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Microbiota-mediated colonization resistance: mechanisms and regulation

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Abstract

A dense and diverse microbial community inhabits the gut and many epithelial surfaces. Referred to as the microbiota, it co-evolved with the host and is beneficial for many host physiological processes. A major function of these symbiotic microorganisms is protection against pathogen colonization and overgrowth of indigenous pathobionts. Dysbiosis of the normal microbial community increases the risk of pathogen infection and overgrowth of harmful pathobionts. The protective mechanisms conferred by the microbiota are complex and include competitive microbial–microbial interactions and induction of host immune responses. Pathogens, in turn, have evolved multiple strategies to subvert colonization resistance conferred by the microbiota. Understanding the mechanisms by which microbial symbionts limit pathogen colonization should guide the development of new therapeutic approaches to prevent or treat disease.

Introduction

The mammalian intestine is colonized by trillions of microorganisms, including bacteria, viruses, fungi, archaea and protozoa, that have co-evolved with the host in a symbiotic relationship. The collection of microbial populations that reside within the host is commonly referred to as the microbiota. Microbial symbionts colonize mammalian hosts immediately after birth (Box 1). In adult individuals, Gram-negative Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes) and Gram-positive Bacillota (formerly Firmicutes) that include Clostridiales and Lactobacillales represent the major phyla among intestinal eubacteria[1]. The microbial communities differ between the small intestine and the large intestine, and between mucosa-associated and luminal communities at the same intestinal segment[2,3], which reflect different nutritional and metabolic requirements of individual bacteria. In the gut, resident bacteria have adapted to the local environments and developed complex interactions with host niches and other

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symbionts to acquire nutrients for survival[4]. In addition, microorganisms compete with each other for space and available nutrients, and this intermicrobial competition not only regulates the composition of the community but also limits the ability of indigenous and foreign microorganisms to colonize the gut[5].

Millions of years of co-evolution between the host and microorganisms have led to a mutualistic symbiosis in which the microbiota contributes to host metabolic functions and the host, in turn, provides nutrients and a hospitable space for the microorganisms. In addition to metabolic benefits, the microbiota provides the host with several functions that promote the intestinal epithelial barrier, immune homeostasis and protection against pathogen colonization[6]. The notion that certain beneficial members of the microbiota could outcompete harmful ones was first proposed by Elie Metchnikoff in the early twentieth century[7]. Later, studies with antibiotics and germ-free (GF) animals revealed that the symbiotic bacteria have a major role in limiting pathogen colonization in the gut[8,9]. This phenomenon that is detected within a few hours after pathogen inoculation was later coined ‘colonization resistance’[10]. In addition to limiting pathogen colonization, the microbiota can also inhibit the expansion of indigenous but potentially dangerous ‘pathobionts’, as well as colonization of innocuous species, such as probiotics[11]. There are a wide variety of mechanisms now known to participate in colonization resistance[6]. Many involve direct interactions between microbial cells, whereas others regulate host physiology, and largely host immune responses that limit pathogen colonization and expansion inside and outside the gut. In this Review, we focus on our current understanding of how the microbiota limit colonization by pathogens as well as the strategies used by pathogens to counter colonization resistance.

Direct mechanisms

Direct mechanisms of colonization resistance take place between bacteria, with the host acting as the environment in which competition takes place. Bacteria can directly inhibit the growth of each other by producing inhibitory compounds (‘interference’ competition in ecological terms) or by competing for resources (‘exploitative’ competition)[12], in both the gut (Fig. 1) and outside the gastrointestinal tract (Box 2). In the gut, the available nutritional resources are largely provided by the host either from ingested food or from secretions such as mucus. Additional nutrients are also provided by other microorganisms that inhabit the gut.

Spatial competition

The concept of ‘niche’ of a species, and the idea that two species cannot occupy the same niche, has developed from observations made by naturalists, including Darwin, to a fleshed out mathematical concept[13]. For the purpose of this Review, the niche can be thought of as the combination of many variables that include both physical location and other factors such as oxygen concentration and availability of various nutrients, which define the actual place of a species in an ecosystem. It has long been assumed that two species with identical niches cannot coexist for long. This ‘competitive exclusion’ can be demonstrated when two identical bacterial strains, only differing by a neutral marker, are sequentially given to GF

mice. The first strain will take up the entire niche, and the second, identical strain will be unable to colonize[14,15]. Giving one strain a new functional ability can alter its niche and allow it to coexist with its former competitors. For example, acquisition of a high-affinity transporter for a sugar (gluconate) allowed two otherwise similar *Escherichia coli* strains to coexist[16]. Comparing different natural variants of *E. coli*, which partially overlap in their sugar preferences, suggests that nutrient use may be a large factor in competition between strains[17]. Freter et al.[18] formulated the 'nutrient niche' hypothesis, which proposed that the relative abundance of a species in the gut is controlled by levels of just a few nutrients. In general, species compete directly with those species with which they share the most gene functions, usually being their closest evolutionary relatives, for example, *Klebsiella*, *Citrobacter* or *Clostridioides* species can prevent colonization with their often more pathogenic cousins[19–21].

The mammalian gut is not a homogeneous environment. There are many different microenvironments that effectively separate it into more niches with different characteristics. Oxygen concentration and pH are both reduced in the large intestine, and many nutrients, such as simple sugars and amino acids are absorbed by the host before reaching the large intestine. The sugars displayed on host cells and mucus can vary in local patches along the intestine[22], as can respiratory electron acceptors produced from host inflammation[23], which could divide up the physical space into different niches and potentially increase diversity or help maintain species or strains that would otherwise be outcompeted. This has led to a new variation of the nutrient niche idea by Freter that incorporates this spatial heterogeneity of nutrients[24]. Adhesion of bacteria to host cells, mucus or food particles could be a key factor in their continued survival in the gut. Adhesion can have different effects, either promoting washout or retention[15,25,26]. Competition for adhesion sites may therefore be another contributor to colonization resistance (Fig. 1). Some bacteria can also construct their own niches by modifying the environment in the host. For instance, some species can induce the host to produce different sugars, which can serve as nutrients or adhesion sites[27], or to trigger the induction of specific antibodies, which might enable better colonization[15].

