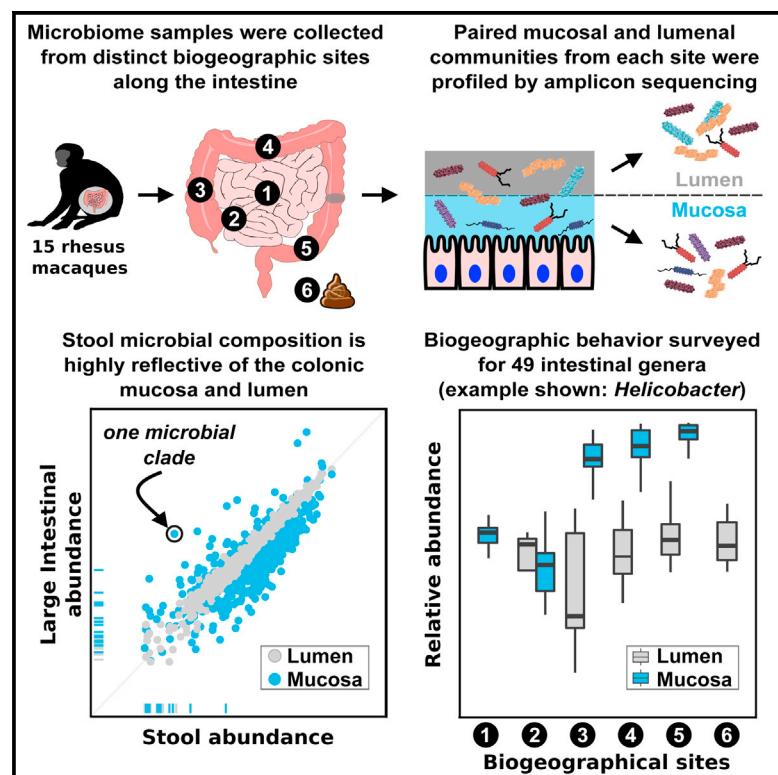


Short Article

Cell Host & Microbe

Biogeography of the Intestinal Mucosal and Luminal Microbiome in the Rhesus Macaque

Graphical Abstract



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In Brief

Stool is commonly used for microbiota assessments, but how accurately it represents the gut is unknown. Yasuda et al. characterize biogeographic relationships in the rhesus macaque intestinal microbiome and find that stool is highly representative of the colonic lumen and mucosa, which are respectively enriched in obligate and facultative anaerobes.

Highlights

- Macaque stool microbiota is highly representative of the colonic lumen and mucosa
- Small intestinal proteobacteria are particularly under-detected in stool
- Facultative and obligate anaerobes are respectively enriched in the mucosa and lumen

Accession Numbers

PRJNA259224



Biogeography of the Intestinal Mucosal and Luminal Microbiome in the Rhesus Macaque

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<http://dx.doi.org/10.1016/j.chom.2015.01.015>

SUMMARY

The gut microbiome is widely studied by fecal sampling, but the extent to which stool reflects the commensal composition at intestinal sites is poorly understood. We investigated this relationship in rhesus macaques by 16S sequencing feces and paired luminal and mucosal samples from ten sites distal to the jejunum. Stool composition correlated highly with the colonic lumen and mucosa and moderately with the distal small intestine. The mucosal microbiota varied most based on location and was enriched in oxygen-tolerant taxa (e.g., *Helicobacter* and *Treponema*), while the luminal microbiota showed inter-individual variation and obligate anaerobe enrichment (e.g., *Firmicutes*). This mucosal and luminal community variability corresponded to functional differences, such as nutrient availability. Additionally, *Helicobacter*, *Faecalibacterium*, and *Lactobacillus* levels in stool were highly predictive of their abundance at most other gut sites. These results quantify the composition and biogeographic relationships between gut microbial communities in macaques and support fecal sampling for translational studies.

INTRODUCTION

Gut mucosal and luminal microbial communities are distinct (Eckburg et al., 2005; Morgan et al., 2012; Stearns et al., 2011), and diseases such as colorectal cancer and inflammatory bowel disease induce site-specific epithelial inflammation at which the microbiota are disrupted relative to adjacent normal tissue (Darfeuille-Michaud et al., 2004; Kostic et al., 2013a; Sobhani et al., 2011). Understanding the relationship between stool and the

mucosal microbiome is thus of great interest, but large-scale human health-related studies typically focus on the stool microbiota due to technical limitations (Human Microbiome Project Consortium, 2012; Qin et al., 2010, 2012; Yatsunenkov et al., 2012).

