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Cause or effect? The spatial organization of pathogens and the gut microbiota in disease

Jen Nguyen [a, b], Deanna M. Pepin [a], Carolina Tropini [a, b, c, *]

[a] Department of Microbiology & Immunology, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada
[b] School of Biomedical Engineering, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada
[c] Humans and the Microbiome Program, Canadian Institute for Advanced Research (CIFAR), Toronto, ON, M5G 1M1, Canada

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ABSTRACT

The human gut hosts a dense and diverse microbial community, spatially organized in multiple scales of structure. Here, we review how microbial organization differs between health and disease. We describe how changes in spatial organization may induce alterations in gut homeostasis, concluding with a future outlook to reveal causality.

1. Microbial spatial organization in the gut

Spatial organization is a pervasive feature of natural ecosystems. Hidden within us is our gut microbiota — a collection of bacteria, fungi and viruses associated with our intestinal tract — organized in a complex structure that changes alongside variations in the gut habitat. Distinct spatial niches of the gut — the intestinal lumen, the mucus layer that separates the luminal contents from the gut epithelium, and the microscopic folds of the epithelium (crypts) — are associated with distinct microbial communities. For instance, Proteobacteria account for roughly a third of the bacteria found in both the mucus and crypts in healthy guts, yet only 1% of the fecal microbiota [1]. These gradients in bacterial abundance are formed by gradients in chemical (e.g., pH, metabolite concentrations), physical (e.g., mucosal coverage, fluid flow), and biological (e.g., microbial species composition, abundance) factors, which control and distinguish microbial function and have been recently reviewed [2–4].

In this review, we focus on the localization of intestinal pathogens and the function of microbial spatial organization in community dynamics and human disease (Fig. 1). We first zoom in to how microscale organization dictates disease–relevant interactions among individual bacteria and between bacteria and host. We then zoom out to increasingly larger interactions, describing how organization at each scale contributes to either the mechanisms of homeostatic gut health, or those underlying disease onset and exacerbation. Finally, we provide an outlook on important future directions to move the field towards an understanding of the onset, progression and recovery from complex intestinal diseases.

2. The 1 μm scale: direct cell-to-cell interactions affect colonization of pathogens

The smallest scale of spatial organization in microbial communities is that of single cells. The most fundamental change in spatial localization is the presence or absence of bacteria, which can define larger-scale gradients in microbial biogeography. Absence arises through cell death or displacement without replacement, while presence is commenced or reinforced by immigration from other locations or cell growth and division. Bacteria frequently enter the gut by oral consumption and are mixed within the gut by peristalsis. In this highly dynamic gut environment, individual bacteria often physically encounter other species and compete for nutrients and space. These single-cell interactions not only occur between commensal bacteria but are also a key step in pathogen invasion (Fig. 2).

* Corresponding author. Department of Microbiology & Immunology and School of Biomedical Engineering, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada.
E-mail address: carolina.tropini@ubc.ca (C. Tropini).

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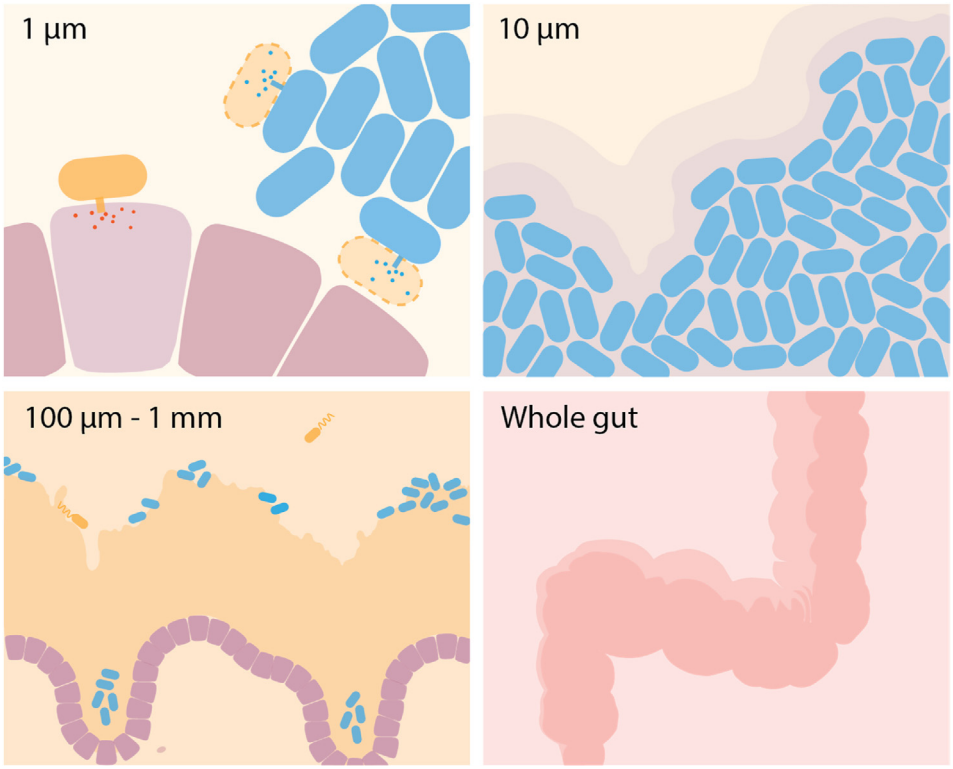

Fig. 1. Bacterial organization in the gut determines the scales at which bacteria interact with each other and their host. Microscale organization dictates disease–relevant interactions between individual bacteria, and between bacteria and host. The smallest scale we consider (1 µm) is that of physical interactions between individual bacterial (blue and orange) and epithelial (pink) cells. One order of magnitude larger (10 µm) are local hotspots of nutrients or antimicrobials (purple), generally highest in concentration at their source. Larger still are mesoscale interactions (100 µm–1 mm), at which bacteria interact with gradients maintained by the mucus layer lining the gut epithelium. Finally, at the scale of whole gut compartments, intestinal motility and biochemistry play further roles in colonization resistance, host health and the onset of disease.