Nutritional competition

The gut microbiota is generally stable over time and is presumed to be a climax community that has reached equilibrium, unless disturbed by antibiotic administration or other major events. Therefore, under homeostatic conditions, it is assumed that most of the available niches are filled and that this is a major factor behind colonization resistance. Although bacterial taxa, as defined by 16S rRNA gene sequences, can vary considerably between individuals, the gene functions present are less variable[28], at least based on our limited ability to accurately predict the function of bacterial genes. This suggests that the same functional niches are being filled in most individuals. A main consequence of this would be that the gut microorganisms scavenge most available nutrients, keeping them at low levels at the steady state, which limits pathogen expansion (Fig. 1). Indeed, GF or antibiotic-treated mice have increased amounts of sugars, amino acids and other nutrients in the gut lumen and a concomitant reduction in colonization resistance[8,29,30]. In line with this, a genetic screen revealed that depletion of dietary amino acids by the microbiota is a major factor underlying colonization resistance for the pathogen *Citrobacter rodentium*[29]. Notably,

administration of a high-protein diet to conventionally raised mice enhanced pathogen colonization by ~3 logs[29]. To achieve complete colonization resistance against a generalist bacterium such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium, a diverse microbial community is probably required to saturate all niches. The altered Schaedler flora, consisting of eight murine bacterial strains that stably colonize and recapitulate some aspects of a normal microbiota, provides little resistance to *S.* Typhimurium[31]. A larger collection containing 12 strains from 5 different phyla that dominate mammalian guts provided better protection against *S.* Typhimurium but not to the extent of a full complex microbiota[32]. To determine what could be missing from the community, the gene content was examined by metagenomic sequencing. There was a reduction of respiration-related genes in the 12 strains compared with a conventional microbiota. Three additional facultative anaerobes, which can respire oxygen, were able to improve resistance to *S.* Typhimurium to levels similar to a complete microbiota[32]. This has also been observed in another study in which native *E. coli* in mice correlated with protection against *S.* Typhimurium, and oxygen respiration was required for the protection[33]. Therefore, oxygen or anaerobic respiratory electron acceptors may be a limiting resource that can mediate resistance to *S.*Typhimurium. Essential metal cofactors such as iron, zinc and manganese are also limited in the gut and are actively sequestered by the host during inflammation. Competition for iron and zinc contributes to the ability of the probiotic *E. coli* Nissle strain to colonize the gut and provide resistance to *S.* Typhimurium[34,35].

Prokaryotic antimicrobial peptides

Nutrient competition is certainly a powerful determinant of community composition and colonization resistance in the gut. However, there are also many examples of active growth inhibition or interference that contribute to colonization resistance (Fig. 1). Bacteriocins are a heterogeneous class of peptides produced by bacteria, which have bacteriostatic or bactericidal activity against symbiotic bacteria and pathogens[36]. In line with the competitive exclusion concept, they often have a narrow target range and, in general, act on species closely related to the producer. They can inhibit growth or kill by various mechanisms; some require a specific receptor, whereas others do not. Bacteriocin-producing strains of *E. coli* outcompete non-producers in a mouse model[37]. A bacteriocin also contributes to the ability of the probiotic *E. coli* Nissle strain to protect from other Enterobacteriaceae[38]. The Bacteroidota phylum is highly represented in the mammalian gut and its members can produce various types of secreted antibacterial proteins, typically acting within the same genus[13,39]. In at least one case, a toxin producer was able to outcompete a sensitive strain in vivo[40]. *Enterococcus faecalis* and *Enterococcus faecium* are usually harmless gut residents that can become pathogenic when they acquire antibiotic resistance and become, for instance, vancomycin-resistant enterococcus (VRE), a major problem in the clinic[41]. An *E. faecalis* bacteriocin-producing strain was able to colonize mice, presumably by killing native enterococci, and cleared a pathogenic strain from the gut as well[42]. Gut resident enterococci can also target pathogenic *E. faecalis* via a different type of secreted small peptides called pheromones[43]. *Blautia producta*, a species in the Clostridia class, was identified as part of a minimal group of strains that could resist and eliminate VRE from mice[44]. The mechanism has been subsequently found to be a secreted bacteriocin, which

inhibited growth of VRE as well as various other Gram-positive species[45]. However, the contribution of prokaryotic antimicrobial peptides (AMPs) to VRE colonization resistance in the intact microbiota of animals or humans remains unclear.

Contact-dependent inhibition

Although bacteriocins and similar molecules can diffuse some distance from the producing cell, some inhibitory mechanisms require direct cell–cell contact (Fig. 1). A cell contact-dependent growth inhibition system, termed contact-dependent inhibition (CDI), was discovered in *E. coli* and later in other Pseudomonadota[46,47]. Contact-dependent inhibition requires a specific receptor protein on the target cell and can encode different toxic effector domains with various modes of inhibition[48]. The genes are usually accompanied by an immunity protein that neutralizes the toxin to protect the producing cell[48]. A more elaborate contact-dependent system was first discovered in *Pseudomonas aeruginosa*[49].

The type VI secretion system (T6SS) is a large multiprotein complex that can ‘spear’ nearby cells and inject toxic proteins, without the need for a receptor. These systems are found in many Gram-negative bacteria, especially Pseudomonadota and Bacteroidota[50]. The toxic effectors can have various functions and are usually found with a cognate immunity protein. Clusters of immunity genes have also been found to accumulate on their own, suggesting that protection from T6SS attack is beneficial in vivo[51], and other mechanisms have also been developed to protect from T6SS killing[52]. The T6SS can have a role in competition among species of the Bacteroidales order in the human and mouse gut and prevent invasion of a pathogenic strain[53–56]. Distinct T4SS and T7SS in Gram-negative and Gram-positive species, respectively, have also been demonstrated to kill cells via translocated toxic proteins[57,58]. Thus, secreted or cell contact-dependent antagonism probably has an important role in competition in the gut, especially among closely related species in confined physical niches, although their contribution to pathogen colonization resistance remains unclear.

Inhibitory metabolites

Metabolic byproducts produced by bacteria can exert inhibitory activity on other gut bacteria (Fig. 1). Bile acids (BAs) are synthesized by the host and conjugated to taurine or glycine in the liver, stored in the gallbladder and released into the intestinal tract to aid in the emulsification and absorption of dietary lipids[59]. Although most conjugated primary BAs are reabsorbed in the small intestine, a small proportion can be deconjugated by bacterial bile salt hydrolases and reach the distal intestine where they undergo several modifications, including 7α/β-dehydroxylation by rare bacteria such as *Clostridium scindens*, to generate secondary BAs[59]. Although primary BAs promote spore germination, secondary BAs such as deoxycholic acid and lithocholic acid inhibit the growth of many Gram-positive bacteria, including vegetative forms of *Clostridioides difficile*[60–63]. In mice and humans, the presence of secondary BAs is associated with resistance against *C. difficile* infection and development of colitis[63]. Likewise, administration of *C. scindens* to gnotobiotic mice colonized with a simplified microbiota lacking 7α-dehydroxylation activity partially restores secondary BA production, limiting *C. difficile* infection[64]. Conversely, selective disruption of the microbiota composition by antibiotics increases susceptibility to *C. difficile*, correlating with

reduced levels of secondary BAs[65]. The tolerance levels of different clinical *C. difficile* isolates to lithocholic acid correlate positively with the disease severity in murine models of infection[66,67]. Notably, *C. scindens* can also protect against *C. difficile* infection even in the absence of detectable levels of secondary BAs, suggesting a BA-independent mechanism of protection[68]. Therefore, additional studies are needed to fully decipher the specific contribution of BAs to *C. difficile* and other infections.

Short-chain fatty acids (SCFAs) including acetic, propionic and butyric acid are produced by microbial fermentation of plant-derived dietary polysaccharides by obligate anaerobes in the large intestine. SCFAs provide an energy source for intestinal epithelial cells (IECs), but also can contribute to colonization resistance[5,6]. For example, SCFAs inhibit the growth of pathogenic *E. coli*, *C. rodentium* and *C. difficile* and limit *Salmonella* species colonization in the mouse gut[69–72]. SCFAs including propionic acid diffuse freely across bacterial membranes (when protonated) and exert their inhibitory activity by disrupting intracellular pH homeostasis[73]. However, the overall contribution of SCFAs to protection against intestinal pathogen colonization remains unclear owing to the inability to specifically remove these molecules from an otherwise intact microbial environment.