Furthermore, human biopsy samples are near universally collected after bowel preparation (Whitlock et al., 2008), which itself alters the mucosal community (Ahmed et al., 2007); paired stool data are rarely available. Previous studies of human gut biogeography have included only samples from different individuals and/or time points (Darfeuille-Michaud et al., 2004; Gevers et al., 2014; Huse et al., 2014; Morgan et al., 2012) or used a very small number of individuals (Eckburg et al., 2005; Stearns et al., 2011). While the mucosa and luminal contents of mice are readily accessible for biogeographic studies, neither the pelleted, sparse nature of their colonic contents nor their native microbial composition are totally representative of the human gut (Kostic et al., 2013b). The captive rhesus macaque (*Macaca mulatta*), widely used in biomedical research due to its genetic and physiological similarities to humans (Bauer et al., 2011; Handley et al., 2012; McKenna et al., 2008; Vallender and Miller, 2013), is an excellent model for detailed biogeographic study of the mucosal, luminal, and stool microbiota. It further avoids confounding due to sample collection and manipulation methods (no colon preparation is required upon autopsy) or diet (synchronized meals).

In this study, we investigated (i) the extent to which the stool microbiome reflects the composition of other intra-intestinal sites, (ii) the biogeography of the composition of the rhesus macaque gut microbiome, and (iii) predictability of microbiota in the gut. Our results indicate that the stool microbiota community is a good proxy of the large intestinal (LI) lumen and mucosa and is surprisingly well correlated with the small intestine (SI). The LI mucosa was highly enriched in *Helicobacter*, which is flagellated and facultatively anaerobic. In contrast, obligate anaerobic Firmicutes were primarily localized to the intestinal lumen. This study thus provides the quantitative relationship between

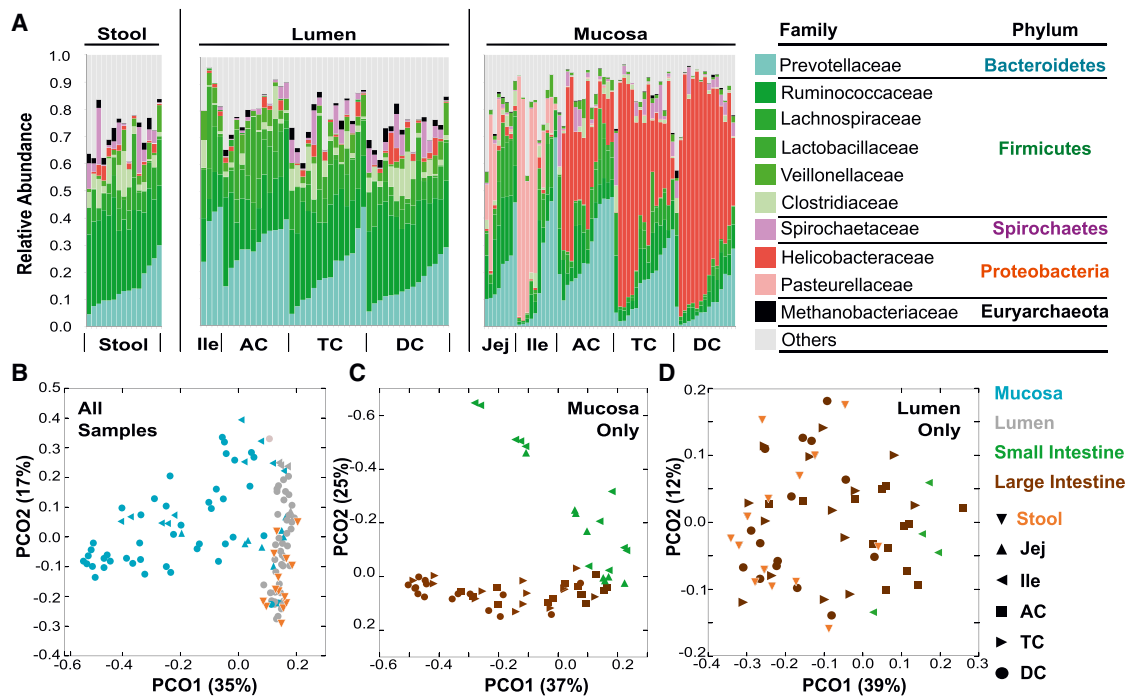


Figure 1. Biogeographic Influences on Macaque Gut Microbial Composition

(A) Family-level relative abundance of intestinal microbiota in the stool (left), lumen (middle), and mucosa (right) of 15 healthy rhesus macaques.

(B) Principal coordinate analysis (PCoA) of all samples by weighted UniFrac distance.