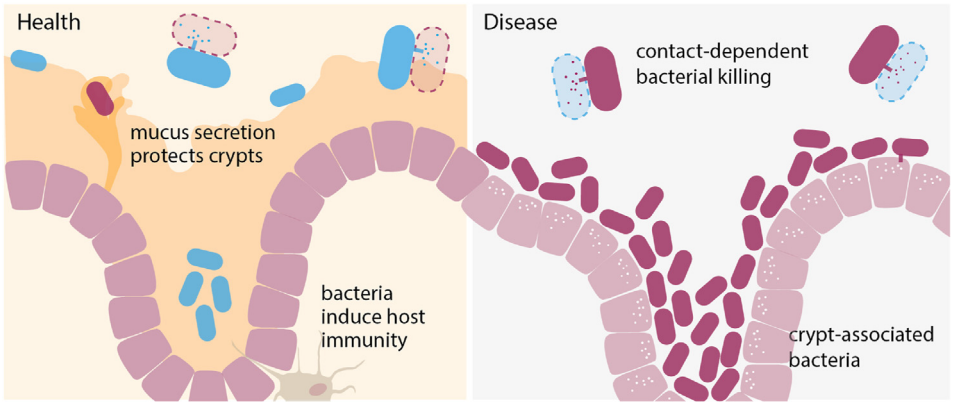

Fig. 2. Micron-scale contacts between commensals, pathogens and host cells. Physical contact between cells can elicit a variety of responses. Commensals and pathogens (blue and red cells, respectively) can displace one another through contact-dependent bacterial killing. Bacterial contact with the host epithelium is rare in health and broadly observed in disease. In health, mucus segregates most bacteria from the epithelium, which upon sensing bacteria, can secrete mucus (orange) to expel them. Pathogens can induce physiological changes in host cells (pink cells), such as inflammation. Specific bacteria can also induce host immune cells (brown cell) by physically attaching to the host epithelium.

2.1. Contact-dependent induction of disease

Most intestinal pathogens require direct contact with the gut epithelium to cause disease and trigger inflammation [5], thereby altering the gut environment and favoring their proliferation. In mouse models of colitis, *Salmonella enterica* serovar Typhimurium mutants unable to trigger inflammation are ultimately outcompeted by the commensal microbiota [6]. This competition between pathogens and commensals is largely dependent on spatial organization, including proximity to the host, as we will now discuss in depth.

Physical contact with the host enables pathogens to inject causative factors of several human diseases directly into host cells (as thoroughly reviewed in Deng et al. [7]), or to drive physiological alterations in the gut that favor pathogen growth and long-term colonization [5]. For example, diverse Gram-negative bacteria express type III secretion systems (T3SS), which enable bacteria such as *Salmonella*, *Shigella*, and *Chlamydia* species to invade host cells and induce inflammation to promote their long-term colonization. Pathogens can also compete with commensal bacteria by modulating host cells via physical contact. In the guts of larval zebrafish,

Vibrio cholerae uses a type VI secretion system (T6SS) directly on host epithelial cells to modulate gut peristalsis and the motility of luminal contents [8]. By inducing faster peristaltic contractions, *V. cholerae* T6SS activity creates stronger than normal flow fields that expel slower swimming or non-motile commensals from the gut. Like humans, zebrafish are natural hosts of *V. cholerae*, and it is plausible that this contact-dependent means of pathogenic modulation of host motility is also an explanation for the altered composition of intestinal microbiota associated with cholera diarrhea [9].

2.2. Contact-dependent host defenses

Upon sensing bacteria, some epithelial cells secrete mucus that pushes potential pathogens away from the gut epithelium [10]. Mucus is made up of highly glycosylated proteins, which bind water and form a gel that slows transport and diffusion along the lining of the gut [11]. In the human colon, the epithelium secretes mucus at an average rate of four microns per minute, maintaining the sterility of the mucus closest to the host tissue: a region penetrated only in disease [12]. Mice lacking Muc2, the most abundantly secreted gastrointestinal mucin, develop inflammation and colorectal cancer [13] and display bacterial colonization at the epithelium, specifically in crypts [11]. To protect crypts from pathogen colonization, goblet cells positioned at the upper entrance of crypts sense bacterial presence via toll-like receptor (TLR) binding to bacterial ligands, including flagellin (a protein component of bacterial flagella) and lipopolysaccharide (LPS, a major component of Gram-negative outer membranes) [10]. TLR binding induces these goblet cells to trigger Muc2 secretion by adjacent epithelial cells, expelling nearby bacteria.

Physical contact between host and bacteria can also induce responses from immune cells. In mice, the localization and attachment of segmented filamentous bacteria (SFB) to intestinal epithelial cells induces the production of intestinal T helper cells (Th17 cells) [14]. SFB antigen and luminal colonization alone cannot cause Th17 cell induction [15]. Rather, the physical adherence of SFB to the host epithelium is required to elicit such a specific immune response [15]. Intestinal colonization with SFB also increases host expression of inflammatory and anti-microbial defenses, enhancing gut resistance to colonization of the intestinal pathogen *Citrobacter rodentium* [14]. Thus, the spatial localization of SFB to the host epithelium enables this commensal bacterium to prevent pathogen colonization via contact-dependent modulation of murine immunity and disease. Intriguingly, this response is also induced by adherent extracellular pathogens such as enterohemorrhagic *Escherichia coli* (EHEC), *Candida albicans*, and *C. rodentium* [15]. Colonizing germ-free mice with a cohort of adherent bacteria isolated from ulcerative colitis (UC) patients also induces Th17 production [15]. That a broad range of bacteria can elicit such responses from the host immune system highlights the importance of bacterial-host contact in intestinal health.