Indirect mechanisms

In addition to the direct inhibition of pathogen colonization by symbionts or their products, there are also indirect mechanisms of defence that limit the invasion and/or expansion of enteric pathogens in the gut (Fig. 2). These include the mucus layer and oxygen gradients, as well as innate and adaptive immune responses, that are induced or maintained by the gut microbiota to limit pathogen colonization.

The mucosal barrier

The mucus layer forms a physical barrier that limits pathogen interactions with the underlying epithelium (Fig. 2). Pathogens such as *C. rodentium, Salmonella* species and some pathogenic *E. coli* strains require attachment to the intestinal epithelium for the initiation of their virulence programmes and effective colonization. Because the mucus layer in GF animals is thinner than in conventionally raised mice[74,75], microbial symbionts may limit pathogen colonization by fortifying the mucosal barrier. Mice lacking mucin 2, a major constituent of the intestinal mucus, exhibit increased pathogen burden and disease severity after infection with *C. rodentium*, *S.* Typhimurium and *Listeria monocytogenes*[76–78]. Similarly, thinning of the mucus layer by administration of a fibre-free diet to gnotobiotic mice harbouring a simple bacterial consortium leads to enhanced *C. rodentium* colonization and epithelial invasion[79]. However, in conventionally raised mice fed a comparable fibre-free diet, the increase in pathogen colonization is only marginal and temporal[80]. These results suggest that a diverse microbiota is important to promote mucus barrier function and colonization resistance in the presence of a disrupted mucus layer.

Under homeostatic conditions, the gut microbiota also releases compounds from the mucus layer and the surface of IECs, which can affect pathogen colonization (Fig. 2). For example, fucosylated proteins shed from IECs can be metabolized by gut bacteria to release L-fucose that reduces pathogen virulence and decreases susceptibility to *C. rodentium*[22]. By

contrast, disruption of the microbiota by antibiotics induces microbiota-dependent release of carbohydrates from the mucus layer that can fuel pathogen growth. For instance, *Bacteroides thetaiotaomicron* deploys mucin-degrading enzymes to release and metabolize sialic acid, which can be directly utilized by *C. difficile* and *S.* Typhimurium to support their own expansion in the gut[81]. Thus, the microbiota inhibits or enhances pathogen colonization by promoting the release of specific sugars from the mucus layer or IECs via different mechanisms.

Oxygen limitation

The gut microbiota promotes changes in the gut environment that indirectly lead to colonization resistance against enteric pathogens and pathobionts. A clear example of this effect is the establishment and maintenance of intestinal hypoxia by symbiotic bacteria to limit the expansion of pathogenic facultative anaerobes (Fig. 2). For instance, Clostridia species produce the SCFA butyrate that promotes aerobic respiration in IECs through β-oxidation, reducing the oxygen concentration at the epithelial surface[82,83]. Accordingly, depletion of Clostridia increases oxygen levels in the gut, which in turn fuels aerobic expansion of *S.* Typhimurium[82,84]. Likewise, intestinal inflammation and dysbiosis often coincide with a reduction of butyrate and butyrate producers, which increases luminal oxygen and expansion of pathogenic facultative anaerobes[85]. Symbiotic facultative anaerobes can in turn limit pathogen colonization by competing for or sequestering residual oxygen in the gut, impairing oxygen-dependent virulence gene expression in *Shigella flexneri*[86] and aerobic respiration in *C. rodentium* and *S. enterica* subsp. *enterica* serovar Enteritidis[87,88]. In addition, other symbionts such as *Mucispirillum schaedleri* can also compete for anaerobic respiration substrates such as nitrate in the absence of oxygen, limiting the expansion of *E. coli* and *S.* Typhimurium that rely on nitrate respiration to thrive in the inflamed gut[89–91].

Antimicrobial peptides and proteins

The microbiota can stimulate host epithelial cells to produce various AMPs that can target symbionts and pathogens (Fig. 2). These inhibitory compounds are positively charged and disrupt bacterial membranes by interacting with negative charges on the bacterial surface[92]. RegIIIβ and RegIIIγ are AMPs contained in the granules of Paneth cells and are released into the intestinal lumen where they promote the spatial segregation of bacterial symbionts from IECs[93,94]. Impaired production of RegIIIβ and RegIIIγ increases susceptibility to infection with various enteric pathogens in animal models[95,96]. For example, microbiota depletion by antibiotic administration reduces intestinal expression of RegIIIγ resulting in defective control of VRE[97], whereas stimulation with resiquimod (R848), a synthetic ligand for Toll-like receptor 7 (TLR7), restores expression of RegIIIγ in the gut and promotes eradication of VRE in antibiotic-treated mice[98]. Similarly, NOD1/2 signalling through the receptor-interacting serine–threonine-protein kinase 2 (RIPK2) limits *C. rodentium* colonization through stimulation of RegIIIβ production at the early phase of infection[99]. NOD2 stimulation by microbiota-derived peptidoglycan also induces expression of cryptdins, another class of AMPs produced by Paneth cells that promote *L. monocytogenes* clearance in mice[100]. In addition to their direct activity against pathogens,

AMPs also regulate gut microbiota composition and abundance[101], which can also influence colonization resistance.

Lipocalin 2 (LCN2) is another important antimicrobial protein, which sequesters bacterial iron-scavenging siderophores such as enterobactin to prevent pathogen iron acquisition[102]. LCN2 expression is induced upon inflammation and is dependent on the presence of the gut microbiota[103,104]. LCN2-deficient mice exhibit elevated levels of enterobactin-expressing gut bacteria and a distinct bacterial community compared with wild type animals, suggesting a role of LCN2 in regulating microbiota composition as well[103,104]. Like LCN2, calprotectin (a protein released by neutrophils and IECs during inflammation) exerts antimicrobial activity against pathogens by sequestering essential divalent metals such as iron, zinc, calcium and manganese[105]. However, whether the gut microbiota influences expression and/or release of calprotectin to confer colonization resistance against pathogens remains unclear. Therefore, the gut microbiota has a key role in the production and regulation of host AMPs and proteins that directly inhibit pathogens through different mechanisms. In turn, these molecules can also modify the gut microbiota and thus its colonization resistance effects.

Cytokines

Some cytokines are induced by the microbiota and can exert a protective role in colonization resistance against enteric pathogens (Fig. 2). For example, induction of IL-22 is associated with the presence of segmented filamentous bacteria (SFB) and increased resistance to *C. rodentium* infection[106]. Similarly, inoculation of murine norovirus, a persistent and asymptomatic colonizer of the mouse gut, protects young mice against *C. rodentium* infection in an IL-22-dependent manner as well[107,108]. IL-22 acts against the pathogen by promoting intestinal barrier function and altering the composition of the gut microbiota[109–111], but can also contribute to protection against *C. difficile*. For example, colonization of human microbiota into GF mice lacking adaptive immunity induces IL-22 production in the gut, which in turn promotes changes in host glycosylation and growth of *Phascolarctobacterium* species that compete with *C. difficile* for succinate[112]. Notably, IL-22 does not confer protection against *S.* Typhimurium intestinal colonization[113] and may even confer this pathogen an advantage over bacterial symbionts in the gut[114]. Likewise, SFB colonization limits murine rotavirus infection in the gut independently of IL-22, probably by directly altering virus infectivity and/or activity on IECs[115]. Thus, the role of IL-22 in protection against pathogen colonization is not universal.