(C) PCoA of mucosal-only samples.

(D) PCoA of lumen and stool-only samples. See also [Figure S1](#) and [Table S1](#).

mucosal and luminal microbial communities as assessed using stool.

RESULTS

The Macaque Intestinal Mucosa Is Dominated by Non-Pathogenic Pasteurellaceae and Helicobacteriaceae

Similarly to humans ([Eckburg et al., 2005](#); [Human Microbiome Project Consortium, 2012](#); [Qin et al., 2010](#)), the macaque intestine was colonized primarily by Bacteroidetes, Firmicutes, and Proteobacteria ([Figure 1A](#)). In contrast, the Actinobacteria and Verrucomicrobia were rare in macaques, and *Spirochaetes* and *Helicobacter* were much more abundant. To assess our data in the context of other human ([Human Microbiome Project Consortium, 2012](#); [Yatsunenkeno et al., 2012](#)) and macaque ([Handley et al., 2012](#); [McKenna et al., 2008](#)) microbiome studies, we combined these data sets, calculated Bray-Curtis dissimilarity and weighted UniFrac distance, and performed principal coordinate analysis ([Figures S1D](#) and [S1E](#); [Supplemental Experimental Procedures](#)). Despite differences in sequencing technology, the three macaque studies were similar to one another and more similar to the Malawian and Amerindian than to the US microbiomes.

We used univariate ([Segata et al., 2011](#)) and multivariate analyses ([Morgan et al., 2012](#)) to identify bacterial taxa significantly enriched (FDR $q < 0.2$) in the mucosa or lumen; multivariate analysis included location, sample type, weight, age, and primate center of origin as covariates ([Table S1](#)). Relative to mucosa,

stool and lumen were enriched for obligately anaerobic, short-chain fatty-acid-producing clades such as the Lachnospiraceae, Clostridiaceae, and Prevoellaceae ([Duncan et al., 2007](#)). In the mucosa, facultatively anaerobic clades were more abundant; these were mostly Proteobacteria, such as *Helicobacter* in the LI and *Pasteurella* in the SI ([Figure 1A](#)). This likely reflects the higher host-derived oxygen content in the mucous layer compared to the lumen. *Helicobacteraceae* in particular was strongly associated with mucosa ($q < 10^{-21}$) and the ascending LI ($q = 0.0011$). While *H. macacae* has been previously associated with chronic diarrhea and intestinal adenocarcinoma ([Fox et al., 2007](#); [Lertpiriyapong et al., 2014](#); [Marini et al., 2010](#)), our animals showed neither any evidence of tumorigenesis nor excess inflammation upon routine histopathologic examination of the ileal, cecal, and colonic tissues.

All the animals in our study were housed at the New England Primate Research Center (NEPRC) for 2 years prior to sample collection, but eleven animals came from Oregon National Primate Center, where they were housed outdoors. Research center was not associated with major systematic variation in microbial diversity but was significantly associated with 23 operational taxonomic units (OTUs) (effect size -0.05 to 0.04 ; $q < 0.2$; [Table S1](#); [Figure S1A](#)). Most of these OTUs were Ruminococcaceae and Lachnospiraceae, which are primarily lumenally enriched taxa. However, several mucosally enriched taxa, including *Treponema*, *Desulfovibrio*, and *Corynebacterium*, were enriched by primate center, suggesting that their presence in the colonic mucosa may be highly influenced by early exposure.

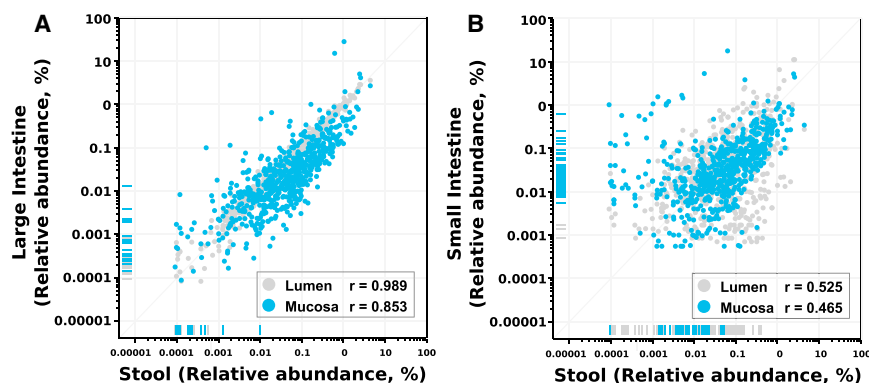


Figure 2. Stool Microbial Composition Mirrors that of the Colonic Lumen

Each dot corresponds to the average relative abundance of an OTU across 15 animals for each of four intestinal regions (SI mucosa, SI lumen, LI mucosa, and LI lumen). To measure correlation, Spearman's r was calculated between stool and mean region OTU abundance. Marks on the x axis (vertical lines) or y axis (horizontal lines) margins represent OTUs with zero measured abundance at one site but non-zero abundance at the other. See also Figure S2.

