MICROBIOME

Gut biogeography of the bacterial microbiota

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Abstract | Animals assemble and maintain a diverse but host-specific gut microbial community. In addition to characteristic microbial compositions along the longitudinal axis of the intestines, discrete bacterial communities form in microhabitats, such as the gut lumen, colonic mucus layers and colonic crypts. In this Review, we examine how the spatial distribution of symbiotic bacteria among physical niches in the gut affects the development and maintenance of a resilient microbial ecosystem. We consider novel hypotheses for how nutrient selection, immune activation and other mechanisms control the biogeography of bacteria in the gut, and we discuss the relevance of this spatial heterogeneity to health and disease.

Microbiota
The collection of microorganisms (including bacteria, viruses, fungi and single-celled eukaryotes) that inhabit a particular habitat, such as an animal.

Syntrophic interactions
Metabolic relationships in which one member provides nutrients to another.

Secreted immunoglobulin A
(sIgA). By far the most abundant isotype of antibody found in the gut.

Prebiotic
A molecule that serves as a nutrient which stimulates the growth of commensal or mutualistic gut bacteria. In many cases, prebiotics can be specific for discrete groups of bacteria on the basis of their metabolic (that is, nutritional) requirements.

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Humans and other mammals harbour a complex gastrointestinal microbiota that includes all three domains of life (Archaea, Bacteria and Eukarya). This extraordinary symbiosis, formed by a series of exposures to environmental factors, is initiated on contact with the maternal vaginal microbiota during birth[1]. Abrupt changes during the first year of life follow a pattern that corresponds to gestational age in both mice[2] and humans[3], which suggests that strong deterministic processes shape the composition of the microbiota during development. These population shifts may be explained by influences from the diet, the developing immune system, chemical exposures and, potentially, the founder effects of initial colonizers. Founder effects are not well understood in the mammalian gut, but the profound changes in host gene expression that occur in response to microorganisms and also the great potential for syntrophic interactions between bacteria suggest that early colonizers would have long-term effects on the establishment of the microbiota. The immune system imposes selective pressure on the microbiota through both innate and adaptive mechanisms, such as antimicrobial peptides[4], secreted immunoglobulin A (sIgA)[5] and other contributing factors[6] (see below). However, current research suggests that diet has the greatest impact on microbiota assembly.

Before weaning, breast milk plays a crucial part in shaping the microbial community composition via transmission of the milk microbiota to the infant gut[7], protection from harmful species by secreted maternal antibodies[8] and selection for certain species by milk oligosaccharides, which can be used by microorganisms as carbon sources[9]. For example, in *in vitro* competitive growth experiments, *Bifidobacterium longum* benefits from its ability to use the fucosylated oligosaccharides in human milk to outgrow other bacteria that are usually present in the gut microbiota, such as *Escherichia coli* and *Clostridium perfringens*[10]. Several species of *Bacteroides* can also utilize fucosylated oligosaccharides as carbon sources[11], suggesting that their colonization of the gut is aided by the prebiotic properties of milk. Accordingly, children of mothers with non-functional fucosyltransferase 2 (FUT2), an enzyme required for the fucosylation of milk oligosaccharides, display lower levels of faecal *Bifidobacterium* spp. and *Bacteroides* spp.[12]. The importance of diet in determining the composition of the microbial community in the gut is also highlighted by the observation that the transition to solid foods coincides with the establishment of a microbiota similar to that found in adults.

The adult intestinal microbiota consists of hundreds to thousands of species, dominated by the Bacteroidetes and Firmicutes phyla[13]. This ecosystem is distinct from those of any other microbial habitats that have been surveyed[14] and includes many species that exist nowhere else in nature, indicating that co-evolution of the host with its gut microbial symbionts (including commensals and mutualists) has generated powerful selective mechanisms. A recent study of how different microbial communities colonize gnotobiotic animals showed that deterministic mechanisms (presumably host–microorganism interactions) led to reproducible shaping of the microbiota regardless of the source of the input community[15].

The adult intestinal microbiota is also partially stable, as a core of ~40 bacterial species (accounting for 75% of the gut microbiota in terms of abundance) persists for at least 1 year in individuals[16]. A more extensive longitudinal study found that 60% of all bacterial strains within an individual persisted for 5 years[17]. During severe perturbations such as antibiotic treatment, the faecal community is depleted to a low-diversity consortium, but after

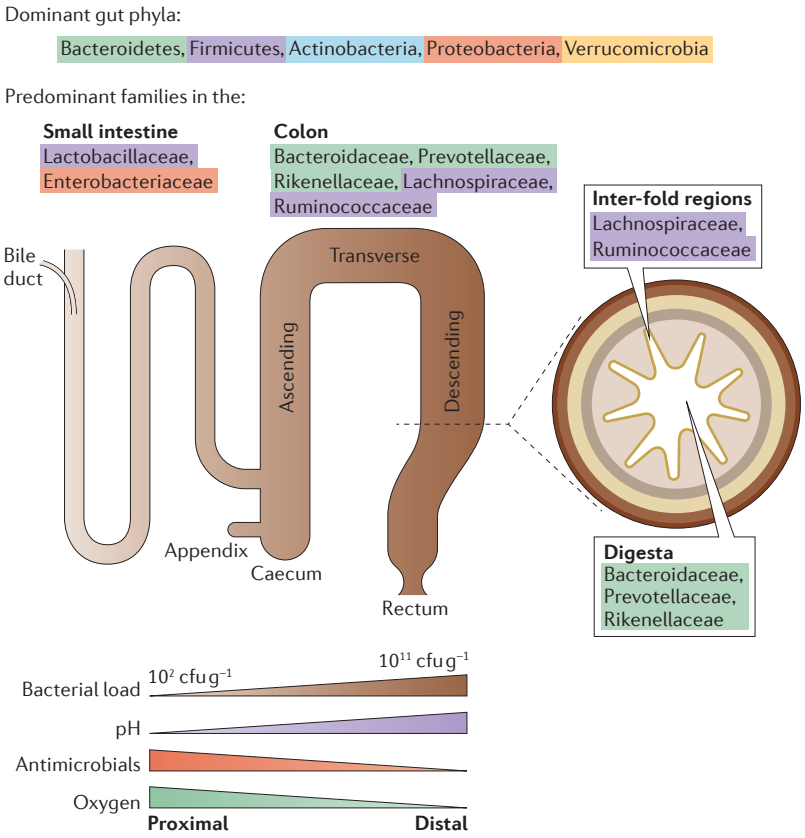

Figure 1 | **Microbial habitats in the human lower gastrointestinal tract.** The dominant bacterial phyla in the gut are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the gut. For example, a gradient of oxygen, antimicrobial peptides (including bile acids, secreted by the bile duct) and pH limits the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. In the small intestine, the families Lactobacillaceae and Enterobacteriaceae dominate, whereas the colon is characterized by the presence of species from the families Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae (colours correspond with the relevant phyla). A cross-section of the colon shows the digesta, which is dominated by Bacteroidaceae, Prevotellaceae and Rikenellaceae, and the inter-fold regions of the lumen, which are dominated by Lachnospiraceae and Ruminococcaceae. cfu, colony-forming units.

Symbionts
In ecology: organisms that participate in a close relationship with other organisms. The term encompasses organisms that participate in different types of relationship, including mutualists, commensals and parasites.

Commensals
In ecology: organisms that participate in a symbiotic relationship in which one party benefits from the other without affecting the other party. Historically, commensals is also used as a term for the resident gut bacteria, although many of these may be mutualists.

a recovery period, membership and relative abundance largely resemble the pretreatment state[18]. Some species that are depleted to undetectable levels in stool are later recovered[18], indicating that there may be reservoirs of bacterial cells that can re-seed the intestinal lumen.