The gut microbiota also induces the production of IL-1β in resident macrophages, which primes these cells to respond more rapidly to *S.* Typhimurium and *P. aeruginosa* through conversion of pro-IL-1β into IL-1β that enhances pathogen clearance through the recruitment of neutrophils[116]. Similarly, colonization of GF and antibiotic-treated mice with a defined Bacteroidota consortium protects against *Klebsiella pneumoniae* via IL-36 signalling[117]. Butyrate-producing bacteria can also limit pathogen colonization by regulating tight-junction protein expression via IL-10 signalling[83,118]. Therefore, different cytokines induced by the microbiota can contribute to colonization resistance through multiple mechanisms.

Adaptive immunity

The production of immunoglobulins that contribute to pathogen clearance is also influenced by the gut microbiota (Fig. 2). For example, certain bacterial symbionts induce production of polyreactive, low-affinity IgA antibodies, which can cross-react with antigens expressed by other bacterial species, including enteric pathogens[119–121]. Therefore, a large proportion of gut symbiotic bacteria are coated with IgA antibodies[120,122]. Notably, homeostatic secretory IgA derived from human milk or colostrum is protective against necrotoxigenic *E. coli* infection in GF newborn piglets in a manner dependent on IgA-associated glycans, highlighting the polyreactivity and protective effect of secretory IgA across species[123].

Although mucosal antibody responses can also shape microbiota composition in the gut, the degree to which this influences colonization resistance against enteric pathogens remains controversial or largely unexplored. For example, the levels of homeostatic IgA reactive against *S.* Typhimurium are different between commonly used inbred mouse strains, which correlate with differences in survival after pathogen challenge[124]. By contrast, mice lacking the polymeric immunoglobulin receptor that cannot transport IgA or IgM into the gut lumen showed increased or reduced susceptibility to *S.* Typhimurium infection in different studies[125,126]. Although the reasons for these discrepancies are unclear, they might be explained by the differences in microbiota composition and/or their associated immunoglobulin repertoires in different mice.

Although antibody binding is usually associated with an exclusionary function against pathogens, IgA can also promote the survival of the human symbiont *Bacteroides fragilis* in monocolonized mice[15]. Similarly, metagenomic analysis of IgA-bound gut bacteria in healthy individuals and IgA-deficient individuals revealed an unexpected depletion of beneficial symbionts associated with IgA deficiency[127]. Furthermore, IgA promotes mutualistic interactions between the host and potentially pathogenic fungal symbionts by targeting and suppressing virulent morphotypes, in both mice and humans[128]. These studies suggest that IgA limits pathogen colonization by promoting the expansion of beneficial symbionts, retaining them in favourable niches or preventing their killing by inhibitory molecules.

Serum IgA and IgG induced by members of the gut microbiota also protect against systemic infection in mice[129,130]. Notably, depletion of secretory IgT, an ancient immunoglobulin class specialized in mucosal immunity, leads to dysbiosis of the gill microbiota and impaired resistance to mucosal parasite infection in the rainbow trout[131]. This evidence suggests that steady-state antibody responses against the microbiota emerged early in vertebrate evolution and contribute to host protection against different types of infections.

Pathogen evasion of colonization resistance

Successful pathogens have evolved multiple strategies to overcome colonization resistance. These often involve the use of pathogen virulence programmes to alter the gut environment to better suit them or to overcome the nutritional limitations imposed by the microbiota (Fig. 3). The importance of pathogen virulence in countering colonization resistance is highlighted by the observation that the major virulence programme of *C. rodentium* is

essential for gut colonization in conventionally raised mice, but not in GF mice[132]. Multiple environmental signals are integrated to decide when and where to express virulence genes after a pathogen enters the gut[133]. The microbiota can modify some of these signals, and bacterial products such SCFAs[134–136], indole[137] and 1,2-propanediol[138] can also regulate virulence gene expression.

Niche construction via virulence factors

Pathogens depend on virulence factors for successful infection of a host (Fig. 3). Prime examples are the T3SS used by *Salmonella* species to inject effector proteins into host cells and to modify their physiology, or the toxins secreted by *C. difficile* or *Vibrio cholerae*. Most of the virulence programmes used by enteric pathogens are dedicated to modifying the niche to better enable the growth of the pathogen, also known as ‘niche construction’. *C. rodentium*, *S.* Typhimurium and other Enterobacteriaceae are facultative anaerobes that are not only able to grow aerobically but also anaerobically by fermenting or respiring various substrates. Most of the symbiotic bacteria in the large intestine, by contrast, are fermenters and highly oxygen-sensitive. Thus, facultative anaerobe pathogens gain a growth advantage if oxygen or other respiratory substrates became available. For instance, on infecting a mouse, *C. rodentium* attaches to the caecal or colonic epithelium, where oxygen is highest. Using its T3SS, the pathogen injects effectors into host epithelial cells, which results in the characteristic hyperplasia of crypt cells. This changes the overall metabolism of the epithelium, resulting in decreased oxygen consumption. The excess oxygen can then be respired by *C. rodentium*[87]. In fact, even before hyperplasia develops, *C. rodentium* can respire hydrogen peroxide produced by epithelial NADPH oxidase (NOX1) during early T3SS-mediated attachment[139]. To circumvent the amino acid limitation imposed by the microbiota, this pathogen induces amino acid biosynthesis genes to make its own amino acids, which is essential for gut colonization[29]. *C. rodentium* can also use alternative carbon sources for its initial expansion in the gut, including dietary-derived metabolites that are made available by commensal bacteria activity[140]. In addition, this pathogen uses its T3SS to localize to or near the epithelium, avoiding spatial and nutritional competition from the microbiota as well[132]. Furthermore, enteropathogenic *E. coli* and presumably *C. rodentium* might use their T3SS to extract nutrients from epithelial cells when attached to IECs to subvert nutritional limitations imposed by the microbiota[141].

S. Typhimurium uses its Spi1 T3SS to trigger inflammation in the ileum and caecum of mice after overcoming initial colonization resistance[142]. Spi1 effectors cause the host to produce reactive oxygen and nitrogen species, which create the byproducts tetrathionate and nitrate that *S.* Typhimurium can respire[143,144], as well as oxidized sugars that it can metabolize[145]. This pathogen also uses oxygen that becomes more abundant when inflammation represses certain native anaerobes and disrupts epithelial cell oxygen consumption[84]. Certain carbon sources in the gut are able to be metabolized by *S.* Typhimurium in the presence of appropriate respiratory substrates, for example, host-derived ethanolamine and the bacterial metabolite 1,2-propanediol[146,147]. Hydrogen (H_2) produced by certain symbionts can be used by the pathogen as a respiratory electron donor[148]. Notably, *Salmonella* species can even make use of some of the bacterially produced compounds that normally inhibit its growth, such as the SCFAs propionate and butyrate, to promote its survival[149,150]. Moreover,

S. Typhimurium has evolved ways to outcompete the host and other bacteria for scarce and essential metals such as iron, zinc and manganese, including high-affinity transporters and siderophores that are resistant to host factors[151–153]. Thus, enteric pathogens can deploy an arsenal of specialized strategies to overcome the colonization resistance imposed by the microbiota.