The Mucosal Microbiota Is Most Influenced by Location, while The Luminal Microbiota Is Most Influenced by Individual

The largest covariation within microbial community structure (as assessed by weighted UniFrac dissimilarity) (Lozupone and Knight, 2005) was explained by mucosal/luminal sample origin (Figure 1B). When mucosal and luminal/stool samples were separated, the largest source of variation in mucosal samples corresponded to SI versus LI sample origin (Figure 1C), but no such pattern was observed for luminal samples (Figure 1D). As observed in previous human studies (Eckburg et al., 2005; Fox et al., 2000), both the stool and lumen showed high inter-individual variation; the latter was not substantially influenced by biogeographical location (Figure S1B). The Bray-Curtis dissimilarity (based on species in common between sites) between stool and each of the other sites showed that stool was equally dissimilar to all mucosal sites regardless of anatomical proximity (Figure S1C); in contrast, luminal dissimilarity increased with colonic distance. This suggests that, despite the close anatomical proximity of distal mucosa and stool, luminal flux of microbiota occurs more readily than transfer of microbiota between mucosa and lumen.

Stool Microbial Composition Accurately Reflects the Colonic Lumen and Mucosa

We assessed the extent to which the mucosal and luminal community of each individual was reflected in the stool by measuring the Spearman correlation between stool and the four major subdivisions of the distal gut (SI mucosa, SI lumen, LI mucosa, and LI lumen), thus accounting for both OTU rank order and the magnitude of relative abundances between the two sites being compared. Stool composition was highly correlated with the LI lumen (Spearman's $r = 0.98$; $p < 0.001$) and LI mucosa ($r = 0.85$, $p < 0.001$; Figure 2A). Stool composition was also surprisingly correlated with the SI mucosa ($r = 0.465$, $p < 0.001$) and lumen ($r = 0.525$, $p < 0.001$; Figure 2B). We examined these OTUs for a systematic taxonomic bias (Figure S2) and found that mucosal OTUs that do not appear in stool.

In the SI lumen and LI mucosa and lumen, over 97% of observed OTUs were also detected in stool, and stool-undetected OTUs had very low relative abundances ($<10^{-3}$) in the mucosa and lumen, and thus may have been detected with deeper

sequencing of stool. In contrast, 10% of SI mucosal OTUs were stool-undetected despite relative abundance typically $>10^{-3}$; thus, increasing stool read depth may not improve the detection of these OTUs. Fusobacteria, β - and γ -proteobacteria are particularly likely to be stool undetected (Figure S2; Table S2).

Nearly all (95%) OTUs detected within the LI mucosa lumen and in stool were detected in stool within within two orders of magnitude (10^{-1} to 10^1) of their luminal and mucosal relative abundances; this was only true for 50% of SI content and 66% of SI mucosal OTUs. Stool is therefore an excellent proxy for the LI lumen and mucosa, as it contains nearly all OTUs at preserved proportions.

Most OTUs Are Shared between Adjacent Sites, but Each Site Has a Small, Site-Specific Community

We found that $\sim 40\%$ – 70% of OTUs are typically shared between adjacent mucosal and luminal sites (Figure 3). It is unclear to what extent these overlapping taxa are persistent, metabolically active residents of the mucosa, rather than luminal residents incidentally trapped on the mucosal surface (or vice versa). Although luminal communities were generally more homogeneous than those of the mucosa (Figure 2D), 20%–30% of OTUs were unique to each luminal segment. As each mucosal sample contained a similar distribution of organisms within higher-order taxa, the variation we observed here at the genus or species level may be the result of colonization resistance by the more abundant members within similar functional groups. Whether the gut microbiota undergoes such nonrandom assembly remains unclear.