2.3. Contact-dependent bacterial warfare

Cell-to-cell contact can affect pathogen invasion not only when it occurs between the pathogen and host cells, but also when it takes place between a pathogen and commensal bacteria. Cell-to-cell mediated bacterial killing is an important protective function of the microbiota, promoting colonization resistance against pathogenic invasion [16]. Contact-dependent mechanisms of bacterial killing are spatially dependent, necessitating that bacterial killers and victims be within a micrometer of each other [17]. Diverse secretion systems (i.e., T4SS, T6SS, T7SS), cell surface filaments, nanotubes, and even the exchange of outer membrane material are among the observed mechanisms by which bacteria deliver bacteriocins, toxic bacterially produced peptides, to inhibit or kill neighboring bacteria in a strain-specific manner [17]. This form of bacterial warfare can occur in any densely occupied compartment of the gut and cause attack of pathogens by commensals, or vice versa [18]. For example, *S. Typhimurium* requires a type VI secretion system (T6SS) to target and outcompete select members of the gut microbiota, including *Klebsiella oxytoca*, and establish within a host gut [19].

In summary, physical contact between pathogens and the epithelium is necessary for the onset of some diseases and heavily implicated in others. Both host and commensals have mechanisms of sensing and combating bacterial contact, underscoring the importance of bacterial spatial organization at the micrometer and sub-micrometer scales in health and disease.

3. The 10 μm scale: local competition restricts biogeography of pathogen growth

Metabolite and protein gradients control bacterial spatial organization at length scales on the order of tens of micrometers (Fig. 3). These local gradients form via the secretion, degradation and consumption of molecules by both bacteria and host cells. For example, some gut commensal bacteria can degrade polysaccharide-rich mucus [20], releasing simple sugars into the local environment. These sugars are an important nutrient source for mucus-degraders and non-degraders alike, and many potential pathogens (e.g., *E. coli, S. Typhimurium*, *C. rodentium*) require access to simple sugars to expand in the gut and induce disease [21,22]. The high competition for these sugars in a densely populated gut likely limits gradients of mucus-derived sugar to the 10 μm nearest the interface of the mucus and the lumen, where potential pathogens and gut commensals compete for them [4].

3.1. Colonization resistance through competition for nutrients

Competition for nutrients in a densely populated commensal gut microbiota presents a mode of protection against potential pathogens, by reducing the locations where pathogens can replicate. Local nutrient hotspots can promote spatial gradients in growth. For example, the mucus can harbor concentrations of certain nutrients (such as mucin glycans) that are orders of magnitude higher than in the lumen (see Section 4.2). In a healthy gut, commensal bacteria outnumber potential pathogens around these nutrient sources, enabling commensals to outcompete nearby pathogenic competitors [23]. By sensing the proximity of competitors (potentially as a decrease in local nutrient concentration), gut bacteria can enhance their ability to compete for the mutually desired nutrient by increasing their capacity to consume it. For example, some bacteria upregulate nutrient−uptake pathways, such as ABC transporters or phosphotransferase systems, in response to another species co-occurring in the same human microbiota [24]. This heightened consumption in crowded environments constrains nutrient hotspots to the roughly 10 μm immediately surrounding their sources by reducing the local nutrient concentration (along with diffusion and fluid flow) [25].

In the case of some nutrients, such as iron, pathogens must compete not only with the commensal microbiota but also the host. Intestinal iron concentrations are highest in the lumen as host-secreted iron binding proteins are concentrated in the mucus, diminishing the iron available for mucus-associated bacteria [26]. To scavenge iron, some bacteria secrete siderophores: high-affinity iron chelators that bind extracellular ferric iron and transport it across cell membranes. Siderophore synthesis and transport are upregulated in mucus-associated *E. coli* compared to luminal *E. coli*

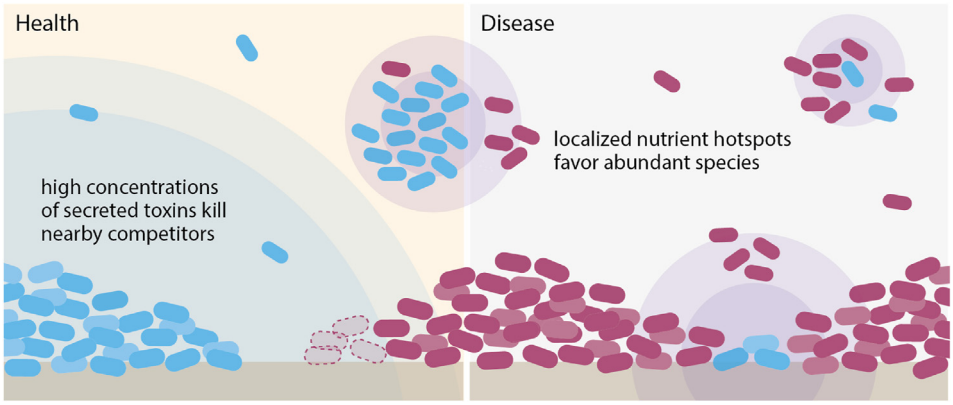

Fig. 3. Local hotspots affect competition and colonization resistance. Nutrient hotspots (purple diffusive patches) are overwhelmingly colonized by commensal bacteria (blue cells), which outnumber pathogens (maroon cells) in healthy guts, whereas pathogen abundances are substantially higher in disease. Secreted toxins (blue diffusive patch) also create local hotspots, that kill competitors within a range of high concentration (dashed maroon cells).

[26]. Mucus-associated *E. coli* are also associated with faster growth rates [26], potentially due to a lowered diffusivity of siderophores within mucus that concentrates siderophores around their producers. Certain siderophore systems, such as yersiniabactin, are enriched in *E. coli* isolated from Crohn's disease patients [26], suggesting a role for siderophores in pathogenesis that may be constrained in healthy guts by diffusion. Thus, the spatial organization of nutrients — maintained in health by commensal bacteria as well as the host — presents a challenging ecosystem for pathogen colonization.

