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Diversity of the autochthonous colonic microbiota

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A longstanding hypothesis in intestinal microbial ecology is that autochthonous microbes (resident) play a role that is distinct from allochthonous microbes (transient microbes in the fecal stream). A challenge has been to identify this pool of microbes. We used laser capture microdissection to collect microbes from the mouse ascending colon. This area contains transverse folds that mimic human intestinal folds and contains a distinct population of intestinal microbes that is associated with the mucosa. Our analysis of bacterial 16S rRNA genes showed that this area was enriched for Lachnospiraceae and Ruminococcaceae. In this addendum, we further compare this community to studies of mucosa-associated microbes in humans. This analysis reveals common phylogenetic groups of bacteria that are present in both mouse and human. However, we found microorganisms at the genus and species levels including *Faecalibacterium prausnitzii* which appears to be specific for humans. We propose that this examination of the mucosa-associated microbes in wild type and genetically modified mice will be a valuable component to define host microbial interactions that are essential for homeostasis.

Key words: intestine, microbiota, spatial, Lachnospiraceae, Ruminococcaceae, mucosa-associated, *Faecalibacterium prausnitzii*

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Intestinal Microbiota and Host Homeostasis

The intestinal microbiota plays a pivotal role in local and systemic host physiology. Studies in germ-free mice demonstrated that intestinal colonization by commensal bacteria contributes to the development of adaptive lymphoid tissue,[1] innate immune responses[2-4] and intestinal angiogenesis.[5] Also, at the level of the whole organism, the intestinal microbiota plays an active role in host's energy harvesting and storage[6-8] as well as impacts the pathogenesis of autoimmune[9] and metabolic diseases.[10,11] In contrast, our understanding of the biological basis controlling selection, colonization, persistence and function of intestinal microbes remains limited.

Specialized Microbial Niches in the Intestinal Mucosa

One strategy to discern factors that drive the selection, diversity and function of microbial populations is the study of their spatial distribution. Studies in soil,[12] hypersaline mats[13] and sewage biofilms[14] have shown that microbial communities are spatially organized in predictable patterns determined by energy source gradients (reviewed in ref. 12–14). However, the application of the techniques used in these environmental studies is challenging for the intestinal microbiota due to the difficulties to collect samples without disturbing the structure of the microbial populations and their nearby environment.

In the intestine, the spatial distribution of intestinal microbes occurs along two basic axes, (1) longitudinally from proximal to distal and (2) radially from the central lumen to the mucosal surface.[15] Many studies used culture-independent methods (e.g., PCR-based techniques) to document the microbial density and composition in different regions along the length of the intestine. These comprehensive analyses of microbial populations showed alterations in the diversity and density of bacterial 16S rRNA genes

