade, Marco Aurélio Takita, Alessandra Alves de Souza, Nian Wang, and Marcos Antonio Machado. The ATP-dependent RNA helicase HrpB plays an important role in motility and biofilm formation in Xanthomonas citri subsp. citri. *BMC microbiology*, 16:55, mar 2016.

[59] Stella Oun, Peter Redder, Jean-Philippe Didier, Patrice François, Anna-Rita Corvaglia, Elena Buttazzoni, Caroline Giraud, Myriam Girard, Jacques Schrenzel, and Patrick Linder. The CshA DEAD-box RNA helicase is important for quorum sensing control in Staphylococcus aureus. *RNA biology*, 10(1):157–165, jan 2013.

[60] Stéphane Hausmann, Diego Gonzalez, Johan Geiser, and Martina Valentini. The DEAD-box RNA helicase RhlE2 is a global regulator of Pseudomonas aeruginosa lifestyle and pathogenesis. *Nucleic Acids Research*, 49(12):6925–6940, jul 2021.

[61] Geoffrey D Hannigan, David Prihoda, Andrej Palicka, Jindrich Soukup, Ondrej Klemprir, Lena Rampula, Jindrich Durcak, Michael Wurst, Jakub Kotowski, Dan Chang, Rurun Wang, Grazia Piizzi, Gergely Temesi, Daria J Hazuda, Christopher H Woelk, and Danny A Bitton. A deep learning genome-mining strategy for biosynthetic gene cluster prediction. *Nucleic Acids Research*, 47(18):e110–e110, oct 2019.

[62] Linggang Zheng, Yang Tan, Yucan Hu, Juntao Shen, Zepeng Qu, Xianbo Chen, Chun Loong Ho, Elaine Lai-Han Leung, Wei Zhao, and Lei Dai. CRISPR/Cas-Based Genome Editing for Human Gut Commensal Bacteroides Species. *ACS Synthetic Biology*, 11(1):464–472, jan 2022.

[63] Mark Mimee, Alex C Tucker, Christopher A Voigt, and Timothy K Lu. Programming a Human Commensal Bacterium, Bacteroides thetaiotaomicron, to Sense and Respond to Stimuli in the Murine Gut Microbiota. *Cell systems*, 1(1):62–71, jul 2015.
[64] Guo Chun-Jun, Allen Breanna M., Hiam Kamir J., Dodd Dylan, Van Treuren Will, Higginbottom Steven, Nagashima Kazuki, Fischer Curt R., Sonnenburg Justin L., Spitzer Matthew H., and Fischbach Michael A. Depletion of microbiome-derived molecules in the host using Clostridium genetics. *Science*, 366(6471):eaav1282, dec 2019.

[65] Eric A Franzosa, Xochitl C Morgan, Nicola Segata, Levi Waldron, Joshua Reyes, Ashlee M Earl, Georgia Giannoukos, Matthew R Boylan, Dawn Ciulla, Dirk Gevers, Jacques Izard, Wendy S Garrett, Andrew T Chan, and Curtis Huttenhower. Relating the metatranscriptome and metagenome of the human gut. *Proceedings of the National Academy of Sciences of the United States of America*, 111(22):E2329—-E2338, jun 2014.

[66] Stavros Bashiardes, Gili Zilberman-Schapira, and Eran Elinav. Use of Metatranscriptomics in Microbiome Research. *Bioinformatics and biology insights*, 10:19–25, apr 2016.

[67] Kristen A Earle, Gabriel Billings, Michael Sigal, Joshua S Lichtman, Gunnar C Hansson, Joshua E Elias, Manuel R Amieva, Kerwyn Casey Huang, and Justin L Sonnenburg. Quantitative imaging of gut microbiota spatial organization. *Cell Host and microbe*, 18(4):478–488, 2015.

[68] Mark Welch Jessica L., Hasegawa Yuko, McNulty Nathan P., Gordon Jeffrey I., and Borisy Gary G. Spatial organization of a model 15-member human gut microbiota established in gnotobiotic mice. *Proceedings of the National Academy of Sciences*, 114(43):E9105–E9114, oct 2017.

[69] Hao Shi, Qiaojuan Shi, Benjamin Grodner, Joan Sesing Lenz, Warren R Zipfel, Ilana Lauren Brito, and Iwijn De Vlaminck. Highly multiplexed spatial mapping of microbial communities. *Nature*, 588(7839):676–681, dec 2020.

[70] Ryan R Wick, Louise M Judd, Claire L Gorrie, and Kathryn E Holt. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLOS Computational Biology*, 13(6):e1005595, jun 2017.

[71] Mao Qin, Shigang Wu, Alun Li, Fengli Zhao, Hu Feng, Lulu Ding, and Jue Ruan. LRScf: improving draft genomes using long noisy reads. *BMC genomics*, 20(1):1–12, 2019.

[72] Mengyang Xu, Lidong Guo, Shengqiang Gu, Ou Wang, Rui Zhang, Brock A Peters, Guangyi Fan, Xin Liu, Xun Xu, and Li Deng. TGS-GapCloser: a fast and accurate gap closer for large genomes
with low coverage of error-prone long reads. *GigaScience*, 9(9):giaa094, 2020.

[73] Pierre-Alain Chaumeil, Aaron J Mussig, Philip Hugenholtz, and Donovan H Parks. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics*, 36(6):1925–1927, mar 2020.

[74] Tatiana Tatusova, Michael DiCuccio, Azat Badretdin, Vyacheslav Chetvernin, Eric P Nawrocki, Leonid Zaslavsky, Alexandre Lomsadze, Kim D Pruitt, Mark Borodovsky, and James Ostell. NCBI prokaryotic genome annotation pipeline. *Nucleic acids research*, 44(14):6614–6624, aug 2016.

[75] Jenni Firrman, LinShu Liu, Pieter Van den Abbeele, Ceylan Tanes, Kyle Bittinger, and Peggy Tomasula. Applying advanced in vitro culturing technology to study the human gut microbiota. *JoVE (Journal of Visualized Experiments)*, (144):e59054, 2019.

[76] Francesco Beghini, Lauren J McIver, Aitor Blanco-Míguez, Leonard Dubois, Francesco Asnicar, Sagun Maharjan, Ana Mailyan, Paolo Manghi, Matthias Scholz, Andrew Maltez Thomas, Mireia Valles-Colomer, George Weingart, Yancong Zhang, Moreno Zolfo, Curtis Huttenhower, Eric A Franzosa, and Nicola Segata. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *eLife*, 10:e65088, 2021.