The mucus layer, the crypts of the colon and the appendix are examples of privileged anatomical sites that are protected from the faecal stream and accessible only to certain microorganisms. In this Review, we highlight relevant features of the spatial heterogeneity of bacterial species and communities in the gut microbiota, and we discuss the impact of microbial localization on engendering specific and stable colonization with profound implications for health and disease.

Microbial composition of the gut

The mammalian lower gastrointestinal tract contains a variety of distinct microbial habitats along the small intestine, caecum and large intestine (colon). Physiological variations along the lengths of the small intestine and colon include chemical and nutrient gradients, as well as compartmentalized host immune activity, all of which are known to influence bacterial community composition. For example, the small intestine is more acidic, and has higher levels of oxygen and antimicrobials than the colon (FIG. 1). Therefore, the microbial community of the small intestine is dominated by fast-growing facultative anaerobes that tolerate the combined effects of bile acids and antimicrobials while still effectively competing with both the host and other bacteria for the simple carbohydrates that are available in this region of the gastrointestinal tract. Bile acids, secreted through the bile duct at the proximal end of the small intestine, are bactericidal to certain species owing to their surfactant properties and are known to broadly shape the composition of the microbiota, especially in the small intestine. For example, feeding mice excess bile acids generally stimulates the growth of Firmicutes and inhibits the growth of Bacteroidetes[19]. In addition, the shorter transit time in the small intestine compared with in the colon (an order of magnitude shorter, despite the greater length of the small intestine) is thought to make bacterial adherence to tissue or mucus an important factor for persistent colonization of the small intestine.

In ileostomy samples from humans, the small intestine was found to exhibit lower bacterial diversity than the colon and was highly enriched in certain *Clostridium* spp. and certain members of the phylum Proteobacteria[20]. Furthermore, a metatranscriptomic analysis revealed that, compared with faecal samples, ileal samples showed much higher expression of genes involved in central metabolism and in pathways responsible for the import of simple sugars by facultative anaerobes[20]. In mice, Proteobacteria (especially members of the family Enterobacteriaceae) and members of the family Lactobacillaceae are enriched in the small intestine[21]. Although bacteria in the small intestine are potentially competing with the host for nutrients, host-derived bile acids and antimicrobial peptides limit bacterial growth to low densities in proximal regions. Only at the distal end of the small intestine, in the terminal ileum, do bacterial densities reach saturating levels similar to those found in the large intestine (FIG. 1).

The caecum and colon cultivate the most dense and diverse communities of all body habitats. Mice, like most herbivorous mammals, have a large caecum between the small and large intestine, where plant fibres are slowly digested by the microbiota. Humans have a small pouch-like caecum with an attached appendix, which is a thin tube-like extension (FIG. 1). In the caecum and colon, microorganisms are responsible for the breakdown of otherwise 'resistant' polysaccharides that are not metabolized during transit through the small intestine. Lower concentrations of antimicrobials, slower transit time and a lack of available simple carbon sources facilitate the growth of fermentative polysaccharide-degrading anaerobes, notably the Bacteroidaceae and Clostridia. In the mouse, the caecum is enriched in species of the families Ruminococcaceae and Lachnospiraceae,

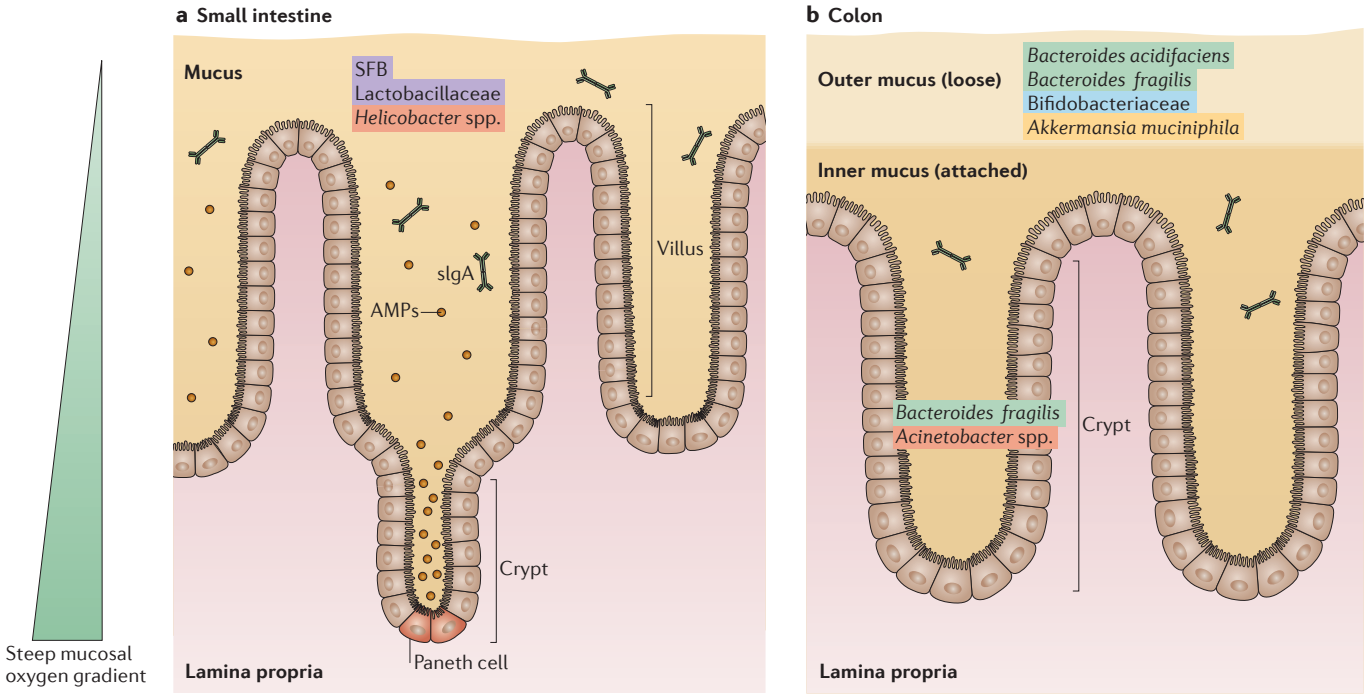

Figure 2 | **The mucus layers of the small intestine and colon.** Several factors limit the ability of gut bacteria to access host cells, such as the mucus layers in the small intestine and the colon; antimicrobial peptides (AMPs) in the small intestine, including those produced by Paneth cells at the base of the crypts; secreted immunoglobulin A (sIgA) in both the small intestine and colon; and a steep oxygen gradient that influences which bacteria are capable of surviving close to the epithelial surface. **a** | The surface of the small intestine is shaped into villi and crypts and is colonized by certain adherent species, including segmented filamentous bacteria (SFB), Lactobacillaceae and *Helicobacter* spp. **b** | The colon has two distinct mucus structures: the loose outer layer is colonized by mucin-degrading bacteria and is characterized by the presence of *Bacteroides acidifaciens*, *Bacteroides fragilis*, Bifidobacteriaceae and *Akkermansia muciniphila*; the tightly adhering inner mucus layer and the crypts are penetrated at low density by a more restricted community that includes *Bacteroides fragilis* and *Acinetobacter* spp.

Mutualists
In ecology: organisms that participate in a symbiotic relationship in which both parties benefit.

Gnotobiotic animals
Formerly germ-free animals that now carry a defined microbiota. The composition of the microbiota in these animals is usually determined experimentally.

Digesta
The bulk of dietary fibres that is digested as it transits through the gastrointestinal tract.