Direct antagonism of native bacteria

Just as the native microbiota uses direct inhibition against outsiders, pathogens can also use direct methods to compete with the native microbiota and carve out a niche to invade (Fig. 3). The T6SS is used by pathogens as well as by symbionts to compete with one another by injecting various toxic effectors into nearby cells. *V. cholerae* can use its T6SS to kill competing *E. coli* in a neonatal mouse model, allowing it to better infect and cause disease[154]. *S.* Typhimurium also uses its T6SS to colonize adult mice, in which it can kill a competing Enterobacteriaceae strain: *Klebsiella oxytoca*[155]. *Shigella sonnei*, which causes dysentery in humans, also has a T6SS that can kill the related species *S. flexneri* or *E. coli*, which enhances its colonization in the mouse gut, although it is still rapidly cleared by the microbiota[156]. The T6SS of *C. rodentium* has been recently shown to have a role during early invasion, through killing of, and avoiding killing by, symbiotic *E. coli* that also possess a T6SS[157].

Conclusions and future perspectives

Symbiotic microorganisms colonize the gut and every epithelial surface in humans and animals and provide many benefits to the host including protection against pathogen colonization. In fact, the native gut microbiota can be harnessed to prevent or treat infections (Box 3). The different mechanisms by which the microbiota mediate colonization resistance are influenced by a wide range of factors including the baseline composition of the microbiota, diet, sex, age and circadian rhythm, as well as pathogen dosage and developmental stage of the pathogen. The protection against pathogen colonization is accomplished largely through multiple mechanisms that indigenous symbionts have evolved to compete with related microorganisms for niches in the gut and other epithelial surfaces. Thus, the direct mechanisms underlying colonization resistance do not target pathogens specifically, in that they are used for constant microbial–microbial competition and microbial warfare in the gut. Although most of the mechanisms of direct colonization resistance are not pathogen-specific, they often exhibit narrow specificity for their targets. This is the case for bacteriocins or contact-dependent killing such as the T6SS. In addition, the microbiota can produce metabolites such as secondary BAs that kill Gram-positive bacteria. Much of the evidence supporting a role for particular microorganisms or microbial molecules in colonization resistance has relied on the use of simplified consortia of microorganisms in gnotobiotic mice. Although the use of such consortia has provided important mechanistic insights in that biological context, the contribution of particular microorganisms or microbial molecules to colonization resistance in the presence of a complete microbiota is unclear. Thus, studies to assess the role of such microorganisms or molecules in conventionally raised animals or humans are needed. Future studies should also provide new insight into the complexity of microbial interactions in their natural niches,

particularly those that are occupied by incoming pathogens or opportunistic pathogens that reside in the gut. In addition, the precise location in the intestine where the inhibition of pathogens by the resident microorganisms takes place remains unknown and warrants further investigation. The presence of a dense and diverse population of gut microorganisms that consume most available nutrients, keeping them at low levels at steady state, also limits pathogen colonization and expansion. The limitation of nutrients and physical space is likely to have a broad and important role in colonization resistance against various pathogens. It is also to be expected that consumption of different diets may affect protection against enteric pathogens by altering the amounts of nutrients, although the effect of diet on pathogen colonization resistance remains poorly understood and warrants further investigation as well. Indirect mechanisms of colonization resistance involve mechanisms by which the microbiota limits pathogen colonization primarily through the induction of immune responses in the host. Such mechanisms are not pathogen-specific either, in that epithelial-derived AMPs or polyreactive antibodies can also target bacterial symbionts. Further understanding of the mechanisms of colonization resistance can guide future approaches for preventing and treating infections.

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Glossary

Bacteriocins	Antimicrobial peptides produced by bacteria to inhibit the growth of similar or closely related bacteria
Colonization resistance	Mechanisms by which the intestinal microbiota limits the colonization of pathogens and pathobionts
Cytokine	Small immunoregulatory protein released by cells that mediates cell-to-cell communication in immune responses
Germ-free (GF) animals	Animals that are born and raised in isolators without exposure to microorganisms
Pathobionts	Microorganisms that under normal circumstances live as non-harming symbionts, but under certain conditions, usually involving environmental or genetic alterations, induce disease
Secretion system	Molecular structure assembled by bacteria that enables them to inject effector proteins directly into the host cytoplasm or other microbial cells
Prebiotics	Compounds in food that promote the growth or activity of beneficial symbionts

Probiotics	Live microorganisms that upon administration could confer a health benefit on the host
Secretory IgA	(sIgA). A dimer of IgA molecules bound by a joining peptide (secretory component). sIgA is the predominant immunoglobulin in mucosal surfaces, including the gut
Symbionts	Organisms of different species living together in a long-term relationship or symbiosis. Many microbial symbionts are mutualistic in that they and the host benefit from each other, whereas commensals receive benefits from the host, but the host is not helped or harmed

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Box 1

Microbiota maturation and early-life colonization resistance

The early composition of the microbiota is heavily influenced by maternal transmission, dietary factors and the mode of delivery[158,159]. Acquisition of an intact microbiota during vaginal delivery seems important because microbial transfer via caesarean section has been associated with increased colonization by pathobionts that can cause opportunistic infections[158]. Unlike the microbiota of the adult intestine that is dominated by obligate anaerobes, the microbiota of neonates and infants is less diverse and contains large numbers of facultative anaerobes[159,160]. The difference in microbiota composition between adults and infants reflects a major role for nutrition in shaping microbiota communities, as cessation of breastfeeding is required for maturation into an adult-like microbiota[160]. This transition coincides with dietary changes from simple sugars and oligosaccharides present in the maternal milk to a more diverse diet rich in complex polysaccharides. Infants are highly susceptible to infections caused by enteric pathogens[161]. The reason for this increased susceptibility seems multifactorial, including the absence of a mature immune system[162]. However, the neonatal microbiota is unable to prevent colonization by two common bacterial pathogens independently of the age of the host[113]. The lack of colonization resistance was caused by the absence of Clostridiales in the neonatal gut, as administration of Clostridiales restored protection against pathogen colonization independently of the host immune system[113]. However, whether the lack of Clostridiales can account for overall increased susceptibility to enteric infection in neonates remains unclear. The microbiota can also promote the protection of neonates against pathogens indirectly through the stimulation of immune responses. For example, dysbiosis induced by treatment with antibiotics increases the susceptibility to sepsis by impairing IL-17-mediated neutrophil homeostasis[163]. In humans, perinatal antibiotic treatment augments the incidence of early-onset sepsis, which was associated with a reduction in lactobacilli in the neonatal gut and the vagina of the mother[164]. Consistent with these findings, bacterial strains from the neonatal microbiota such as those belonging to the *Lactobacillus* genus and *Escherichia coli* can protect neonatal mice from late-onset sepsis, but the mechanism remains unknown[165]. One possibility is that certain bacteria residing in the neonatal gut can elicit immune responses, including production of protective IgG, that can also target pathogens[130]. Consistent with this notion, the maternal gut microbiota can induce IgG that protects newborn babies from pathogens and enteric *E. coli* infection[166].