Using Logistic Regression to Distinguish Mucosally and Lumenally Enriched Taxa and to Predict Bacterial Flow

To understand microbial niches and potential bacterial flow within individuals, we used logistic regression to model the extent to which the abundance of a genus (Figure 4A) at one site was predictive of its abundance at an adjacent site (Figure 4B; see Experimental Procedures). Of 56 genera identified in our cohort, 30% were mucosally enriched, 40% were lumenally enriched, and 30% showed no consistent enrichment. The proteobacteria comprised none of the lumenally enriched taxa but one third of the mucosally enriched taxa (6/17 genera). Conversely, nearly 70% of the lumenally enriched taxa were Firmicutes. The mucosally enriched genera were primarily

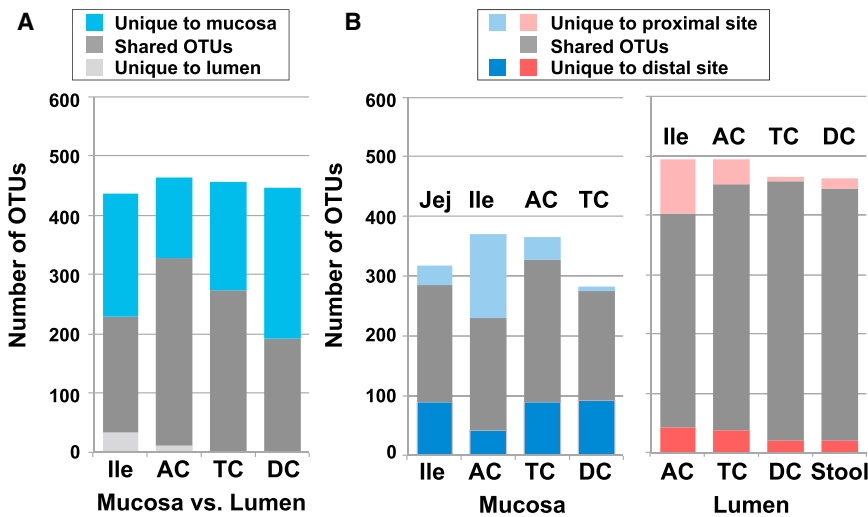


Figure 3. Microbial Overlap between Adjacent Mucosal and Luminal Sites

(A) Mean total, shared, and unique OTUs between the mucosa and lumen across all individuals at each paired site.

(B) Mean total, shared, and unique OTUs between adjacent mucosal (left) and luminal (right) sites, averaged across individuals. Most luminal taxa are shared with the adjacent mucosa and luminal sites, with a gradient of unique mucosal taxa occurring along the intestine. See also Table S2.

Gram-negative (13/17 genera) and frequently facultatively anaerobic (8/17 genera), while the lumenally enriched taxa were primarily Gram-positive (16/22 genera) and obligately anaerobic (19/22 genera). Most obligately anaerobic genera were not abundant in the mucosa; only *Treponema*, which is well-adapted to oxidative stress, showed a modest mucosal preference (Giacani et al., 2013; Jovanović et al., 2000). This suggests that oxygen availability is a major, but not sole, determinant of mucosal composition (Albenberg et al., 2014).

Most mucosally enriched genera identified here were not identified by univariate analysis because, with the exception of *Actinobacillus*, they were enriched only in either the SI or LI mucosa, but not both (e.g., *Klebsiella* in the LI and *Gemella* in the SI) (Figure S3); univariate analysis only detected mucosal enrichment consistent in both locations. The SI lumen was represented only by ileal samples, but most genera strongly enriched in the ileal lumen relative to the ileal mucosa (e.g., *Lactobacillus* and *Slackia*) were also strongly enriched at multiple locations along the LI lumen relative to the LI mucosa (Figures 4 and S3). This luminal community similarity may be partially explained by pH similarity between ileum (7.0–7.4) and colon (6.6–7.0) (Mercier et al., 2007) (Figure S4C).

Figure 4B summarizes the relationships of β values (regression slopes) between adjacent sites in the macaque gut. We observed four main patterns of microbial enrichment and potential flow: (1) SI mucosally enriched taxa, (2) LI mucosally enriched taxa, (3) SI and LI lumenally enriched taxa, and (4) clades following no consistent pattern. *Actinobacillus* and *Pasteurella* exemplify the pattern typically observed in SI mucosally enriched clades (Figure S3). They are most abundant in the ileal and jejunal mucosa, much less abundant in LI than SI, and more abundant in the LI mucosa than lumen. The differences in abundance between sites are very consistent, so the abundance at one site can be used to predict the abundance at another. Similarly, *Brachyspira* and *Helicobacter* are most abundant in the LI mucosa, and stool is highly predictive of their abundance elsewhere in the LI. *Lactobacillus*, *Ruminococcus*, and *Dialister* are enriched throughout the lumen and predictably present in the mucosa at much lower abundance. Finally, several clades had predictable but atypical abundance patterns. For example,

Klebsiella was only present in the distal LI. In summary, using logistic regression modeling allowed us to group bacterial taxa that followed similar predictable ecological patterns across the intestine, and in some cases, we were able to predict where taxa may have originated within the intestine when observed in the stool.