Goblet cells
Specialized epithelial cells throughout the gastrointestinal tract that secrete gel-forming mucins. Goblet cells can also be present in other mucosal epithelial surfaces throughout the body.

whereas the colon is enriched in members of the families Bacteroidaceae and Prevotellaceae[21]. Species from the family Rikenellaceae are prominent in both the caecum and the colon[21]. As well as the variation in microbial community composition longitudinally within the gut, various host factors drive community differences over the cross-sectional axis of the gut. The entire wall of the colon folds over itself, creating compartments between folds (inter-fold regions) that are distinct from the central lumenal compartment (FIG. 1). In mouse studies that used laser capture microdissection to profile the composition of the microbial communities in discrete regions, significant differences were observed between the central lumenal compartment and the inter-fold regions[22,23]. Specifically, the Firmicutes families Lachnospiraceae and Ruminococcaceae were enriched between folds, whereas the Bacteroidetes families Prevotellaceae, Bacteroidaceae and Rikenellaceae were enriched in the digesta[22]. Relative to the digesta, the inter-fold regions are likely to contain greater amounts of mucus, which can serve as a nutrient source for certain bacteria.

Gut microhabitats: mucus and colonic crypts. Throughout the human small intestine and colon, specialized epithelial cells called goblet cells secrete a mucus layer of varying thickness that partially or fully covers the epithelium (depending on the region), creating a boundary between the gut lumen and the host tissue (FIG. 2). The small intestine harbours a single, tightly attached mucus layer (FIG. 2a), whereas in the colon, mucus is organized into two distinct layers: an outer loose layer and an inner denser layer that is firmly attached to the epithelium (FIG. 2b). As mentioned above, bacterial densities are much higher in the colon than in the small intestine, and examination of the colon by fluorescence *in situ* hybridization (FISH) has shown that the inner mucus layer appears to be essentially sterile compared with the densely populated outer layer[24]. In addition to mucus density itself serving as a physical obstacle for microorganisms, antimicrobial molecules and oxygen secreted from the epithelium accumulate at high local concentrations within the mucosa, especially in the small intestine, greatly restricting potential microbial inhabitants.

Mucus is continuously secreted, and the outer layers are sloughed off, generating 'islands' of mucus that are carried into the faecal stream[25]. In mice, a viscosity gradient of the gel-forming mucus increases from the proximal colon (which includes the caecum and the ascending and transverse colon) to distal colonic sites (which include the descending colon and the sigmoid colon connecting to the rectum). Accordingly, there are more mucus-associated bacteria in the proximal region

than in the distal sites[26]. Mucosal biofilm formation in the proximal colon is conserved from mammals to amphibians[27], suggesting an ancient, evolutionarily conserved origin of this region for interactions with bacteria. Therefore, the mucus layers of the gastrointestinal tract create environments that are distinct, protected habitats for specific bacterial ecosystems that thrive in proximity to host tissue.

Divergence between the mucosal and digesta-associated colonic communities has been observed in several mammals, including humans[28], macaques[29], mice[30], cows[31] and flying squirrels[32]. More specifically, human colon biopsy and swab samples have revealed a distinct mucosal community enriched in species from the phyla Actinobacteria and Proteobacteria, compared with the lumen community[33]. Certain species are highly enriched in colonic mucus, such as the mucin degraders *Bacteroides acidifaciens* in mice[34], *Bacteroides fragilis* in macaques[29] and *Akkermansia muciniphila* in mice and humans[34,35] (FIG. 2b). Human mucosal communities in colonic biopsy[36–38] and lavage[39] samples exhibit significant variability between sample locations less than 1 cm apart, which suggests that mucosal microbial populations occur in patches. Interestingly, an imaging study using approaches that carefully preserve the structure of faeces also identified discrete patches; individual groups of bacteria were found to spatially vary in abundance from undetectable to saturating levels[25]. This spatial niche partitioning in faeces may be reflective of aggregates of interacting microorganisms, heterogeneity of nutrient availability in plant fibres, or microenvironments in mucosal communities that imprint the digesta as it transits through the gut. Therefore, microbial profiling of faecal samples, which is the most common strategy employed in microbiome studies, represents an incomplete and skewed view of even the colon, which has distinct mucosal communities and a spatial heterogeneity that is lost on sample homogenization.

Some bacteria completely penetrate the mucus and are able to associate directly with the epithelium, within the crypts of the colon. Crypt-associated microorganisms were first described using electron microscopy[40,41]. Many subsequent imaging studies probably failed to observe or underestimated the number of tissue-associated bacteria because common washing and fixing methods can remove mucosal biofilms[42]. This led to the hypothesis that the mucosal surface is largely devoid of microbial colonization in healthy individuals. However, imaging studies using Carnoy's fixative, which is known to preserve the mucosal layer, found that there are bacteria in a significant fraction of colonic crypts in healthy mice[43] and humans[44]. More recent work using laser microdissection and sequencing to profile mouse crypt-associated communities revealed that these communities are especially dominated by *Acinetobacter* spp. and are generally enriched for Proteobacteria capable of aerobic metabolism[23] (FIG. 2b). Evasion of immune responses and particular metabolic activities are likely to be required for crypt occupancy by microorganisms that are specialized to reside in close proximity to the host. A well-characterized example of this adaptation is the ability of the human symbiont *B. fragilis* to enter crypts of the proximal colon of mice via a process requiring both modulation of the immune system[45] and utilization of specific host-derived nutrients[46] (see below). Although a dogma has emerged that microorganisms contact mucosal surfaces exclusively in disease states, it seems that in fact life-long physical associations between specific members of the microbiota and their hosts represent symbioses forged over millennia of co-evolution.

Biofilm
An aggregation of bacteria that are colocalized in a matrix and reside on a surface. Biofilms may include single species of bacteria or polymicrobial communities.

Mechanisms responsible for gut biogeography

Several factors influence the biogeography of bacteria within the gut, including diet, antimicrobials, mucus, adherence and the host immune system.

Diet and nutrients. Bacterial metabolism in the gut is likely to contribute to the localization of particular groups of microorganisms. Because fatty acids and simple carbohydrates from food are absorbed and depleted during transit through the small intestine, sustainability of the colonic bacterial ecosystem requires growth by the fermentation of complex polysaccharides, the principal carbon sources that reach the colon. Best studied in this regard are *Bacteroides* spp., which are able to catabolize polysaccharides derived from the diet and from the host[47]. Compared with other gut bacteria, *Bacteroides* spp. have the largest number and greatest diversity of genes involved in polysaccharide degradation[48]. This extensive array of polysaccharide utilization systems is dominated by those resembling the starch utilization system (Sus), originally described in *Bacteroides thetaiotaomicron*[49]. Sus systems consist of lipid-anchored enzymes that are either secreted or displayed on the bacterial cell surface and can catabolize particular complex glycans into smaller oligosaccharides, which are then imported through a dedicated outer-membrane transporter (FIG. 3a). In the gut, *Bacteroides* spp. use Sus-like systems to break down dietary polysaccharides and host-derived mucin glycans[50]. The genome of *B. thetaiotaomicron* encodes 88 Sus-like systems presumably with different glycan specificities, providing remarkable metabolic flexibility[51]. On the basis of these findings, *Bacteroides* spp., and *B. thetaiotaomicron* in particular, are sometimes referred to as 'generalists', as they are capable of occupying a variety of metabolic niches depending on the availability of diverse polysaccharide nutrients.