Box 2

Colonization resistance outside the gut

In addition to the intestine, other body cavities and the skin that are exposed to the external environment are colonized by a diverse population of microbial symbionts. The surface of the epidermis, the outermost multilayer aspect of the skin, consists of the protein-rich and lipid-rich cornified layer dotted with pilosebaceous units and sweat glands. These structures provide the skin not only protection against pathogen invasion but also a unique metabolic niche for microbial survival[167]. Despite being relatively nutrient-poor and acidic, the skin harbours millions of bacteria, fungi and viruses that can limit pathogen colonization and invasion[167,168]. Although most skin symbionts are considered commensal or mutualistic, some microorganisms including *Staphylococcus aureus* and *Staphylococcus epidermidis* can induce the expression of virulence genes and become pathogenic depending on host or microbial factors that remain poorly understood. Similar to other microbial-rich host sites, skin symbionts have developed strategies to limit competition with other bacteria and to promote their colonization. For example, coagulase-negative *Staphylococcus* (CoNS) species that are abundant in the skin can reduce the colonization of pathogenic *S. aureus* through the production of bacteriocins and other antimicrobial peptides (AMPs)[169,170]. These peptides produced by some strains of CoNS can synergize with host AMPs to inhibit *S. aureus* growth[170–172]. Some competitive interactions between skin bacteria are not based on prokaryotic antimicrobials. For example, *Corynebacterium accolens* produces the LiS1 lipase that acts on skin surface triacylglycerols to release antibacterial free fatty acids that inhibit the growth of the opportunistic pathogen *Streptococcus pneumoniae*[173]. CoNS can also antagonize other CoNS species and *S. aureus* through the accessory gene regulator (*agr*) quorum-sensing system. The *agr* system is used by all staphylococci to coordinate cellular behaviour in response to bacterial density through the production of a cyclic peptide known as the autoinducing peptide (AIP)[174]. Every staphylococcal species contains an *agr* locus and produces a unique stimulatory AIP molecule, but non-cognate AIPs can inhibit quorum-sensing activation in other species[175]. Such mechanisms of *agr* crosstalk can be used to inhibit *S. aureus* in models of intradermal skin infection and atopic dermatitis[176,177]. Some members of the skin microbiota can also target pathogens indirectly by inducing AMP production in keratinocytes reducing pathogen invasion[178].

Like the skin, the nasal cavity harbours a diverse microbiota that includes opportunistic pathogens such as *S. aureus* that is present in the nares of up to 40% of healthy individuals[179]. Nasal CoNS species produce multiple antibiotics against competitors that could regulate pathogen colonization[180]. Likewise, *Staphylococcus lugdunensis*, another CoNS that inhabits the nasal cavity, produces the cyclic peptide lugdunin that inhibits *S. aureus* colonization[181]. Furthermore, nasal carriage of *S. lugdunensis* is negatively correlated with that of *S. aureus* in humans[181]. *S. epidermidis* can also interfere with *S. aureus* virulence and colonization through the production of the serine protease Esp that can degrade proteins that are important for *S. aureus* biofilm formation and nasal colonization[182,183]. A negative correlation between abundance of *S. epidermidis* and several opportunistic pathogens including *S. aureus* and *Moraxella catarrhalis* was also

observed in the human nasal cavity[184], suggesting interbacterial competition. Further analyses revealed that *S. epidermidis* can stimulate host AMP production that can kill and outcompete *S. aureus* and *M. catarrhalis*[184]. Although the physiological relevance of these intermicrobial interactions in the skin and nasal cavity remains unclear, these studies suggest that CoNS and other bacteria can antagonize pathogens via different mechanisms.

In most women, the cervicovaginal community is dominated by *Lactobacillus* species[185]. The replacement of the *Lactobacillus* species-dominated microbiota by a diverse community with a greater abundance of anaerobes, a clinical condition called bacterial vaginosis, is a risk factor for several common sexually transmitted infections[186]. The mechanism by which a diverse, non-*Lactobacillus* species-dominant microbiota increases the risk of infection is poorly understood. The presence of lactic acid-producing *Lactobacillus* species reduces the pH in the vagina, which can reduce pathogen colonization including bacteria associated with urinary tract infections, for which the optimal pH for growth is neutral[187]. Furthermore, some lactobacilli can also produce H_2O_2 and AMPs[188,189]. Bacterial vaginosis has been associated with increased cytokine production, which could also promote pathogen growth; however, increased inflammatory responses were not observed in some studies[190]. Further studies are needed to understand the relationship between microbiota dysbiosis and increased risk for infection in bacterial vaginosis.

Box 3

Harnessing the microbiota to fight infections

The use of faecal microbiota transplantation and other microbiota-based therapeutics to treat infectious and inflammatory disorders has been recently reviewed[191]. The concept that the intestinal microbiota can be harnessed to treat infectious disease was first tested in the 1950s and later demonstrated to be effective in the treatment of recurrent colitis caused by *Clostridioides difficile*[192,193]. Infection with *C. difficile* is associated with the use of broad-spectrum antibiotics that remove symbiotic bacteria that mediate colonization resistance against the bacterium in the gut. The engraftment of the donor microbiota can be predicted largely from the abundance and phylogeny of bacteria in the donor and recipient faeces[194]. Although faecal microbiota transplantation is highly effective in treating recurrent *C. difficile* infection, the variable nature and potential pathogen transmission of the faecal microbiota make the implementation of the approach in the clinic challenging. Thus, current efforts are devoted to identifying and characterizing defined consortia of bacterial isolates that mediate protection against *C. difficile* and other pathogens[195]. The assembly of effective consortia for the treatment of disease is also challenging because of our lack of knowledge about the gut microbial ecosystem. Increased understanding of the metabolic requirements and microbial–microbial interactions should lead to the development of consortia of microorganisms that can function cooperatively to target pathogens more effectively. However, administration of isolated live microorganisms in the clinic is problematic because probiotics typically have limited effects on the overall composition of the microbiota, as these exogenous bacterial species are often unable to persistently colonize hosts[11]. Concurrent supplementation with prebiotics may be useful in that regard because it can enhance the colonization of exogenous microorganisms, including enhanced colonization resistance against *C. difficile*[196]. Furthermore, understanding of the mechanisms that are used by symbionts to outcompete pathogens may lead to the genetic engineering of microbial species with increased capacity to limit pathogen colonization. For instance, symbiotic bacteria can be engineered to gain a competitive edge over related resident bacteria enhancing their colonization in the gut[197]. Such approaches could improve our armamentarium to treat intestinal infections such as those caused by *C. difficile*. Diet and prebiotics can alter the composition of the microbiota and could potentially be effective in the treatment of infectious disease as well. However, such approaches can only be effective if sufficient protective microorganisms are present in the microbiota of the patient, which is not always the case in clinical situations.