Differences in Mucosal and Luminal Community Function Correspond to Oxygen and Nutrient Availability

In order to understand the functional differences between communities at distinct biogeographic sites and their relation to community composition, we used PICRUSt (Langille et al., 2013) to infer community metabolic potential (Supplemental Experimental Procedures) and then used LEfSe (Segata et al., 2011) to identify functions that differed significantly between sites (see Experimental Procedures; Figure S4D; Table S1). PICRUSt is particularly useful for determining mucosal microbial community function due to the high host DNA content of mucosal metagenomes.

Many functional differences between mucosa and lumen, such as increased capacity for riboflavin biosynthesis, could be explained by difference in *Helicobacter*. While *Helicobacter* and Gram-positive bacteria both have fused *ribAB* genes in their riboflavin biosynthesis operons, *Helicobacter* also have an additional copy of the *ribA* gene (Fassbinder et al., 2000). Likewise, glutamate/aspartate transport capacity was mucosally increased; *H. pylori* preferentially consumes amino acids for energy (Stark et al., 1997), and its glutamate and aspartate transport and deamidase activity are essential for mouse colonization (Leduc et al., 2010). Glycolysis capacity was correspondingly increased in the lumen, where *Helicobacter* was not dominant. The SI enrichment in the mannose-specific phosphotransferase system is likely explained by *Pasteurella* (Binet and Bouvet, 1998) (Figure S4D).

DISCUSSION

In this study, we comprehensively examined the composition of the macaque gut microbiome at ten different biogeographic locations within 15 individuals. The most similar previous study cross-sectionally compared the gut microbiota of healthy and

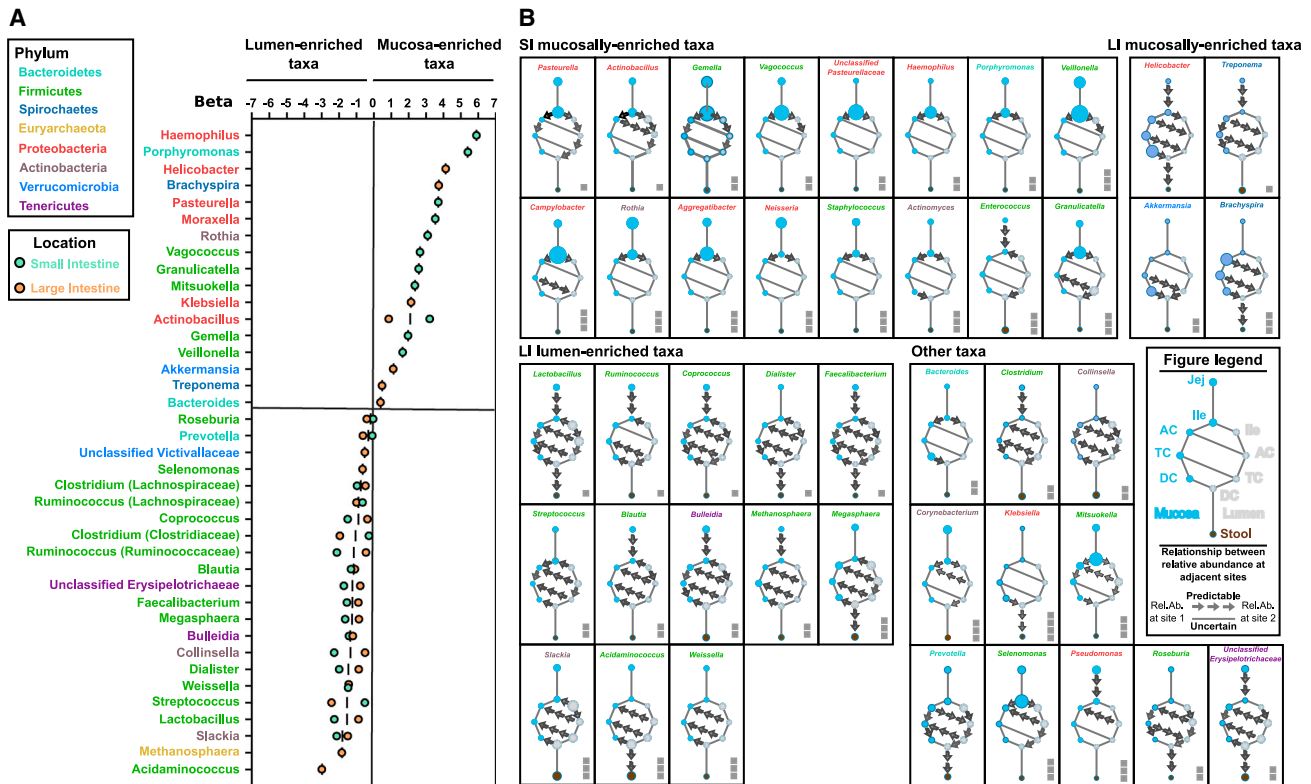


Figure 4. A Logistic Regression Model of Bacterial Taxa Site Enrichment and Flow through the Macaque Gut