Diet-derived polysaccharides control microbial community composition in the lumen of the colon. Unsurprisingly, the influence of the diet is readily apparent in studies that profile the faecal community. A study of humans who completely switched between plant- and animal-based diets showed that the microbiome abruptly shifts with diet[52]. Over small timescales this effect is reversible, suggesting that these changes represent transient ecosystem adaptations via blooms of particular species in the lumen while the mucosal reservoir remains unchanged. Many studies of *Bacteroides* spp. glycan metabolism in mice have shown that restricting the polysaccharide content of the mouse diet allows selection for species (or strains) that are capable

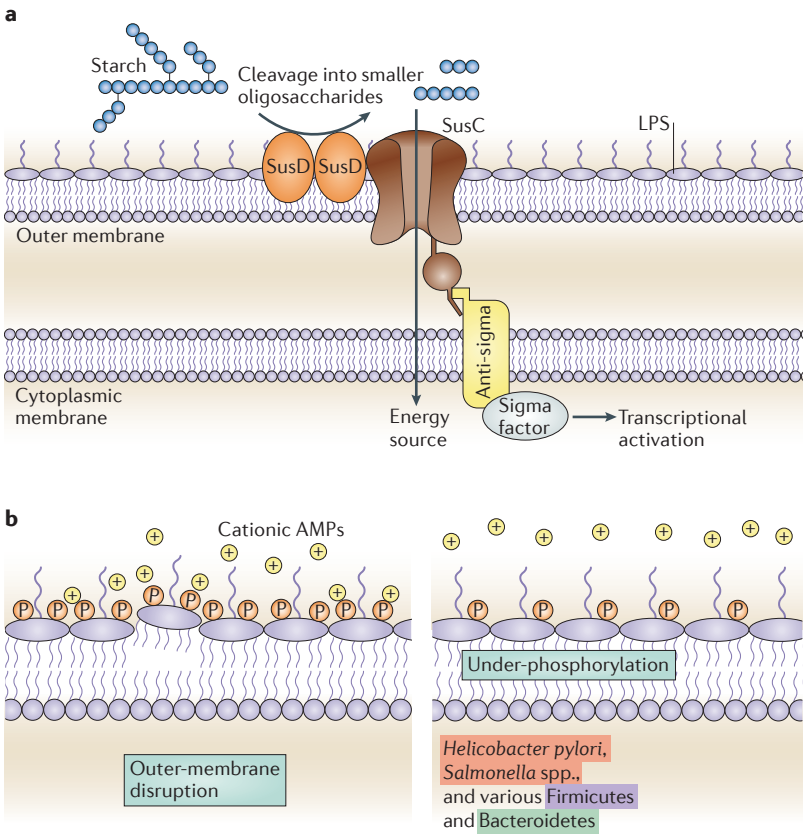

Figure 3 | **Bacterial colonization determinants.** Several factors affect the localization of bacteria within the gastrointestinal tract, including the ability to utilize different glycans and to resist antimicrobial peptides (AMPs). **a** | Starch utilization system (Sus)-like systems in *Bacteroides* spp. allow the utilization of complex polysaccharides from the diet or the host. The figure shows a generalized schematic of a Sus-like system. Homologues of SusD and other outer-membrane lipid-anchored enzymes bind and cleave the glycans (such as starch) into smaller oligosaccharides, which are then imported by the SusC-like outer-membrane transporter. Interaction with the cognate glycan often leads to transmembrane signalling to activate gene regulatory mechanisms, such as a two-component system or a transmembrane anti-sigma factor that releases and activates a sigma factor. Downstream transcriptional regulation allows *Bacteroides* spp. to respond to the local availability of glycans. **b** | Cationic AMPs in the small intestine, which also pass into the colon via the faecal stream, disrupt bacterial outer membranes by interacting with negative charges on their surface. By removing phosphate groups (P) from the lipid A of lipopolysaccharide (LPS), pathogens and commensals alike — such as *Helicobacter pylori*, *Salmonella* spp. and various Firmicutes and Bacteroidetes members — reduce the negative charge on their membranes and evade attack by cationic AMPs.

of metabolizing the complex glycans present, such as fructans[53], human milk oligosaccharides[11], fucosylated mucin glycans[54] and mannan[55]. Presumably, the variety of Sus-like systems present in the genomes of *Bacteroides* spp. provides the metabolic plasticity required to persist in the gut despite short- and long-term changes in nutrient availability. However, even in terms of monosaccharide and disaccharide utilization, there is a hierarchy of bacteria that are more efficient consumers, which helps explain how diet can dramatically and rapidly change the composition of the faecal community. Importantly, the nutrient environment of the gut lumen may be in a dynamic state of flux owing to potential meal-to-meal variability, especially in omnivorous mammals.

In contrast to the variable conditions in the gut lumen, mammals probably maintain a more consistent nutrient balance in the mucosa, which serves as a stable positive selection factor for certain species of bacteria. Mucus degradation and metabolism by gut microorganisms provides access to privileged spatial niches and therefore a competitive advantage over other species, both indigenous organisms and invasive species. For example, several studies have shown that the ability to grow in an *in vitro* mucus culture is generally predictive of the ability of a bacterial species to colonize the mouse gut[56,57]. Mucin 2 (MUC2) alone is coated with more than 100 different *O*-linked glycan structures in humans[58]. These glycans differ between mice and humans[59], and the difference in the complex glycan 'preferences' of various bacterial species is a suggested mechanism of host-specific selection of a characteristic microbiome profile. In agreement with this theory, computational models have shown that positive selection at the epithelium via the ability to metabolize specific nutrients can be a more powerful mechanism for shaping host-associated microbial communities than negative selection driven by antimicrobials[60].

A. muciniphila, a prominent symbiont in many mammals, is one of the most effective mucin degraders *in vitro*[35] and is consistently found at high abundance in the mucus layer in humans[35] and mice[34]. Consumption of mucus glycans as a carbon and energy source allows *A. muciniphila* and other mucin degraders to colonize the gut irrespective of the host diet, providing a clear advantage to these bacteria during conditions of nutrient deprivation. Accordingly, levels of *A. muciniphila* increase in fasting Syrian hamsters[61] and hibernating ground squirrels[62]. Similarly, during intestinal inflammation in mice, the community metatranscriptome indicates increased mucin utilization with a corresponding increase in abundance of the mucin-degrading species *B. acidifaciens*[63]. In gnotobiotic mice, restriction of complex polysaccharides in the diet causes the generalist *B. thetaiotaomicron* to shift its metabolism to utilize mucin glycans[50]. Further work in *B. thetaiotaomicron* has revealed that mutations affecting Sus-like systems involved in mucin glycan utilization cause a defect in competitive colonization and in the vertical transmission of bacteria from mother to pup[64]. Therefore, the ability to utilize mucus as a carbon and energy source contributes to the ability of some microorganisms to stably colonize the host and transfer to offspring across generations. Not surprisingly, genetic manipulation of enteric mucus production in mice changes microbial community composition[54,65]. In turn, gut bacteria affect transcription of mucin-encoding genes in mice[66]. Overall, the development of a healthy mucosa is a collaborative, bidirectional event between the host and the gut microbiota, creating an environment that allows the specific members to establish persistent colonization via the utilization of host-derived glycans.

In some cases, the ability of a bacterium to colonize the gut may be determined by its ability to utilize a specific, but limiting, nutrient. Bacterial species-specific carbohydrate utilization systems termed commensal

Indigenous organisms
Organisms that are native to a particular habitat (also termed autochthonous), as distinct from organisms that are simply passing through a habitat (allochthonous)

Mucin 2
(MUC2). The most abundant mucin protein in the human gut; the mouse homologue is also the most abundant mouse gut mucin.