3.2. Colonization resistance through diffusible toxins

In addition to nutrients, bacteria produce local gradients of antibacterial toxins. Secreted bacteriocins, released by diverse bacteria into the extracellular environment, are a potential danger for bacteria in close proximity to one another [17]. *In vitro*, toxin-producing colonies of *E. coli* are able to effectively kill neighboring colonies of susceptible *E. coli* located tens of micrometers away, while susceptible cells located farther away are safe [27]. The potency of killing may be local, but the implications of secreted toxins on colonization resistance affect whole-gut physiology, as the presence of bacteriocin-producing commensals in the gut are associated with human resistance to disease [16]. For example, the Nissle 1917 strain, a commensal *E. coli* famously isolated from a soldier particularly resistant to dysentery, can outcompete closely related pathogenic strains (such as adherent-invasive *E. coli* and *S. enterica*) by secreting microcin M and microcin H47 [28]. Many commensal and pathogenic bacteria can wield bacteriocins [17]. However, commensals likely dominate toxin-mediated competitions in healthy guts by outnumbering colonizing pathogens.

Overall, low abundance pathogens face tough local competition in the dense and diverse microbiota associated with healthy guts. Multiple factors that promote or inhibit bacterial growth, such as nutrient or bacteriocin concentration, are organized into hotspots that exist on the order of tens of microns. In a healthy gut, the spatial organization of these factors combined with the high abundance of competing commensals poses a barrier to pathogen colonization. Pathogens thus generally capitalize on disturbances, such as antibiotic treatment, that dramatically decrease the abundance and activity of the microbiota, leading to a heightened availability of nutrients that allows pathogens to expand [5].

4. The 100 μm–1 mm length scale: bacterial motility, gradients and aggregation

The heterogeneous structure of the gut environment implies that the niches favoring pathogens in the presence of a dense and diverse microbiota ecosystem are few and far between, promoting host health. If a nutrient hotspot is densely crowded or protected by combative commensals (see Section 3), the ability to search elsewhere may increase a pathogen's chance of success. It is therefore not surprising that many known pathogens are flagellated and highly motile, while some abundant commensals (e.g., some *Bacteroides* species) are able to be competitive without motility. Pathogens in particular must overcome challenges on length scales on the order of 100 μm in order to reach the epithelium and induce physiological changes in the host that favor colonization (Fig. 4).

4.1. The mucus layer as an exclusive physical barrier

Intestinal mucus is a major driver of bacterial organization in the gut. It plays a critical role in the homeostasis of gut health by keeping the microbiota hundreds of microns away from human epithelia [11]. The mucus that contacts the lumen directly is looser and more permeable than the innermost mucus occupying the 100 μm closest to the colonic epithelium in humans [29]. While this inner mucus layer is devoid of bacteria, the outer layer houses a mucosal microbiota distinct from the luminal microbiota [30]. In healthy humans, mucus is predominantly colonized by Proteobacteria and Firmicutes (each making up over a third of the mucosal microbiota) and to a lesser degree Actinobacteria and Bacteroidetes (together making up the final third) [1]. The interactions between bacteria and mucus are complex due to the multiple physical and chemical factors that span the viscous mucus separating the lumen from the epithelium.

The spatial organization of bacteria in mucus plays an important role in gut health. Both the taxonomy and proximity of the mucosal microbiota are altered in diseases such as colon cancer [1,31] and inflammatory bowel disease (IBD) [32]. Bacteria are closer to the epithelium in inflamed guts [32], and the intestinal colonization of some ectopic pathogens not normally present in the gut microbiota requires the presence of gut inflammation [33]. Inflammation-associated changes in the mucosal microbiota may arise because mucus in inflamed guts is physically weaker [34], potentially

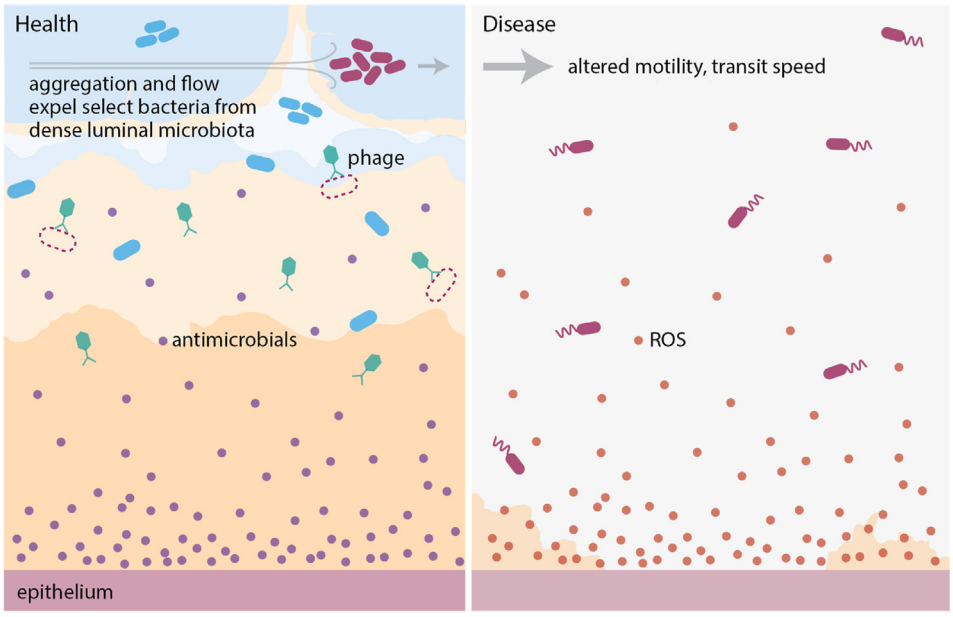

Fig. 4. Meso- and macroscale gradients differ between healthy and diseased guts. In health, a mucus layer (orange) physically and chemically separates the host epithelium from the dense microbiota (blue) in the lumen. The looser outer mucus layer is more permeable to cells, whereas the inner mucus layer is generally bacteria-free. The mucus also contains concentrated levels of host-secreted antimicrobials and bacteriophages, which control which species of bacteria can inhabit it. Bacteria associated with the mucus layer also experience weaker flow rates than in the lumen, where bacteria can become aggregated. These bacterial aggregates range in size, in a strain-specific manner. Conversely, aggregate size affects bacterial interactions with gut motility and flow, determining whether the aggregate is retained or expelled from the gut. Intestinal flow and motility are altered in disease, along with changes in gut biochemistry (e.g. reactive oxygen species, ROS, are increased during inflammation [6]).

providing pathogens easier access to the host epithelium. Increased proximity and access can enhance bacterially mediated damage to the host, including the exacerbation of bacterially induced inflammation, which positively correlates with disease progression including tumor development [35].