(A) Average within-small-intestine and within-large-intestine β values (regression slopes) for each genus in each biogeographic region. β corresponds to the magnitude of a difference in relative abundance between two sites (mucosa and lumen) and its consistency across 15 animals, and thus the enrichment of a taxa in mucosa or lumen. Only genera with at least one significant ($p < 0.05$ and $q < 0.05$) value for β are shown.

(B) Intra-intestinal microbial predictability between adjacent sites for each bacterial genus. Points on each clock-like diagram represent biogeographic sites, and point size corresponds to mean relative abundance across all animals for each genus. Adjacent sites with significant non-zero β (indicating that relative abundance at one site can predict that of the other) are connected with an arrow; arrows always start at the site with higher relative abundance. Solid lines indicate non-significant β , and arrows with significant β that point opposite of the physiological flow of intestinal contents inside the intestine (proximal to distal) are also replaced by a solid line. For visualization, taxon relative abundances were adjusted by a factor of 10^1 to 10^3 ; the adjustment is indicated by the number of squares by each diagram (e.g., *Pasteurella* was adjusted by 10^1 and has one square). See also Figure S3.

sick (e.g., SIV-infected) macaques, although it also included several biogeographical locations drawn from distinct individuals (McKenna et al., 2008). In contrast, our study included only healthy individuals and comprehensively examined the microbiota of the same individual at many biogeographic sites at the same time. We quantified the degree to which microbial composition at one biogeographical location within the gut predicts that of another, particularly the extent to which stool samples reflect the mucosal microbiome. We found that between stool and colonic mucosa, both the conservation of taxa and their rank correlation were remarkably high ($r > 0.85$), supporting the use of stool samples for translational studies of colonic mucosal inflammation in human subjects.

The human, macaque, and mouse gut microbiomes are fundamentally similar in containing Bacteroidetes, Firmicutes, and Proteobacteria (Human Microbiome Project Consortium, 2012; Kostic et al., 2013b; McKenna et al., 2008). In contrast, the macaque gut mucosa was most remarkable for its abundance of ϵ -proteobacteria, specifically *Helicobacter*, which reached up to 80% relative abundance in the LI mucosa of some animals. The macaque gut also included a substantial component of the

Spirochaetes *Treponema* and *Brachyspira*, which comprised ~3% of the mucosal microbiota. A recent study of the gorilla, chimpanzee, and bonobo microbiomes found that they also contained *Brachyspira* and *Treponema*, but no *Helicobacter* was detected in their stool (Moeller et al., 2013). While Spirochaetes carriage is typically associated with intestinal pathology in humans, it can be asymptomatic and is more typically found in stool in residents of non-developed countries (Tsinganou and Gebbers, 2010). Recent studies have detected *Brachyspira* spp. and *Treponema* spp. OTUs in the stool of Malawians, Amerindians, and children from rural Burkina Faso and Bangladesh slums, but not in comparison cohorts from the USA (De Filippo et al., 2010; Lin et al., 2013; Yatsunenko et al., 2012). The disappearance of these taxa in residents of developed countries may be associated with modern sanitation practices, including use of antibiotics and pesticides.

Integrating these results with our prior knowledge of intestinal ecology and microbial metabolism (Human Microbiome Project Consortium, 2012; McKenna et al., 2008; Qin et al., 2010; Stearns et al., 2011; Yatsunenko et al., 2012) refines our insights into the ecological dynamics of the gut microbiome. Relative to the

inter-individual differences observed in human populations, inter-individual differences in this study were minimal, yet they were still a significant source of variation despite all animals being uniformly fed and housed for at least 2 years prior to sampling. Intestinal oxygen content appeared to determine the dominant taxa colonizing the mucosa and lumen, as the mucosa was colonized primarily by facultative anaerobes, while the lumen was colonized mainly by obligate anaerobes. This in turn dictates the main patterns of community functionality, as luminal bacteria (Prevotellaceae, Lachnospiraceae, and Ruminococcaceae) are as a result primarily carbohydrate fermenters (Duncan et al., 2007). There were correspondingly large differences between small- and large-bowel mucosal communities, potentially corresponding to difference in pH, bile salt, and/or mucin composition (Croix et al., 2011; Walter and Ley, 2011), although the difference between the luminal microbiota of the SI and LI was much smaller.