Box 1 | **Colonization resistance**

One of the benefits afforded by the microbiota to the host is colonization resistance against pathogens. Invasive species of bacteria are inhibited from colonizing the gut because they are unable to displace indigenous species that have gained a strong foothold. After years of studying colonization resistance against pathogens in gnotobiotic animals in the 1960s and 1970s, Rolf Freter theorized that the ability of a bacterial species to colonize the gut is determined by its ability to utilize a specific limiting nutrient[135]. This notion has been well supported by studies showing that colonization resistance against pathogens is mediated by the availability of nutrient niches in the cases of *Escherichia coli*[136] and *Clostridium difficile*[137]. But Freter's hypothesis reached even further, suggesting that the relative amounts of limiting nutrients could dictate the abundance of each species in the indigenous community. Correspondingly, the variety of host-derived growth substrates could explain the stable diversity of the gut microbiota, if individual species have evolved to specialize in the uptake and metabolism of specific limiting nutrients, such as in the case of *Bacteroides fragilis*[46]. The concept of spatial niche partitioning being governed by host production of specific and scarce nutrient resources is an attractive one and may help to explain both the long-term persistence and the resilience of the microbiota, as well as colonization resistance against pathogens.

colonization factors (CCFs) have been identified in *B. fragilis* and *Bacteroides vulgatus*, and allow these bacteria to colonize saturable nutrient niches[46]. This discovery was made because of the observation that gnotobiotic mice colonized with a specific *Bacteroides* species were resistant to colonization by the same species, but not to colonization by closely related species. A genetic screen revealed that a set of genes encoding the CCF system was required for this intraspecies colonization resistance phenotype (BOX 1), suggesting that CCFs are responsible for defining the species-specific niche. Accordingly, when the *ccf* genes from *B. fragilis* were expressed in *B. vulgatus*, the resulting hybrid strain gained the ability to colonize an alternative niche. The CCF system was also required for the penetration of *B. fragilis* into colonic crypts and for long-term resilience to intestinal perturbations such as antibiotic treatment and gastroenteritis. Collectively, these data suggest that although metabolic flexibility allows bacterial adaptation in the lumenal environment, the occupation of a narrowly defined tissue-associated niche is probably very important for stable colonization by some bacteria.

Antimicrobials. Specialized epithelial immune cells called Paneth cells reside at the base of the crypts of the small intestine, secreting an array of antimicrobials that restrict the growth of the bacteria which are found near the mucosal surface[4] (FIG. 2a). Many of these molecules are cationic antimicrobial peptides that interact with and disrupt negatively charged bacterial membranes (FIG. 3b). Modifications to lipid A, which is the lipid portion of lipopolysaccharide (LPS) and a major component of the outer membrane of Gram-negative bacteria, are known to confer resistance to cationic antimicrobial peptides in several pathogens[67]. Interestingly, in *B. thetaiotaomicron*, under-phosphorylation of lipid A, a modification shared with the pathobiont *Helicobacter pylori*, was found to be important for resilient colonization during inflammation[68] (FIG. 3b).

The concentration of a variety of antimicrobials is higher towards the proximal end of the small intestine than at the distal end, creating a gradient that leads to a higher abundance and diversity of bacteria in distal locations (FIG. 1). For example, the lectin REGIIIγ is bactericidal to the Gram-positive bacteria that dominate the small intestine, because it binds to and disrupts their exposed peptidoglycan layer. REGIIIγ is required to prevent massive infiltration of the mucosa and microbial invasion of the tissue[69]. In addition to REGIIIγ, the innate immune system deploys many other antimicrobials (such as α-defensins from Paneth cells and β-defensins from neutrophils) with differing specificities to limit access to the epithelium[70], and resistance to these host-derived antimicrobial peptides is a general feature of many indigenous gut species of Firmicutes and Bacteroidetes[68].

In addition to these antimicrobials, gut bacteria, which are largely anaerobic, must contend with reactive oxygen species produced by aerobic host metabolism. Rapid dilution and consumption of oxygen secreted from the host tissue generates a gradient of oxygen that decreases in concentration from tissue to lumen (FIG. 2). Accordingly, the mucosal community is enriched in genes required for resistance to reactive oxygen species[33]. Although all *Bacteroides* spp. are classified as obligate anaerobes, *B. fragilis* can use oxygen as a terminal electron acceptor at nanomolar concentrations[71]. *B. fragilis* and tissue-associated microaerophilic Lactobacillaceae members express catalase, superoxide dismutase and other enzymes to inactivate reactive oxygen species[72].

Altogether, these mechanisms restrict access to the epithelium to a subset of bacterial species that can utilize nutrients found only at the tissue boundary and can survive host antimicrobial strategies as well.

Mucus and adhesion. To access the epithelium, pathogens and commensals alike must contend with the mucus barrier and the immune system (FIG. 4). Secreted MUC2 forms peptide crosslinks to create a viscous gel-like substance[73], serving as a barrier and host defence mechanism[74]. In mice lacking MUC2, the crypts of the colon are filled with bacteria, and the tissue is covered in biofilms[24], indicating that the gel-forming mucus is the primary barrier to tissue association by the microbiota at large. However, certain bacteria are able to penetrate the mucus by swimming or eating their way through.

In the gut, bacterial motility is generally restricted owing to the immunogenicity of flagellin, which is a ligand for Toll-like receptor 5 (TLR5)[75], and the viscosity of mucus, which limits the effectiveness of swimming (FIG. 4). Nonetheless, the enteric pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium depends on flagella and chemotaxis to penetrate the mucus layer and to reach host tissue[76]. *E. coli* and the close relative *Shigella flexneri* opt for an alternative strategy of secreting a mucin-binding serine protease, Pic, which rapidly digests mucus (FIG. 4). Interestingly, Pic also causes hypersecretion of mucus, which may interfere with the ability of indigenous bacteria to compete with these pathogens[77]. Similarly, another family of mucus-degrading proteins, M60-like peptidases, are conserved in pathogens and commensal mucosal bacteria from the Proteobacteria,

Colonization resistance
The prevention of invasion of an exogenous species into a microbial community. In the gut, colonization resistance may be a result of resource competition, spatial exclusion or direct inhibition by commensal microorganisms, or of selection mediated by host factors.

Paneth cells
Specialized epithelial cells that are found at the base of crypts in the small intestine and that secrete antimicrobial peptides.

Pathobiont
A symbiont with the potential to promote pathology under conditions that deviate from homeostasis, such as in immunocompromised or nutrient-deprived individuals.

Microaerophilic
Pertaining to a microorganism: obligately aerobic. These microorganisms thrive only in environments with low oxygen concentrations, such as at the epithelial surface in the gut.

Firmicutes, Bacteroidetes and other phyla[78]. In enterotoxigenic *E. coli*, an M60-like peptidase was required for association with villi in the mouse small intestine[79].

In addition to the ability to penetrate the mucus layer, bacterial adhesion to the epithelium also influences the microbial composition of the gut, especially in the small intestine (FIG. 2a). *Helicobacter* spp. adhere to and colonize the stomach and small intestine tissue via adherence to epithelial surface glycans[80]. Further downstream in the small intestine, segmented filamentous bacteria (SFB) adhere intimately to the epithelial surface, as first described in imaging studies of mice[81]. Host-specific strains of SFB seem to be present in many mammals, including humans[82]. These bacteria were only recently cultured *in vitro* using tissue-cultured enterocytes as a platform to support their growth, reinforcing the idea that they are obligate symbionts with the mammalian gut tissue[83]. Their mechanism of attachment is still a mystery, although the attachment site is marked by the accumulation of actin and leaves a visible indentation on the surface of the epithelial cell following removal of the filaments[83]. By virtue of their intimate host association, SFB shape the host immune response[84] and affect autoimmune disease in mouse models[85,86].