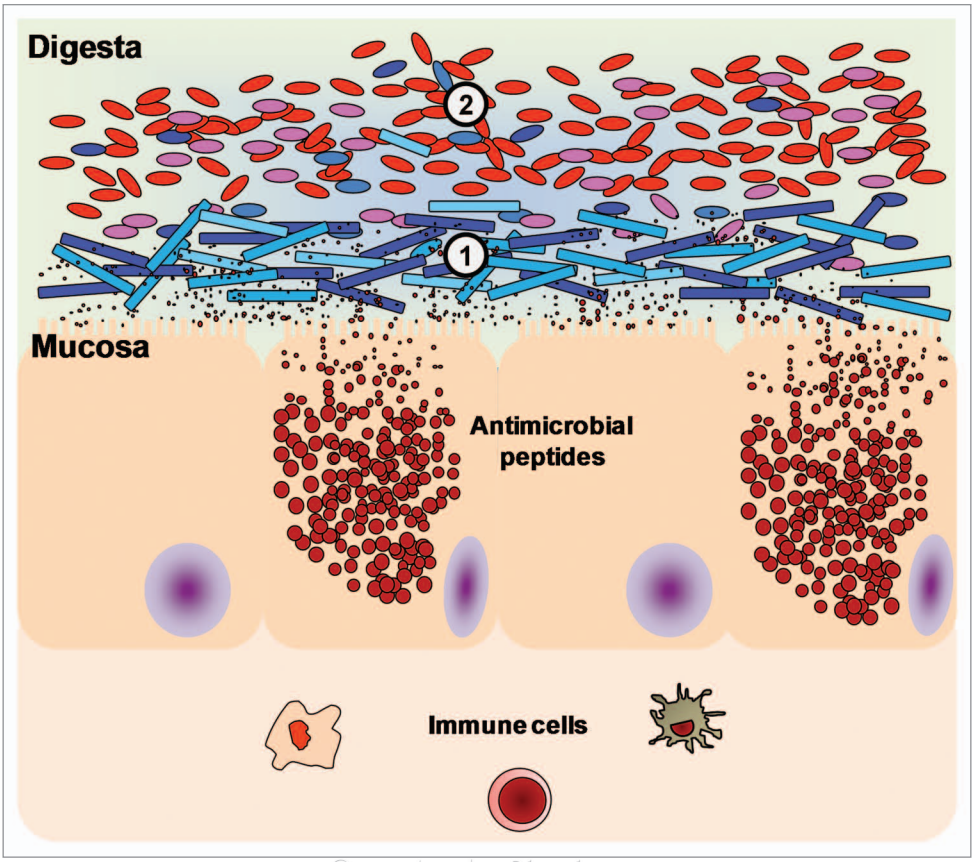

Figure 1. Model of the interaction of host and microbiota in the intestine. The epithelial monolayer produces mucus as well as antimicrobial peptides and proteins that form electrostatic interactions in the intestinal lumen. This forms a specialized niche where autochthonous (resident) microbes appear to reside. Autochthonous microbes (1) are comprised of compact interlacing layers of predominately large, fusiform-shaped bacteria that appeared in close apposition to the apical surface of the colonic epithelium. These microbial communities are distinct, both morphologically and phylogenetically, as compared to allochthonous (transient) microbes (2) that are located in the central lumen (digesta) as part the fecal stream. Allochthonous communities include rod- and coccoid-shaped bacteria associated with undigested food particles.

in ileum, cecum, proximal, distal colon and feces of healthy humans and mice.[16-20] However, comprehensive studies of the spatial organization of microbes across the radial axis of the intestine are scant.

The spatial organization of the intestinal microbiota is of interest as it implies the determination of autochthonous (resident) versus allochthonous (transient) microbes. Resident microbes have been proposed to be closely associated with the intestinal mucosa perhaps in association with a portion of the mucus that overlies the intestinal epithelium, while more transient microbes are thought to be located in the central lumen as part the fecal stream.[21] The anatomy of the intestinal mucosa contains important local variations which may provide a niche for autochthonous microbes. In humans, the small intestine and proximal colon contain complete circular or semi-circular folds (also known as *plicae circularis* or *plicae semilunaris*, respectively) that project approximately 1–2 mm into the intestinal lumen and are perpendicular to the direction of the fecal stream. Their function is unproven, though these structures have been proposed a mechanism to slow transit time.[22]

As the acquisition of microbes from this anatomic location in humans is difficult without manipulation of the luminal contents, we examined a portion of the mouse intestine that contains similar mucosal anatomy. The mouse proximal colon (also known as the ascending colon) contains transverse folds that project ≈1 millimeter into the lumen and are oriented in a direction perpendicular to the fecal stream. This mucosa niche provides an excellent biological system to examine the interaction between host and microbial communities.

Defining the Diversity and Function of Colonic Mucosa-Associated Bacteria in a Specialized Niche

In mammals, the radial organization of intestinal microbes has been a long-standing interest. Morphological examination of the mouse and rat intestine showed that microbes characterized by long spiral rod morphology colonize at high density in select mucosal associated areas. Such niches are in close proximity to the columnar epithelium that lines the intestine whereas coccus-shaped microbes are more prevalent to low density areas, such as the central intestinal lumen.[23-25] These morphologic patterns of spatial structure and niche adaptation by the colonic microbiota were initially proposed in a series of studies by Savage and Dubos.[26-29] Their observations of