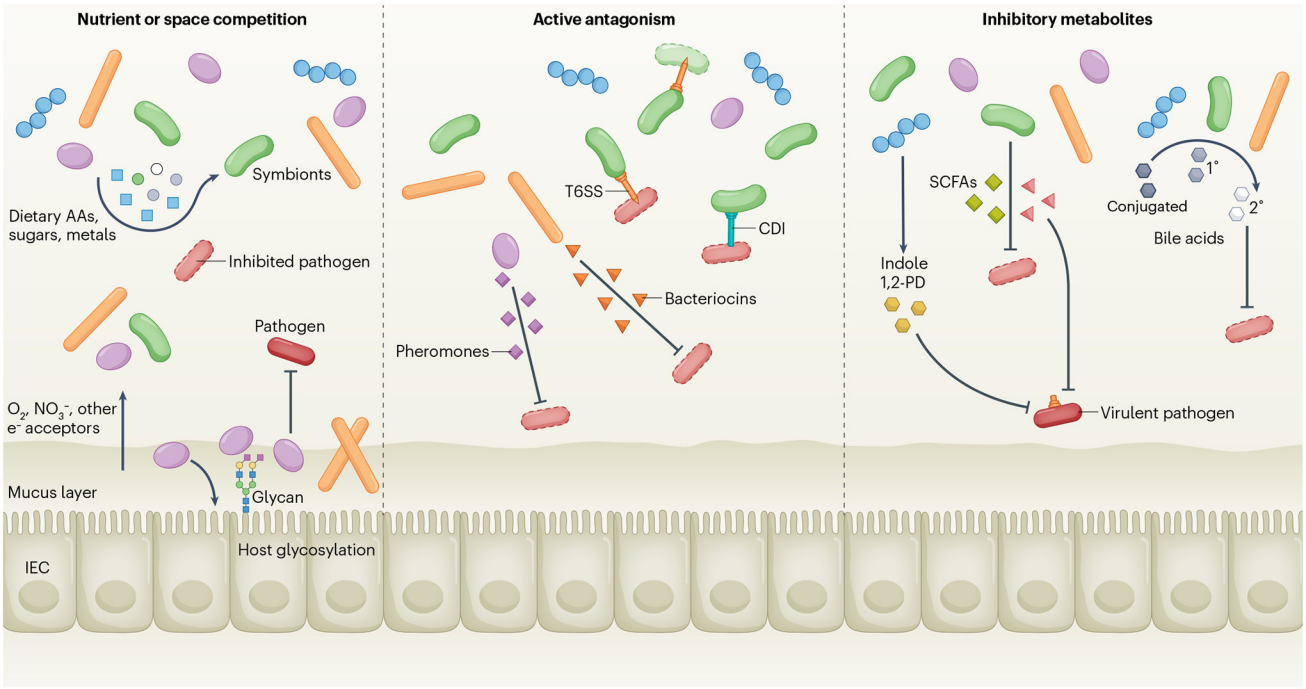

Fig. 1 |. Direct mechanisms of colonization resistance.

Native symbiotic bacteria consume dietary amino acids (AAs), sugars, metals and respiratory electron acceptors such as O_2 and NO_3^-, thus starving the pathogen of essential nutrients and molecules (left panel). At the epithelial surface, bacteria can modify host glycosylation and/or use it as a nutrient for adhesion, creating a new microscopic niche that blocks pathogen access to the epithelium. Other symbionts adhering to sites on the epithelium or in the mucus can also prevent pathogen access. Symbionts can also directly kill pathogens via contact-dependent inhibition (CDI), the type VI secretion system (T6SS) or secreted molecules, including bacteriocins or pheromone peptides (centre panel). Other inhibitory compounds such as acetic, butyric or propionic acids (short-chain fatty acids (SCFAs)), indole or 1,2-propanediol (1,2-PD) are secreted by the microbiota and inhibit growth or suppress virulence factor expression in pathogens (right panel). Many symbionts can modify bile acids by deconjugation, whereas some (for example, *Clostridium scindens*) can convert primary to secondary bile acids that inhibit pathogen growth. IEC, intestinal epithelial cell.

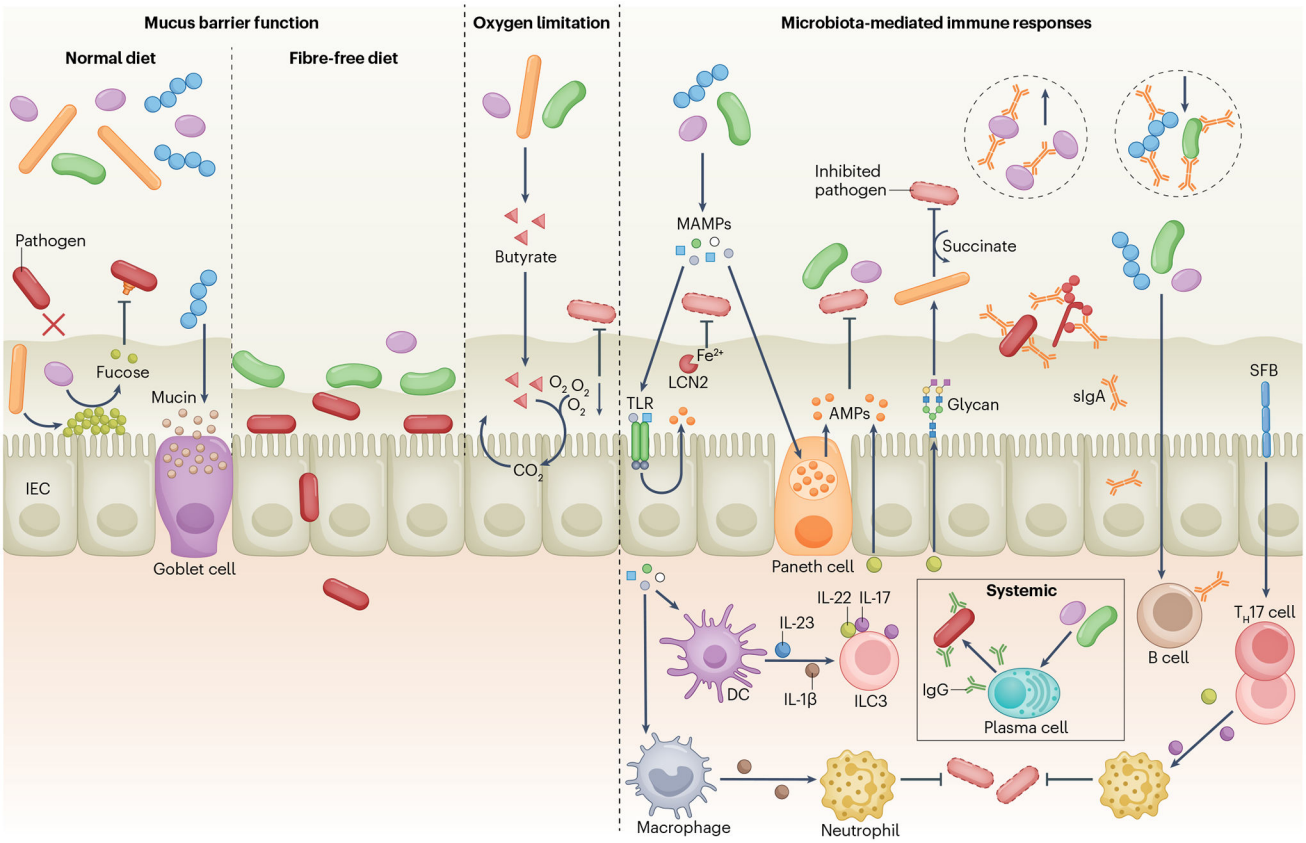

Fig. 2 |. Indirect mechanisms of colonization resistance.