The molecular mechanisms underlying the attachment of microorganisms to host tissue have been well studied in pathogens (reviewed in REF. 87). Although all of these features were initially discovered and described in pathogens, they are also found in many commensal species. Bacteria adhere to mucus and epithelial surfaces by deploying outer-membrane proteins, capsules, lectins, adhesins and fimbriae (also called attachment pili) (FIG. 4). For example, the non-invasive pathogen *Vibrio cholerae* forms a layer of adhered cells on the wall of the small intestine using toxin-coregulated pili[88]. *V. cholerae* also binds mucins using an outer-membrane *N*-acetyl-D-glucosamine-binding protein, which may also facilitate penetration of the mucus and access to the epithelium[89]. Without attachment, these normally plankton-associated marine bacteria are unable to colonize the gut and thus are avirulent. *E. coli* possesses a great number of lectins with diverse sugar specificities, allowing it to bind mucins as well as other glycoproteins and extracellular matrix components of epithelial cells[90]. Invasive pathogens also depend on adherence factors as a preceding step to penetration and infection of the tissue. *Listeria monocytogenes* expresses a surface protein, internalin A (InlA), which binds epithelial cadherin (E-cadherin; a host cell adhesion protein) as a first step before exploiting actin to induce phagocytosis[91]. Studies of *S. typhimurium* also reveal a crucial role of apical surface attachment in inducing neutrophil-mediated inflammation, which appears to paradoxically promote infection[92] by providing a competitive advantage for the pathogen over the resident microbiota[93].

Beneficial microorganisms also adhere to particular regions of the epithelium and can serve to exclude adherent pathogens by occupying limited binding sites, although little is known about the underlying mechanisms or functions of this process (BOX 1). Early imaging studies revealed that *Lactobacillus* spp. which form

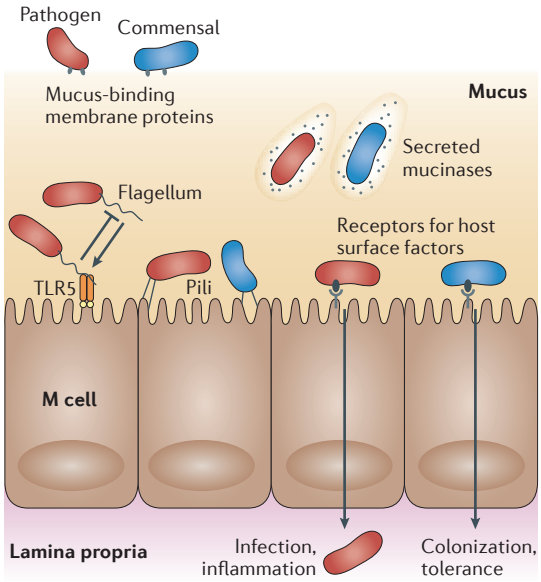

Figure 4 | **Bacterial access to the epithelium.** Both bacterial pathogens and commensals (or mutualists) have the ability to cross the mucus layer and access the gut epithelium. Lectins and other mucus-binding proteins facilitate initial interactions with the mucus layer. Mucinases and proteases are used to degrade mucus, allowing bacteria to 'eat' their way through, whereas some pathogens (such as *Salmonella* spp.) use flagella to swim through the viscous mucus. Toll-like receptor 5 (TLR5) sensing of flagellin effectively leads to inhibition of flagellum biosynthesis for most bacteria in the gut. Adherence to the tissue is achieved by both commensals and pathogens through pili, lectins and other outer-membrane proteins that target ligands on the epithelial cell surface. Adherence facilitates gut colonization for both commensals and pathogens, and also allows tissue invasion by pathogenic bacteria. Microfold cells (M cells) are specialized immune sentinel epithelial cells that detect gut bacteria and are also exploited by many pathogens as a means of translocation across the epithelium.

adherent layers on the epithelium in the rat stomach prevent yeast[94] and staphylococcal[95] adherence to the epithelium. Members of the family Lactobacillaceae (such as *Lactobacillus* and *Lactococcus* spp.) that colonize the small intestine and stomach have become model systems for studying adhesion by commensals, and exopolysaccharides, pili and cell wall-anchored proteins have thus been found to be involved in interacting with mucus, extracellular matrix proteins and other molecular targets on the epithelial cell surface[96]. Notably, cell wall-anchored mucus-binding proteins (MUBs) unique to Lactobacillaceae are known to be involved in both adherence and aggregation[97]. Strain-specific diversity in adherence and aggregation factors underlies the host specificity of *Lactobacillus reuteri*, indicating that tissue-associated biofilm formation is fundamental to colonization by this species[98]. Other means of attachment for commensal bacteria involve mechanisms that are conserved with pathogens, such as the adhesive pili of *Lactobacillus rhamnosus*, which bind mucus[99]. Analogous mechanisms can be found in unrelated species such

as *Bifidobacterium bifidum*, which uses pili to bind extracellular matrix proteins, thus contributing to bacterial aggregation[100].

Collectively, these studies suggest that interactions with mucus and adherence to intestinal epithelial cells seem to be adaptations that are used by pathogens during infection, as well as strategies employed by commensals during persistent colonization (FIG. 4).

Immunomodulation. In order to persist in the gut, non-pathogenic bacteria that intimately associate with host tissue must be tolerated by the immune system. The mucosa is inundated with large amounts of sIgA, which interacts with the microbiota. Many bacteria in the gut are coated in sIgA, and this subpopulation broadly resembles the mucosal population[101]. Certain adherent species such as *Helicobacter* spp. and SFB are especially highly coated in sIgA[102]. Binding of sIgA to commensal bacteria may contribute to mucosal biofilm formation, which serves as a barrier to pathogen adherence[103]. Gnotobiotic studies with *Rag1*-knockout mice (which effectively have no adaptive immune system owing to the lack of V(D)J recombination-activating protein 1 (RAG1)) showed that experimental coating of *B. thetaiotaomicron* with sIgA reduces microbial fitness but also leads to reduced inflammatory signalling and changes to bacterial gene expression[5,104]. Through these mechanisms, sIgA mediates homeostasis between the host and the microbiota, as well as between the host and potential pathogens at mucosal surfaces. Furthermore, natural antibodies have evolved to recognize bacterial capsular polysaccharides; although these have been studied mainly in the context of infectious agents, such antibodies may also represent an evolutionarily conserved strategy used by the host to sense indigenous bacterial species. However, examples of how the immune system can dependably distinguish between harmful and beneficial microorganisms remain limited.

An alternative view is that the immune system is not 'hard-wired' to discriminate between various classes of microorganisms, but rather that specific species have adapted to promote their own immunological tolerance. A few examples of active, species-specific immunomodulation by beneficial microorganisms suggest that some bacteria display signals to ensure they are tolerated by the immune system (FIG. 5). *B. fragilis* is one of the best understood gut bacteria in terms of immunomodulation. A component of its capsule, polysaccharide A (PSA), signals through an antigen-presenting cell intermediary (such as a dendritic cell) to stimulate the production of interleukin-10 (IL-10) by regulatory T cells[105], an anti-inflammatory subset of immune cells, thus contributing to the ability of *B. fragilis* to enter the mucus layer of the colon[45] (FIG. 5). Surface fucosylation of the bacterial capsule also contributes to *B. fragilis* fitness in the gut, perhaps by mimicking the host cell surface to elicit a tolerogenic immune response[106]. Through these specific molecular signals, *B. fragilis* induces an anti-inflammatory immune profile that facilitates its own colonization. Similarly, exopolysaccharides of *Bifidobacterium breve* promote immune tolerance by decreasing the production of pro-inflammatory cytokines and preventing a B cell response[107] (FIG. 5). Through a less well-defined mechanism, *B. breve* also induces IL-10 production by regulatory T cells[108]. Notably, both *B. fragilis* and *Bifidobacterium* spp. are known to closely associate with the host, which may necessitate immunomodulation to prevent an inflammatory reaction against these bacteria. Similarly, adherent SFB stimulate the development of a subset of T helper 17 (T_H17) cells, which are required for normal SFB colonization and also confer resistance to the pathogen *Citrobacter rodentium*[84] (BOX 1; FIG. 5). Clostridia are able to induce regulatory T cells, but a population of many species is much more effective than single isolates or combinations of a few species, suggesting that this induction is a combined effect of the production of different metabolites, such as short-chain fatty acids, by different species (see below)[109] (FIG. 5). Similarly, a defined community of eight mouse gut bacterial species (including several members of the Clostridiaceae and Lactobacillaceae families), referred to as the altered Schaedler flora[110], was also shown to modulate immune responses mediated by regulatory T cells. Therefore, it is likely that many other beneficial microorganisms have co-evolved with the immune system to facilitate stable long-term colonization.