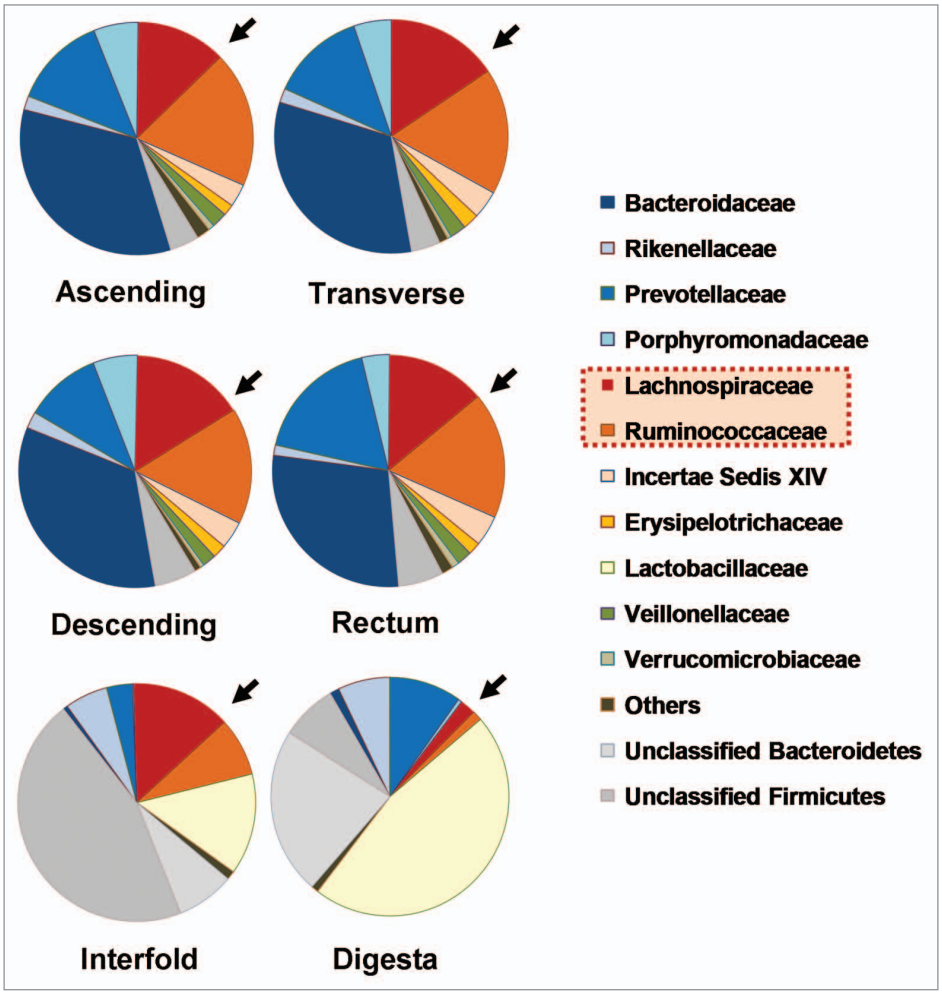

Figure 2. Mucosa-associated autochthonous microbes in the human and mouse colon are enriched for Lachnospiraceae and Ruminococcaceae families. We compared diversity at the family level. Each chart represents the taxonomic composition. Sequences were previously obtained in another study from the ascending-, transverse-, descending-colon and rectum biopsies of healthy humans. This data was extracted from a comprehensive molecular analysis of almost full-length 16S rRNA gene sequences.[17] Pyrosequencing analysis was used to examine microbial diversity between interfold (29,560 reads) and digesta (38,120 reads) regions from the colon of wild-type mice.[30] Lachnospiraceae and Ruminococcaceae are indicated by arrows and outlined with a dotted line to highlight these families. Noteworthy, the enrichment of both Lachnospiraceae and Ruminococcaceae families was also observed in mucosal biopsies obtained from healthy humans who had undergone bowel preparation. These data indicate that detection of these bacterial families can be accomplished regardless the methodology for sample acquisition. Unclassified Bacteroidetes and Firmicutes correspond to sequences not classifiable at family level (as of December 2010). Both data sets were classified using the Classifier version 2.2 at the Ribosomal Database Project.

the proximal colonic mucosa showed that fusiform- and spiral-shaped bacteria were the most predominant resident microbes in mucus layer covering the colonic epithelium; therefore, these microbial populations were defined by Dubos and colleges as autochthonous communities.[29] They suggested that these fusiform-shaped microbes had established symbiotic relationships with their host through evolution; in contrast, microbes ubiquitous in the community should be considered normal or commensal microbiota.[29]

We recently reported in reference 30, morphologically comparable microbial structures in the interfold regions of the colonic mucosa of mice. These microbial communities were comprised of compact interlacing layers of predominately large, fusiform-shaped bacteria (size >5–10 μm) that appeared in close apposition to the apical surface of the colonic epithelium. Conversely, the central lumen (digesta) contained rod- and coccoid-shaped bacteria (size 1–2 μm) associated with undigested food particles (**Fig. 1**). Our contribution was to provide a method to collect microbes in these distinctive locations and perform a comprehensive characterization of microbial communities. Our goal was to identify the resident microbes discovered by Dubos and colleages more than four decades ago.