In addition to inflammation, other perturbations to the mucus barrier can induce the mislocalization of bacteria in mucus or at the epithelium. As an example, low-fiber diets are associated with thinner mucus layers, lessening the distance between bacteria and the intestinal epithelium [36]. In the absence of dietary fiber, mucus-degrading bacteria expand in the gut, depleting the mucus layer and promoting pathogen-induced inflammation [20]. Fiber-rich diets, on the other hand, allow for thicker mucus layers, which enhance microbiome recovery from antibiotics by limiting the loss of antibiotic-susceptible bacteria [37]. This enhancement suggests that, in addition to preventing bacterially induced inflammation, thicker mucus may provide a spatial refuge for commensal bacteria from a luminal source of antibiotic [20,36]. These commensal bacteria may in turn enhance microbiome resilience by competing with pathogens for nutrients and space. Overall, mucus clearly plays a key role in gut homeostasis. The exact mechanisms of homeostasis — e.g., which changes to the mucus-associated microbiota arise from disease, and how they drive disease — are important and active areas of research.

Recent work provides an example mechanism by which changes in mucus lead to disease, showing that even temporary disruptions to mucus can induce lasting changes in microbiota composition [38]. A mild pulse of diarrhea depletes the mucus layer in mice, allowing for direct contact between intestinal bacteria and the epithelium. Mild diarrhea also induces the extinction of major commensal bacterial families and the expansion of *E. coli* and *Enterococcus* species [38], which can be pathogenic in some cases. Some of these community-level changes in the fecal microbiota persist even after the return of normal mucus [38], mirroring the pathogenic expansions associated with disease. Diarrhea also enables *Clostridium difficile* colonization and expansions in humans [39], potentially through mucus depletion. In the year following an episode of diarrhea, patients periodically experience short 1–2 day blooms of *C. difficile* between weeks of undetectable *C. difficile* abundances [39], suggesting that pathogens can colonize and persist within an intestinal niche made available by mucosal disturbance. Reinforcing the mucus barrier, e.g., with a high-fiber diet, not only restores mucus thickness [36], but also suppresses *C. difficile* infection, further highlighting the importance of an intact mucosal barrier to protect against pathogen invasion [40]. Future work can improve our understanding of intestinal pathogenesis by determining where *C. difficile* resides in chronically infected guts with respect to the mucus, and what triggers the reoccurrence of *C. difficile* blooms.

4.2. The mucus layer as a host of selective chemical gradients

The biochemical heterogeneity of the mucus layer controls bacterial spatial organization beyond physical exclusion. We currently know that some phage, antimicrobial peptides and oxygen are concentrated in the mucus, each limiting bacterial access to the epithelium [2,3,41]. In this section, we discuss the biological and chemical gradients that occur across the mucus layer, providing further protection from pathogens on length scales on the order of 100 μm.

4.2.1. Gradients of phage control bacterial localization and population structure

Both the concentration and movement of phage differ between the mucus and the lumen. Compared to the lumen, phage-to-bacteria ratios are much higher in mucus for some phages [41], increasing the likelihood that a susceptible bacterium may encounter its nemesis. Some phages, including T4 phage (which target *E. coli*) can adhere to mucus via interactions between phage capsid proteins and mucosal glycans [41]. This adherence increases

bacterial killing by decreasing phage movement in mucus, concentrating the phage in zones of mucus that increase encounters with bacterial hosts [42]. By slowing T4 diffusion into the mucus layer, adherence to mucus enables phage to remain in the outer layer of mucus for more time [42]. This subdiffusive motion increases phage-bacterial encounters, as *E. coli* are also hindered by mucus en route to the epithelium, thus reducing *E. coli* colonization of the epithelium by over 4000-fold compared to mucus with non-adherent phage [42]. Thus, it appears that some phages have evolved mechanisms to exploit human mucus to increase their chances of encountering prey in the gut. These phages may be important players in combating adherent-invasive *E. coli* (AIEC) and other pathogens that must cross the mucus to cause disease at the epithelium, and are actively being researched as potential phage-therapeutics.

In some cases, sensitive bacteria can penetrate mucus further than the phages that infect them, and this spatial separation of predator and prey can drive the long-term coexistence of phage and phage-susceptible bacteria [43]. For example, localizing to the mucus protects a commensal strain of *E. coli* from predation by phage (specifically P3, P10, and P17 phage), while descendants of these mucus-associated *E. coli* are infected in the lumen [43]. No evidence of phage resistance in this *E. coli* has been observed, suggesting that bacteria in phage-free regions of mucus experience little pressure to evolve resistance and serve as a source of *E. coli* in the lumen. Thus, the spatial organization of bacteria and phage in mucus can restrict bacterial colonization to specific locations in the gut, protecting some bacteria within mucus while preventing the colonization of mucus by others.

4.2.2. Host-derived gradients of antimicrobial peptides

Alongside phage, an array of host secreted defense peptides fortifies the mucus barrier. One key example are cathelicidins: small antimicrobial peptides that disrupt bacterial membranes and thereby control pathogen abundance [44]. Mice deficient in cathelicidin production exhibit increased inflammation, diarrhea, epithelial attachment and infection by pathogens, including *C. difficile*, *C. rodentium* and pathogenic *E. coli* [44]. Constitutively secreted in humans by neutrophils and epithelial cells positioned at the entrance of colonic crypts [44], cathelicidins and several other host defense peptides are concentrated in the mucus, where their antimicrobial activities protect the host with yet another system of defense on the 100-micron scale.