The relationship between the stool and mucosal microbiota is highly relevant to human clinical studies, as disease may localize to specific locations while only stool remains readily accessible (Darfeuille-Michaud et al., 2004). Patient biopsies are the current gold standard for study of human-associated mucosal communities, but invasiveness and expense limit the frequency with which they can be performed. At the same time, an increasing body of data (Gevers et al., 2014; Morgan et al., 2012) underscores the importance of studying the microbiome longitudinally during the development of disease, a near impossibility with mucosal biopsies. Our results quantify the extent to which the stool microbiota reflects the SI and LI mucosa and is highly correlated with the colonic lumen and mucosa. Thus, although biopsies are optimal for studying the SI mucosa, our results in a primate model representative of the human gut microbiome show that stool is still surprisingly representative of the colonic mucosa.

EXPERIMENTAL PROCEDURES

Sample Collection and Sequencing

All animals used in this study were housed at the NEPRC in accordance with all applicable regulations and in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were maintained under an experimental protocol approved by Harvard Medical School's Standing Committee on Animals. Stool and paired intestinal luminal and mucosal samples from ten segments distal to jejunum were collected from 15 clinically healthy female rhesus macaques, and the V4 region of the 16S rRNA gene was sequenced by Illumina MiSeq (Figure S4A; Figure S4B). A mean sequence depth of 29,914/sample was obtained; samples with fewer than 3,000 filtered sequences and those OTUs with fewer than 15 reads were excluded from further analysis. All further details of animal husbandry, sample collection, preparation, sequencing, and bioinformatic processing are outlined in the Supplemental Information.

External Data

For all comparisons to previously published studies, taxonomic or raw sequencing data were obtained from publicly available sources (Handley et al., 2012; Human Microbiome Project Consortium, 2012; Yatsunenko et al., 2012) or directly from the investigator (McKenna et al., 2008). Taxonomic tables were summarized to genus-level clades and merged. Further details are outlined in Supplemental Information.

OTU Overlap between Sites

For each pair of adjacent sites, the number of OTUs observed in both adjacent sites was counted in each individual and subsequently averaged across all 15 animals. To minimize the influence of low prevalence OTUs and differences in

sequencing depth, only OTUs with 15 reads per OTU in 15 animals were considered in this analysis.

Identification of Microbial Taxa Enrichment Sites and Predictability by Logistic Regression

We built a logistic regression model for each taxa between each adjacent biogeographical site pair, as described in Supplemental Experimental Procedures. The regression slope β between each pair of locations is calculated as the contrast between the coefficients of indicator variables corresponding to the locations. p values of all β are adjusted for multiple hypothesis testing using the Benjamini-Hochberg procedure (false discovery rate [FDR] = 0.05). Cytoscape was used to visualize Figure 4B.

ACCESSION NUMBERS

Sequences generated in this study are publicly available (NCBI BioProject ID number PRJNA259224).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2015.01.015>.

AUTHOR CONTRIBUTIONS

K.Y., K.O., S.V.W., K.G.M., E.J.V., G.M., J.K.R., D.G., C.H., and X.C.M. designed the study. K.Y., S.V.W., K.G.M., and D.G. processed samples and facilitated DNA sequencing. K.Y., K.O., B.R., T.L.T., E.A.F., C.H., and X.C.M. analyzed the data. K.Y., C.H., and X.C.M. prepared the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank L. Waldron, E. Schwager, and J. Moon for assistance with statistical analyses; W. Garrett and the Garrett and Huttenhower research groups for comments on this manuscript; S. Macri, E. Curran, L. Kattenhorn, B. Assaf, K.C. Lin, N. Silva, and J. Mackey for assistance with sample collection; and T. Poon for assistance with sequencing. This work was supported by the Robert F. Naka fellowship (K.Y.), Base Grant (P51 RR000168-48) (NEPRC), NIH R01HG005969 (C.H.), NSF DBI-1053486 (C.H.), and ARO W911NF-11-1-0473 and W911NF-11-1-0429 (C.H.).

C.H. is a member of the scientific advisory board of Seres Health. The other authors have nothing to disclose.

Received: October 12, 2014

Revised: December 23, 2014

Accepted: January 9, 2015

Published: February 26, 2015

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