The mucus layer limits pathogen access to the intestinal epithelium (left panel). Microbiota stimulation of host fucosylation protects against pathogens, whereas certain symbionts (such as *Bacteroides thetaiotaomicron*) release L-fucose from the mucus layer, which reduces virulence gene expression in *Citrobacter rodentium*, pathogenic *Escherichia coli* and *Enterococcus faecalis*. Fibre deprivation leads to an increase in mucus-degrading symbionts that erode the mucus layer, promoting *C. rodentium* early access to the epithelium. Clostridia produce butyrate that promotes aerobic respiration in intestinal epithelial cells (IECs), reducing oxygen levels in the gut and limiting the expansion of facultative anaerobe pathogens (centre panel). Microorganism-associated molecular patterns (MAMPs) stimulate Toll-like receptors (TLRs) and NOD-like receptors (not shown) in IECs and Paneth cells, leading to the production of antimicrobial peptides (AMPs) that target symbiotic bacteria and pathogens (right panel). Likewise, the microbiota promotes lipocalin 2 (LCN2) production in IECs, limiting pathogen access to iron. Symbiotic bacteria stimulate IL-22 production by type 3 innate lymphoid cells (ILC3s), which regulates AMP production and glycosylation-dependent expansion of symbionts that compete with *Clostridioides difficile* for succinate. Symbionts also promote pro-IL-1β production by resident macrophages, priming these cells to produce IL-1β in response to *Salmonella enterica* subsp. *enterica* serovar Typhimurium and enhancing the recruitment of neutrophils to fight infection. Symbiotic bacteria induce production of polyreactive secretory IgA (sIgA) that can recognize antigens on enteric pathogens, including *S.* Typhimurium, pathogenic *E. coli* and virulent *Candida albicans*. sIgA also limits or promotes expansion of specific symbionts.

Microbiota-induced IgG confers protection against pathogens primarily at systemic sites. Segmented filamentous bacteria (SFB) induce IL-17 and IL-22 production by T helper 17 (T_H17) cells and ILC3s, promoting neutrophil recruitment and pathogen control. DC, dendritic cell.

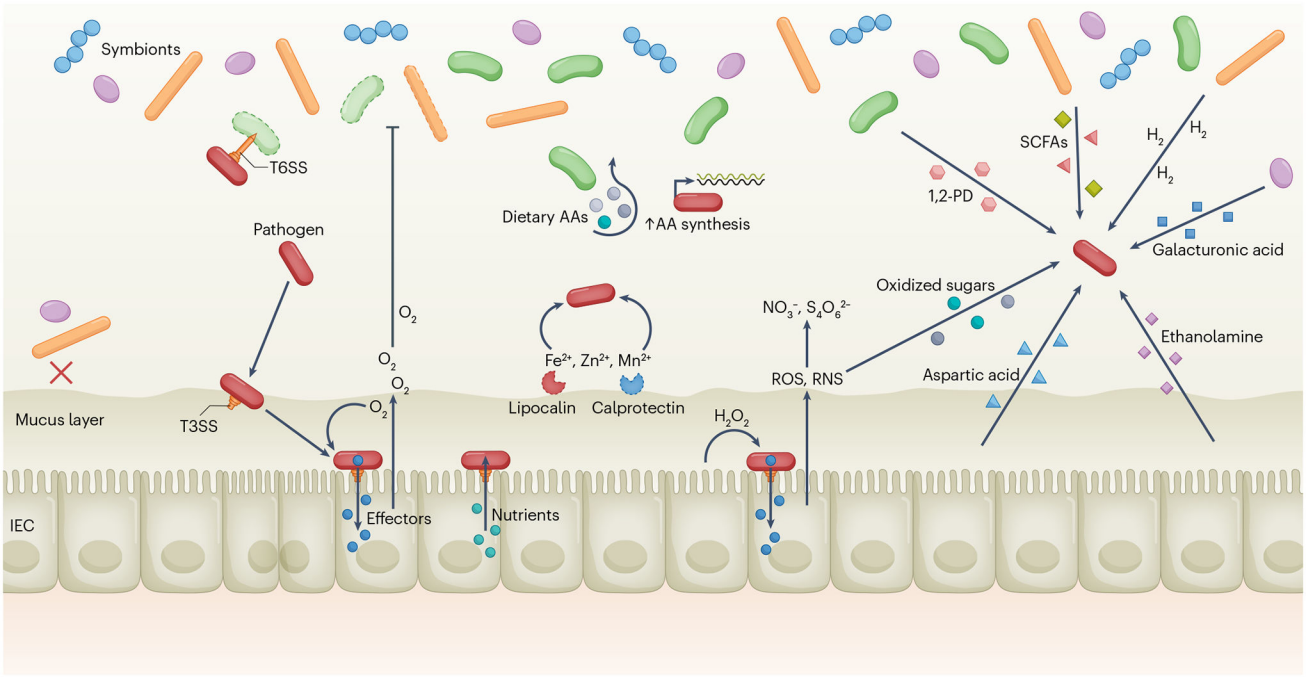

Fig. 3 |. Pathogen evasion of colonization resistance.

Upon entering the gut, some pathogens can use a type VI secretion system (T6SS) to directly kill competitors and open a niche (upper left). Most symbionts and pathobionts are excluded from the inner mucus layer (lower left). Certain pathogens, such as *Citrobacter rodentium*, utilize a type III secretion system (T3SS) to adhere to the epithelium and inject effectors into intestinal epithelial cells (IECs) to modify their physiology, including hyperproliferation of epithelial cells, which causes O_2 to be released and respired by the pathogen while killing oxygen-sensitive symbiotic bacteria. *C. rodentium* can also respire hydrogen peroxide (H_2O_2) produced by IECs during its early, T3SS-mediated access to the epithelium. Enteropathogenic *Escherichia coli* can also use its T3SS to obtain nutrients directly from the host cell cytoplasm. Dietary amino acids (AAs) are depleted by the microbiota, but pathogens, including *C. rodentium*, can synthesize AAs de novo to circumvent this nutrient deficiency (centre). *Salmonella enterica* subsp. *enterica* serovar Typhimurium has evolved resistant siderophores and higher-affinity transporters to overcome lipocalin 2-mediated and calprotectin-mediated metal (Fe^{2+}, Zn^{2+}, Mn^{2+}) sequestration. Injection of effectors by the T3SS also causes host inflammatory responses, releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS), which produce respiratory electron acceptors such as tetrathionate ($S_4O_6^{2-}$) and nitrate (NO_3^-) that can fuel pathogen growth. Oxidized sugars (for example, glucarate and galactarate) are also produced by RNS and used by pathogens. Aspartic acid is released during inflammation and can be utilized for energy production by pathogens. In turn, 1,2-propanediol (1,2-PD), propionic and butyric acids (short-chain fatty acids (SCFAs)), H_2 and galacturonic acid are produced by the microbiota and can be metabolized by certain pathogens, as can host-derived ethanolamine, if the appropriate electron acceptors are present.