We point the reader to an excellent recent review on the several types and activities of host-secreted defense peptides [44] and focus our discussion on the protective functions of host peptides that control the spatial organization of specific microorganisms. For example, the abundant *Bacteroides thetaiotaomicron* induces antimicrobial Ang4 secretion by murine Paneth cells, located at the base of crypts in the small intestine [45]. *B. thetaiotaomicron* is more resistant to Ang4 than other commensals and opportunistic pathogens, suggesting that its proximity to crypts can enhance host resistance to colonization by other bacteria. Conversely, *B. thetaiotaomicron* can also promote the expansion of pathogens by releasing sialic acid from mucus, making nutrients available for pathogenic species such as *C. difficile* and *S. Typhimurium* [46]. Future work exploring the function of these gradients will be important to elucidate the multiple roles of *B. thetaiotaomicron* in human health and disease.

4.2.3. Oxygen gradients across the mucus layer

Oxygen, released from host cells, contributes another gradient in mucus that controls which bacterial species can live close to the host epithelium. While the gut microbiota predominantly consists of obligate anaerobes, oxygen-tolerant bacteria are enriched in the mucus- and crypt-associated microbiota of mice [47] and humans [1]. The anaerobic nature of the lumen derives partially from abiotic reactions with luminal contents that chemically consume oxygen [48]. However, the rate of oxygen consumption is substantially lower in germ-free mice than conventionally raised mice [48], suggesting that the bacterial consumption of oxygen plays an important role in maintaining an anaerobic lumen. Indeed, extremely oxygen intolerant organisms (i.e., *Faecalibacterium prausnitzii*) cannot colonize a germ-free host [49]. Instead, facultative and aerotolerant microorganisms dominate the intestinal ecosystem of human infants, which is more aerobic immediately after birth [50]. Within 2–3 years, a complex community of microbial members, dominated by anaerobic bacteria, proliferates [50].

All in all, multiple diverse mucosal gradients control the spatial organization of bacteria, and the localization of specific bacteria in turn affects the nature of these gradients. These gradients control which bacteria can approach the epithelium: only certain members of the intestinal microbiota are able to colonize mucus and crypts [3]. The microbiota in these locations is altered in disease [1,32] as well as after surgery [51]. How these bacteria find and colonize these locations is an active topic of research [52], and further characterization of the reactive gradients present in intestinal mucus may prove important towards understanding microbial dynamics in these compartments. For example, T cell-dependent IgG1 antibodies have been found to target and be induced by specific gut commensals such as *Akkermansia muciniphila* [53]. Known for its colonization of mucus, the mechanism by which *A. muciniphila* mediates specific T cell responses is unknown, but may be related to its close proximity to the epithelium in healthy mice.

4.3. Bacterial aggregation and fluid flow together cultivate species composition

Some host secreted products regulate gut homeostasis by controlling the spatial organization of bacteria in the lumen, outside of the mucus. Secreted antibodies, particularly secretory IgA (sIgA), bind and enchain bacterial cells in a strain-specific manner [54]. Secretory IgA binding crosslinks bacterial cells to one another, forming multi-cellular aggregates [54]. Larger aggregates are more easily expelled by fluid flow compared to planktonic (unattached, free-living) cells [55] and, therefore, more likely eliminated from the gut [54]. While the molecular determinants of sIgA specificity are still uncertain, IgA binding appears to preferentially target pathogens: the bacterial species most highly coated in sIgA are those associated with the induction of IBD [56]. A recent hypothesis suggests yet another mechanism by which IgA mediates the specific expulsion of pathogens, proposing that faster growing bacteria are increasingly aggregated by sIgA. As pathogens generally exhibit shorter doubling times than slower growing commensals, sIgA-mediated bacterial aggregation is thought to protect gut health by selectively removing potential pathogens [54].

Curiously, recent evidence indicates a contradictory role for IgA: one that promotes the mucosal colonization of *Bacteroides fragilis.* IgA-bound *B. fragilis* was observed to better colonize crypts than *B. fragilis* mutants unable to induce the IgA response [52,54]. It has been hypothesized that IgA may enhance attachment to mucus, though this remains to be directly tested. Another hypothesis can be derived from work in larval zebrafish, where IgA-independent aggregation can promote species maintenance in specific gut locations [57]. In this system, the mass of the aggregates formed by various commensal species predicts the spatial location of that species along the 1–2 mm length of the gut [57]. Single *V. cholerae* cells (a few microns in length) are mostly planktonic and localize

primarily in the upper gut, while symbionts, such as *Aeromonas caviae* and *Aeromonas veronii*, that form intermediately sized aggregates (tens of microns in length) localize between the upper and midgut [57]. *Enterobacter cloacae*, which is found almost entirely in larger aggregates (hundreds of microns in length), localizes predominantly in the midgut [57]. While the IgA-independent observation that larger aggregates are expelled more readily comes from zebrafish, a similar size-dependent mechanism may explain IgA-mediated expulsion from human guts. Species-specific differences in IgA binding may induce differently sized aggregates, thereby expelling some species while promoting colonization of others. For example, IgA-bound *B. fragilis* may accumulate in intermediately sized aggregates that facilitate *B. fragilis* association with mucus and crypts, while larger aggregates localize to the bulk and are more easily lost. These hypotheses remain to be tested.

In addition to regulating bacterial maintenance and expulsion, IgA may also differentially affect bacterial physiology. Some bacteria have been shown to degrade elements of secreted IgA for nutrients [54], and some may alter their metabolism and interaction with the surrounding environment once bound by IgA. Indeed, IgA induces *B. thetaiotamomicron* expression of uncharacterized polysaccharide utilization genes, which are expressed in the mucus-associated bacteria in mice and humans [54]. Further work to dissect precisely how sIgA selectively inhibits or promotes bacterial colonization in the gut will broaden our understanding of bacterial organization in aggregates and the role aggregations play in gut community composition. Overall, bacterial aggregates (with or without sIgA) remain an exciting area in gut microbiota research, with aggregates of mixed species not having yet been observed [54,57]. How different species form aggregates and what ecological advantages are gained from localizing in specific regions remain unclear and are important next steps in characterizing bacterial organization and its interactions with gut motility.