Several nonspecific signals in the gut also promote tolerance towards beneficial microorganisms. Short-chain fatty acids such as butyrate, propionate and acetate are the end products of anaerobic fermentation of sugars, which is the dominant metabolism in the colon. The development of regulatory T cells is stimulated by these molecules[111,112], so this could be a more general way for the immune system to recognize beneficial bacteria or to assess the total fermentative productivity of the community. Mucus is another nonspecific anti-inflammatory signal. When MUC2 is taken up by dendritic cells in mice, it inhibits the expression of pro-inflammatory signals[113], raising the possibility that indigenous mucin degraders induce host tolerance by being co-presented with mucus. Pathogens also have an arsenal of anti-inflammatory mechanisms to suppress the immune system in order to promote infection[114]. It is particularly perplexing that features that are traditionally regarded as virulence factors in pathogens, such as capsular polysaccharides and pili, are also colonization factors in beneficial bacteria. Our notion of the defining characteristics of pathogens has probably been clouded by a historical underappreciation of similar colonization strategies used by beneficial species (FIG. 4). It is not surprising that similar mechanisms of host association (mucus penetration, adherence and immune modulation) are used by pathogenic and commensal bacteria alike; however, a key distinguishing feature is that commensals either have not evolved traits resembling traditional virulence factors, or have evolved additional features or modifications to offset the host response to such factors. This perspective suggests that commensal bacteria have reached an immunological and metabolic 'truce' with their host, enabling the persistent establishment of defined microbial habitats and elaborate microbial biogeographies.

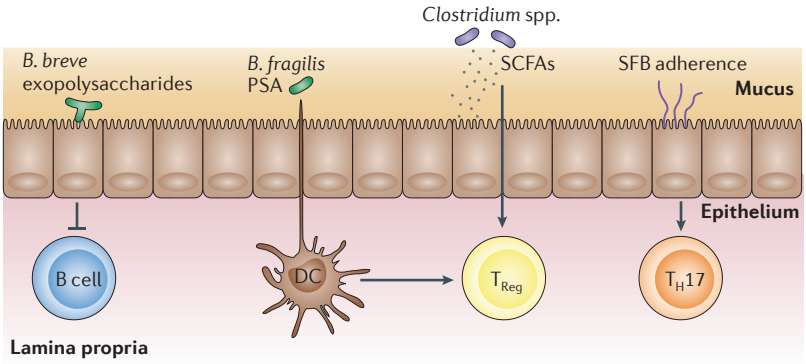

Figure 5 | **Immunomodulation by commensal gut bacteria.** Commensal gut bacteria induce immunomodulation via interactions with epithelial cells and antigen-presenting cells (such as dendritic cells (DCs)), and via the production of signalling metabolites. The exopolysaccharides of adherent *Bifidobacterium breve* reduce the production of inflammatory cytokines to dampen B cell responses. The capsular polysaccharide of *Bacteroides fragilis*, polysaccharide A (PSA), and the short-chain fatty acids (SCFAs) produced by many *Clostridium* spp. (and species of other genera) stimulate the production of the anti-inflammatory interleukin-10 (IL-10) by regulatory (T_{Reg}) T cells. Segmented filamentous bacteria (SFB) intercalate between the microvilli of epithelial cells and stimulate the development of T helper (T_H17) 17 cells, which are important for mucosal immunity to extracellular pathogens.

Micro-biogeography in health and disease

Microhabitats in the gut are likely to contribute to the development and stability of microbial communities because spatially stratified niches facilitate greater diversity. In mouse pups, the faecal microbiota is initially dominated by Proteobacteria, a signature of the small intestine but, following weaning, the dominant species become Clostridia and *Bacteroides* spp., which are characteristic of the adult colon[2]. The sequential development of the microbiota may thus occur from proximal to distal compartments, which makes sense as dispersal in the gut is largely unidirectional along the faecal stream. Because of this restriction on dispersal, the depletion of beneficial species, especially in the colon, could be catastrophic without a mechanism to replenish the community. Therefore, protected regions that are less susceptible to variable conditions in the gut may serve as reservoirs of bacterial cells that can seed growth in the lumen, possibly after an environmental insult (FIG. 6). In the case of *B. fragilis*, mutants that are unable to colonize the crypts of the colon are less resilient to intestinal perturbations such as antibiotic treatment and enteric infection[46]. This reservoir role is also a proposed function of the human appendix, which has a mucus- and bacterium-filled lumen contiguous with the caecum[115]. The appendix is protected from the faecal stream, but harbours a diverse microbial community and a contingent of specialized immune cells. The appendix is also phylogenetically widespread and evolved independently at least twice, providing strong evidence that this is not a vestigial structure, as was once believed[27]. In the rabbit appendix, indigenous bacteria coordinate the education of B cells and T cells, suggesting that these tissue-associated niches are venues for immunomodulation[116]. Microhabitats such as crypts, mucus and the appendix may be crucial to facilitate immune homeostasis, to protect microbial inhabitants from competitors and to repopulate the gut following catastrophic perturbations that alter bacterial community structure or deplete certain species from the lumen.

Micro-biogeography alterations during disease. The adverse effects of dysbiosis on host health have long been appreciated. Increasing clinical evidence links dysbiosis with various immune, metabolic and neurological disorders in both intestinal and extra-intestinal sites. For example, inflammatory bowel disease (IBD) is associated with changes in the gut microbiota, characterized by a decreased abundance of Clostridia[117–119] and an overall reduction in bacterial diversity[118–120]. Childhood asthma is correlated with low intestinal microbial diversity during the first month of life[121]. The obesity-associated microbiota is characterized by reduced microbial diversity and, in some studies, an increased *Firmicutes*/*Bacteroidetes* ratio[122]. In recent years, the role of gut dysbiosis in the pathogenesis of chronic liver diseases[123,124], colorectal cancer[125,126] and even neuropsychiatric dysfunctions[127] has been explored in animal models and humans. For clinical applications, profiling of the faecal microbiota has been widely used as a surrogate for profiling of the gastrointestinal bacterial community owing to the non-invasive and straightforward sample collection; however, faecal populations may be less informative than mucosal biopsies in defining disease-associated dysbiosis[128], a notion that requires additional experimental support. Below, we detail two examples that illustrate the importance of alterations in the micro-biogeography of the gut microbiota during disease: IBD and hepatic encephalopathy.

IBD is characterized by inflammation of the gastrointestinal tract, resulting in pain, vomiting, diarrhoea and other complications, including severe weight loss and behavioural changes. Generally, IBD is categorized into two syndromes: Crohn disease, which may involve inflammation throughout the gastrointestinal tract (mouth to anus); and ulcerative colitis, in which pathology is restricted to the large intestine. For more than a decade, studies have attempted to define a pattern of dysbiosis associated with IBD, but have yielded inconsistent and sometimes contradicting results[129]. Studies that focused on faecal microbiota reported wide inter-individual differences in composition, with overall microbial diversity being reduced in patients with Crohn disease compared with healthy controls[118]. However, in a study in which the human gut microbiota was assessed for the ability to drive colitis pathology in mice, it was found that bacteria contributing to the disease are highly coated in sIgA[102], suggesting that the mucosal or tissue-associated population is most relevant. Human studies based on biopsy samples elucidated several consistent features in line with this hypothesis: compared with controls, patients with IBD had an increased concentration of bacteria on the mucosal surface[130], a decreased microbial diversity[119,120], a decreased abundance of *Clostridium* spp.[117] and an increased number of Enterobacteriaceae (especially adherent, invasive *E. coli*) in their ileal mucosa[131].