We established methodologies to examine these mucosa-associated microbial communities and compare their spatial distribution across the radial axis. To achieve these objectives, we adapted the technology of laser capture microdissection (LCM) to procure intestinal microbes from defined locations within

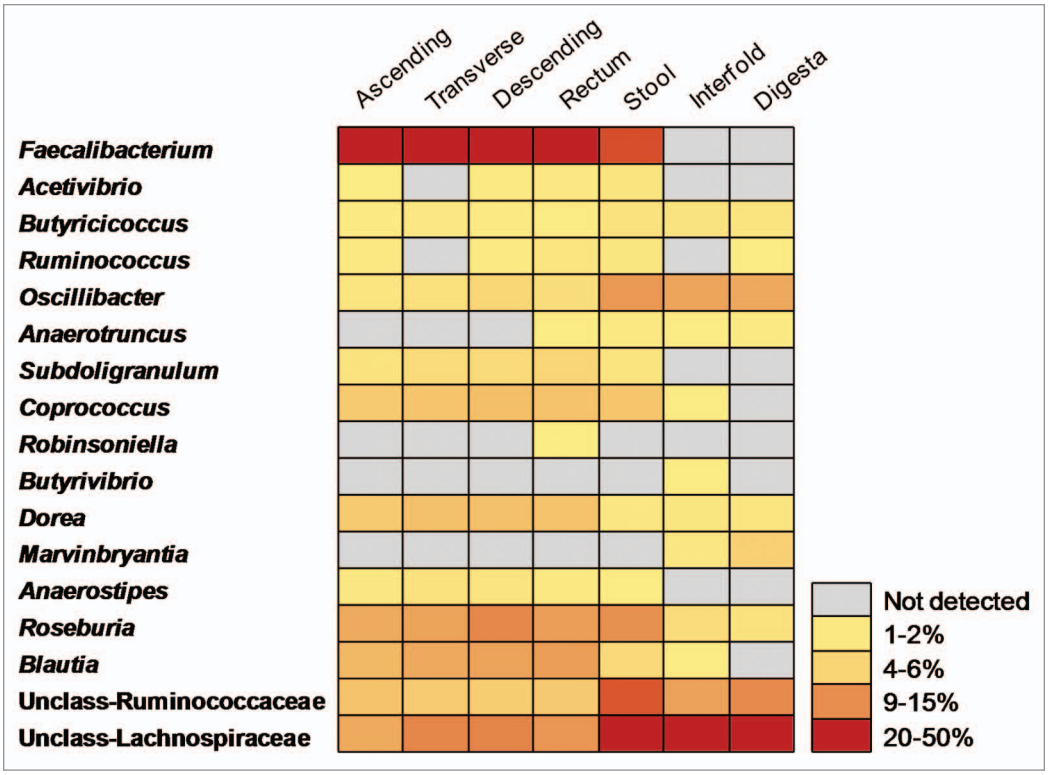

Figure 3. Common and specific phylogenetic genera of autochthonous bacteria associated to the colonic mucosa of both mouse and human. We compared diversity at the genus level. Each heat-map represents the relative abundance of each genus from Lachnospiraceae, Ruminococcaceae and *Incertae Sedis* XIV families (all members of the *Clostridium* cluster XIV). Sequences were obtained from the ascending-, transverse-, descending-colon and rectum biopsies of healthy humans, and interfold and digesta regions from the colon of wild-type mice. Unclassified (Unclass) Ruminococcaceae and Lachnospiraceae correspond to sequences not classifiable at genus level (for details see legend in Fig. 2).

the intestinal lumen. We propose that this technique will be generally applicable and will increase our understanding of the biological basis controlling selection, colonization, persistence and function of intestinal microbes.

As a proof of principle, we examined the diversity and spatial organization of microbes in the proximal murine colon, a region that contains mucosal folds inhabited by the autochthonous microbial communities described by Savage, Dubos and colleagues.[26,29] We used complementary microbial ecology techniques such as tag pyrosequencing, Sanger sequencing, DNA fingerprinting and quantitative PCR targeting bacterial 16S rRNA genes to examine diversity and density of microbes associated with mucosal folds (interfold region) and within the central lumen (digesta region). This approach successfully dissected the radial distribution (mucosa- vs. digesta-associated) of the colonic microbiota. Our study showed that microbial communities in the interfold region were highly enriched for the phylum Firmicutes and, more specifically, for the families Lachnospiraceae and Ruminococcaceae. In contrast, Bacteroidaceae, Enterococcaceae and Lactobacillaceae were all enriched in the digesta region. A comparable enrichment for Lachnospiraceae and Ruminococcaceae, members of the *Clostridium* cluster XIV,[31] have been observed in the colonic mucosa of healthy humans[16,17] (**Fig. 2**).