4.4. Diverse roles for bacterial motility and chemotaxis in colonization

The opposite of bacterial aggregation is bacterial dispersion, which bacteria achieve through swimming motility and chemotaxis (the ability to move in response to a chemical stimulus). Both traits are required for some intestinal bacteria to avoid aggregation and maintain their spatial position while planktonic in the gut. For example, in larval zebrafish, wild-type *V. cholerae* persist in the gut as planktonic individuals, while non-motile and motile but non-chemotactic mutants are mislocalized and aggregated in the gut [58]. These multi-cellular aggregates are eventually expelled from the gut by fluid flow, inhibiting colonization [58].

Motility and chemotaxis are generally considered a means for bacteria to find a favorable niche. Some intestinal bacteria upregulate chemotactic pathways when a particular other species co-occurs in the human microbiome [24]. This increased expression of directional motility may increase the odds of finding a spatial location richer in nutrient and farther from competitors. Motility can also enhance a pathogen's ability to infect the gut. *In vitro*, populations of *Pseudomonas aeruginosa* with swimming cells can disperse millimeters farther than populations without swimmers to colonize greater areas within the gut [59]. This motility-driven enhancement of dispersion enables populations with swimmers to reach higher cell abundances than motility-deficient populations and kill higher fractions of host cells [59].

Chemotaxis is directly implicated in the colonization capacity of several intestinal pathogens, enabling pathogens to target and localize at sites of infection. Upon sensing host hormones, EHEC induce the expression of a pathogenicity island containing motility and chemotaxis genes [60]. EHEC is known to chemotax towards major hormones and polyamines found in the human gut [61], possibly steering pathogenic *E. coli* towards the host epithelium. A pathogen involved in food poisoning, *Campylobacter jejuni*, chemotaxes towards sodium deoxycholate, a major component of bile, and its chemotaxis is required to penetrate the mucosal layer and colonize the jejunal epithelium [60]. Similarly, *V. cholerae* traverse galactose-6-sulfate gradients in intestinal mucus to penetrate towards the host epithelium [60]. Lastly, chemotaxis enables *Helicobacter pylori* to accumulate around a microscopic stomach lesion within minutes of exposure [62].

By organizing bacteria to different spatial locations, motility and chemotaxis can also drive bacterial speciation. Closely related bacteria can diverge into ecologically distinct populations when, over evolutionary timescales, variation in motility and chemotaxis reinforces the physical separation of populations, reducing horizontal gene transfer [63]. The recent discovery of distinct populations of *Ruminococcus gnavus* offers a potential example of spatially segregated diversification in human microbiomes. These populations of *R. gnavus* are differentially associated with healthy individuals and ulcerative colitis patients and separated by sequence-based evidence of recent gene flow [64]. *R. gnavus* cells can be either motile or nonmotile, and new research will be critical for uncovering whether motility-driven spatial organization ecologically distinguishes populations of pathogenic *R. gnavus* from commensal ones.

5. The gut scale: intestinal physiology as a determinant of pathogen success

The largest scale of bacterial spatial organization is defined by intestinal segments (e.g., stomach, intestine, etc.), which are characterized by distinct physical and chemical environments largely controlled by the host [2,3]. However, bacteria also contribute to host physiology within the gut, through means that include the bacterial induction of host motility [8] as discussed in Section 2.1. We now discuss how the physiological context of the whole gut, namely gut motility and chemistry, can feedback on bacterial organization and disease.

5.1. Gut motility: the frequency of fluid flow on microbial colonization

The abundance of any given species in an ecosystem is controlled by its rate of growth, death, and migration (entry and exit) in the intestinal tract [54]. The key regulator of bacterial exit is intestinal motility, which periodically expels bacteria in large aggregates (as discussed in Section 4.3). Mucus- and crypt-associated bacteria may generally be protected from flow; however, mucus and epithelial shedding, as well as disturbances that weaken the mucus (e.g., diarrhea [38] and inflammation [34]), enable the expulsion of bacteria within these compartments.

In addition to determining bacterial presence and absence in the gut, intestinal motility can substantially affect bacterial growth and physiology. Colonic transit time, the time required for ingested food to fully pass through the gastrointestinal tract, has recently been associated with the metabolites produced by the colonic microbiota [65] and is longer in germ-free mice than in mice colonized with a microbiota [66]. Specifically, increased transit time coincides with a shift from carbohydrate fermentation (the dominant microbial function in healthy colons) to protein degradation [65]. These degradation products are associated with diseases such as colorectal cancer, chronic kidney disease and autism — diseases for which constipation is a risk factor [65]. Increased transit time also increases the number of unique species within the fecal microbiota, suggesting that it may promote a form of ecological succession [65].

Prolonging the passage of ingested material in the gut may enable certain species, undetectable from stools passed with shorter transit times, to reach detectable abundances and alter metabolic profiles within their host.

5.2. The role of spatial organization on whole-gut chemistry in health and disease

Some microbial metabolites impede invasion from potential pathogens and influence bacterial organization at the diffusive length-scales, as discussed in Section 3. Microbial metabolites can also impede pathogen invasion at the whole-gut level, particularly metabolites that are widely available and highly concentrated such as short chain fatty acids (SCFAs). SCFAs are the anaerobic fermentation products of dietary fiber, produced by the dominant phyla, Bacteroidetes and Firmicutes. SCFAs such as acetate, butyrate and propionate reach concentrations as high as 70–140 mM in the colon, where they induce peristaltic reflexes [67] and can enhance resilience against enteric pathogens [16].

SFCAs can further protect host health by directly affecting host physiology. SCFAs are absorbed by host cells and stimulate immune system production of the anti-inflammatory factor interleukin-10 [2]. Furthermore, recent evidence has pointed to a role for SCFAs during childhood development, protecting against the early onset of type 1 diabetes [68]. Lastly, Crohn's disease and UC are associated with decreased levels of SCFAs and SCFA-producing bacteria [69]. Thus, through SCFAs, the microbiota can regulate its own composition through regulating gut motility by affecting the interactions between gut motility and bacterial aggregation.