Dysbiosis
A deviation from a normal microbial community, such as an imbalance in the abundance, membership or localization of microorganisms.

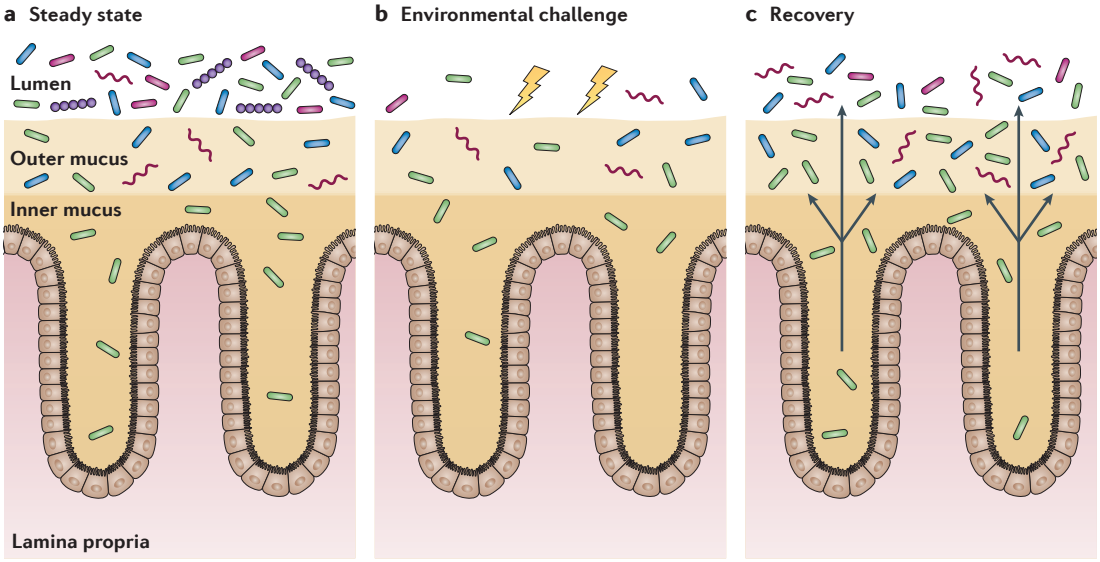

Figure 6 | **Gut microhabitats as reservoirs of bacterial diversity.** Specific niches such as crypts, the inner mucus layer and the appendix may be crucial to facilitate immune homeostasis, to protect commensal species from competitors and to re-seed the gut microbiota after the bacterial community structure is altered or certain species are depleted from the lumen. **a** | A subset of species (green) is able to penetrate the inner mucus layer and enter crypt spaces. **b** | Environmental challenges such as diet perturbations, antibiotic consumption or abnormalities in gastrointestinal motility massively alter the lumen community. However, the more stable mucosal environment and the crypts protect important bacterial species. **c** | The crypts and mucosa serve as reservoirs to repopulate the lumen.

Most recently, both the lumenal microbiota and the mucosal microbiota were profiled in a large cohort of new-onset, treatment-naive paediatric patients with Crohn disease and controls without IBD. Analysis of the mucosal microbiota revealed a significant drop in species richness, an increase in Enterobacteriaceae members, a decrease in Clostridiales order members and significant changes in several other previously unidentified taxa in patients with Crohn disease versus controls. Importantly, these dysbiotic signatures were lost when stool samples were examined[128]. Intriguingly, a laser capture microdissection study of colonic crypt mucus in patients with ulcerative colitis found that they had lower levels of crypt-associated bacteria than controls[132]. Overall, these studies highlight that distinguishing between faecal and mucosal microbial communities is particularly important for finding a reproducible microbial signature of IBD. Moving from correlations to a potential causal aetiology of the microbiota for IBD and other disorders will require further study of mucosal communities, focusing on the interactions between the host and microbiota.

Biogeographical changes in the gut microbiota may also influence liver function. Hepatic encephalopathy is a neuropsychiatric complication of cirrhosis and direct sequelae of gut dysbiosis. As a result of impaired liver function and the presence of portosystemic shunts (bypass of the liver by the circulatory system), toxic metabolites produced by the gut microbiota evade liver catabolism and cross the blood–brain barrier, leading to cerebral toxicity[123]. Interestingly, a comparison of the faecal microbiota of patients with cirrhosis but either with or without hepatic encephalopathy showed minimal differences between patients, whereas analysing the composition of the colonic mucosal microbiota revealed significant changes in patients with hepatic encephalopathy, including a lower abundance of *Roseburia* spp. and a higher abundance of *Enterococcus*, *Veillonella*, *Megasphaera*, *Burkholderia* and *Bifidobacterium* spp.[133]. The bacterial genera that are over-represented in the mucosa of patients with hepatic encephalopathy were also correlated with poorer cognition, higher levels of inflammation and higher clinical severity scores. In summary, dysbiosis in the gut mucosal microbiota, but not in the faecal community, significantly correlates with the severity of chronic liver disease phenotypes, including hepatic encephalopathy.

Conclusion

We have highlighted evidence that the microbiota is biogeographically stratified within the gut on different spatial scales and axes. Progress towards a functional understanding of the microbiota will come from paying greater attention to microhabitats within the gut ecosystem and to the spatial relationships among microorganisms and between microorganisms and the host. Faecal community profiling enabled by next-generation sequencing provides a valuable picture of the diversity, specificity, stability and developmental dynamics of the gut microbiota, but focusing on measurements of abundance in faeces neglects the importance of mucus- and tissue-associated organisms and cannot account for spatial distributions. Similarly, studies in gnotobiotic animals allow a reductionist approach to studying host–microorganism interactions, akin to methods that are traditionally employed by microbiologists studying

pathogens, but this simplified methodology is likely to miss important contributions from interspecies interactions. The functional study of gut microbial ecology using meta-omics techniques enables one to account for the behaviours of the community as a whole, but attributing functions to particular microbial members remains a challenge in community-level ecology. Therefore, testing unifying hypotheses using both reductionist and ecological approaches will be essential to our understanding of the microbiota and its biological functions.

More than half a century ago, in *Microorganisms Indigenous to Man*, the microbiologist Theodor Rosebury lamented on the lack of a general theory for influences that control the composition of the microbiota, the roles of individual members and their functions that affect the host[134]. With the true complexity of the problem revealed recently by sequencing advances, research is only now in a position to fulfil Rosebury's call for a general theory. Rolf Freter's nutrient niche hypothesis[135], which states that limiting nutrients control the population level of species that are particularly adept at utilizing them (BOX 1), provides a metabolic foundation to explain some of the nascent observations in the field. However, when Freter proposed his ideas, we were unaware of the role of immunomodulation by non-pathogens, an aspect that requires these bacteria to have access to the tissue. On the basis of evidence outlined in this Review, we propose that the host presents limiting nutrients as well as attachment sites in privileged locations. Furthermore, the immune system has an active role in allowing only beneficial species to access these locations during homeostasis. Selection for particular species close to the epithelium creates protected, stable reservoirs for microorganisms to persist in the face of rapidly changing conditions in the gut lumen. Thus, through localized, immune-facilitated and adherence-dependent nutrient selection, the host maintains the stability of a diverse community of microbial symbionts.

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The authors declare no competing interests.