Niche Specialization in the Intestinal Folds

Our working hypothesis is that members of the autochthonous microbiota, (e.g., Lachnospiraceae and Ruminococcaceae) have undergone significant genome adaptations to facilitate niche specialization. This type of diversification may involve gain and lose of regulatory genes, interspecies horizontal transfer, gene mutations and genome reduction. Bacterial families such as Vibrionaceae have exploited this genomic plasticity to live as pathogens, symbionts, free-living forms and extremophile microorganisms.[32]

The challenge now is to develop a system to characterize members of the autochthonous microbiota to gain knowledge of different mechanisms involved in niche diversification. One potential strategy is to examine the spatial organization of the intestinal microbiota in different model organisms and perform comparative analysis, including microbial surveys obtained from intestinal biopsies from humans. The outcome of these analyses will facilitate the selection of bacterial targets for isolation and whole genome analysis.

New Challenges and New Horizons in the Study of Intestinal Microbiota

Based on the initial studies by Savage, Dubos and colleagues in the 70's[26,29] and

our new data,[30] we hypothesize that these autochthonous communities, including Lachnospiraceae and Ruminococcaceae, evolved special mechanisms to survive in a hazardous niche that contain high concentrations of endogenous antimicrobial peptides (for a review see ref. 33 and 34) but at the same time remain innocuous to the host. To further support the idea that autochthonous microbes have evolved symbiotic relationships with their host, we have performed additional analysis comparing the diversity of colonic mucosa-associated bacteria in human and mice from our study and the published work of other investigators.[17] We found that the mucosa associated surfaces of the colon of mice and humans is predominantly colonized by similar phylogenetic core of Lachnospiraceae and Ruminococcaceae genera: *Butyricicoccus, Ruminococcus, Oscillibacter, Anaerotruncus, Coprococcus, Robinsoniella, Dorea, Anaerostipes, Roseburia* and *Blautia* (**Fig. 3**).

More importantly, comparisons of microbial diversity between human and mice uncovered indications of species-specificity. For example, genera *Faecalibacterium, Subdoligranulum* and *Acetivibrio* were only observed in the human colon whereas genera *Marvinbryantia* and *Butyrivibrio* were only found in the colonic mucosa of mice (**Fig. 3**). Based on these observations, we conducted a systemic search using one of the most comprehensive, well annotated and frequently updated 16S rRNA gene database.[35] We found that of the 6,429 sequences of *Faecalibacterium* archived in the database (as of December, 2010), only seven have been obtained from mice, a humanized gnotobiotic mouse model.[36] Likewise, of the 319 sequences of *Marvinbryantia* archived in the database (as of December, 2010), none correspond to human intestinal samples. Interestingly, one of these genera, *Faecalibacterium*, was implicated in a human intestinal disease; a decrease in *Faecalibacterium prausnitzii* has been observed repeatedly in the ileal and colonic mucosa of inflammatory bowel disease patients.[16,37,38] Unfortunately, as with many other members of the Lachnospiraceae and Ruminococcaceae families, our knowledge about the biology of the genus *Faecalibacterium* is very limited. Only a few species from this genus (*Faecalibacterium prausnitzii*, *Faecalibacterium* sp. DJF-VR20, Clostridiaceae bacterium DJF-VR09, Butyrate-producing bacterium M21/2 and Bacterium ic1379) have been isolated and cultivated. Taken together, these data provide new experimental frames for the study of autochthonous microbes and uncover their potential role in intestinal health.

In summary, our study has established new insights into the spatial organization and diversity of microbes across the intestinal lumen. The current challenge is to adapt this technology to perform whole metagenomic and transcriptome analyses of these autochthonous communities. This system will provide a great experimental model to uncover factors and mechanisms driving the selection, diversity and function of microbial populations. With the aid of mouse models, these studies will facilitate the discovering and validation of biomedical principles and therapeutic alternatives to improve and restore intestinal health.

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