5.3. The colon as a potentially fragmented ecosystem

Beyond maintenance and expulsion, SCFAs may affect bacterial colonization by regulating bacterial encounters with the colonic mucus. In the human colon, host absorption of SFCAs is coupled to Na^+ and Cl^- absorption, which drive the absorption of roughly 1.5–2 L of water per day [70]. The rate of water uptake can determine colonic microbiota composition [71] and may generate a radial flow of water towards the host epithelium, a possibility to pursue experimentally, as it may potentially aid the encounter of even nonmotile bacteria with the colonic mucus. Colonic water absorption also implies that the lumen is not continuously saturated but fluctuates in hydration status. In soils, the mixed presence of water and air fragments the microbial habitat into disconnected liquid patches, and more fragmented soil ecosystems are associated with increased bulk microbial diversity [72]. We speculate that fecal material and mucus may similarly structure the gut environment, spatially segregating microbes into fragmented niches in a hydration-dependent manner. Under this model, changes in colonic water absorption, such as those associated with IBD [70], may result from changes in the spatial connectivity of bacteria niches, which changes how the microbiota can interact.

Overall, whole-gut physiology has broad consequences for bacterial spatial organization. Changes in these gut-wide parameters are strongly associated with fecal community changes and numerous disease states, including colorectal cancer [31], diabetes [68], IBD [69], and chronic infections [73]. Deciphering the role of diverse gut metabolites (including those yet to be characterized) and how they affect microbial biogeography is an important next step in determining the causes and effects of disease onset. For example, recent work has shown how intestinal inflammation increases nitrate concentration, which increases T3SS-mediated EHEC adherence to host cells and colony formation [74]. Finally, while some metabolites may not directly determine bacterial localization, they still influence small-scale cell-to-cell interactions by altering the environment in which they take place and will ultimately affect gut biogeography.

6. Outlook: novel techniques for a functional understanding of spatial organization in disease

A better understanding of spatial organization of the intestinal microbiome is necessary to advance our insight into the mechanisms that underlie health and disease. Over the past few years, new techniques have advanced the field with tools that enhance our access to gut biogeography. New tools are essential for the temporal and spatial resolution required to assess this complex ecosystem. Here, we discuss some exciting new avenues by which to explore microbial biogeography and highlight areas that would benefit from further development.

6.1. Spatially and phylogenetically resolved maps of biogeography

Because cell-to-cell interactions depend on the spatial proximity between cells, we need micron-scale maps of the locations of the hundreds of microbial species present in complex intestinal communities. To adequately infer function, these maps should also contain high genetic resolution, as functional diversity at the strain-level certainly exists [63,64]. Towards this, a new technique (HiPR-FISH) leverages combinatorial fluorescent barcodes and single-cell segmentation to distinguish hundreds of distinct bacterial taxa coexisting within the same fixed sample [75]. Previously unachievable in complex communities, single-cell and strain-level mapping enables quantitative study of bacterial spatial organization, by measuring the distances between specific species in a host-associated microbiota. These quantitative data, at such high genetic and spatial resolution, are important to answer key questions concerning the behavior of microbial communities: who interacts with whom, and where their interactions take place.

6.2. Spatial expression of function

We need to match host biogeography and function to bacterial localization in order to understand the local environments that control cell behavior. Future efforts should leverage recent developments in single-cell transcriptomics to associate host expression and bacterial expression. Single-cell RNA-sequencing has recently reported global transcriptomes of individual bacteria, impressively capturing gene expression profiles from all RNA classes and genomic regions in different growth conditions [76]. Future efforts should also adapt single-cell metabolic measurements to in situ localization. Single-cell Raman spectroscopy has recently been used to identify previously unexplored mucin-degrading bacteria [77] and nanometer-scale secondary ion mass spectrometry (NanoSIMS) has been used to measure the role of phenotypic heterogeneity metabolism under dynamic conditions [78]. Together with single-host cell transcriptomics, such as the cell type specific data reported in Tabula Muris [79], host and bacterial cell expression data would provide information on the type of interactions as well as the spatial distance over which microbe–microbe and host–microbe interactions occur.

6.3. Temporal expression of function

Spatial and temporal information will together enable the demonstration of causality. Current methods generally rely on antibody staining or FISH probes to report bacterial location in anaerobic environments, but the necessary fixation in these methods severely limits the temporal information often required to observe microbial behaviors. Currently, fluorescent markers for

anaerobic systems are lacking. Recent developments have taken advantage of the ability of some anaerobes to respire aerobically and grow at low (0.10–0.14%) oxygen, enabling live imaging of bacteria that are prominent in the gut (e.g., *Bacteroides*), yet currently represented by exceedingly limited dynamic single-cell data [80]. In situ measurements from larval zebrafish guts have revolutionized our biophysical understanding of bacterial dynamics in real-time [8,57,58]; however similar dynamical data in other animal models remains a challenge.

Improving our ability to quantitatively describe the spatiotemporal dynamics of intestinal bacteria would vastly improve our understanding of microbial physiology and ecology in the gut. One major question concerns how bacteria reach crypts or the epithelium, as only about half of human pathogens are known to possess canonical genetic chemosensory pathways [60]. Visualizations of bacterial movement and behavior, for example via metabolic labeling of gut bacteria [81] or microfluidic mimics of the gut [82], will be critical to understand how these pathogens localize themselves to their target host sites and to uncover new mechanisms by which bacteria modulate their spatial organization within the gut.

Microbiota studies are flourishing into exciting new areas that go beyond enumeration. Just as macroecological concepts are deeply rooted in understanding the movement of species across habitats — not simply the abundances of animals in an ecosystem — the study of microorganisms is also advancing in that direction. Spatial organization is a promising and important avenue towards understanding the onset, progression and recovery from disease. By pursuing the temporal dynamics of bacterial spatial organization, we can come to understand the causality of complex intestinal disease and the mechanisms underlying system resilience.

Conflict of interest

The authors declare no conflicts of interest.

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