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Control of Pathogens and Pathobionts by the Gut Microbiota

Nobuhiko Kamada,
Department of Pathology and Comprehensive Cancer Center, University of Michigan, Ann Arbor 48109, USA

Grace Y. Chen,
Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan, Ann Arbor 48109, USA

Naohiro Inohara, and
Department of Pathology, University of Michigan, Ann Arbor 48109, USA

Gabriel Núñez
Department of Pathology and Comprehensive Cancer Center, University of Michigan, Ann Arbor 48109, USA

Nobuhiko Kamada: nkamada@umich.edu; Grace Y. Chen: gchenry@umich.edu; Naohiro Inohara: ino@umich.edu; Gabriel Núñez: gabriel.nunez@umich.edu

Abstract

A dense resident microbial community in the gut, referred as the commensal microbiota, co-evolved with the host, and is essential for many host physiological processes that include enhancement of the intestinal epithelial barrier, development of the immune system, and nutrient acquisition. A major function of the microbiota is protection against colonization by pathogens and overgrowth of indigenous pathobionts that can result from the disruption of the healthy microbial community. The mechanisms that regulate the ability of the microbiota to restrain pathogen growth are complex and include competitive metabolic interactions, localization to intestinal niches, and induction of host immune responses. Pathogens, in turn, have evolved strategies to escape from commensal-mediated colonization resistance. Thus, the interplay between commensals and pathogens or indigenous pathobionts is critical for controlling infection and disease. Understanding pathogen-commensal interactions may lead to new therapeutic approaches against infectious diseases.

Structural composition of the gut microbiota

Vertebrates harbor a densely populated resident microbial community, which consists of bacteria, viruses, and fungi, particularly in mucosal organs, such as the oral cavity and the intestine. This internal microbial community residing within the host is referred as the microbiota[1]. In healthy individuals, Gram-negative Proteobacteria and Bacteroidetes, and Gram-positive Firmicutes, such as Clostridiales and Lactobacillales, represent the major phyla among intestinal eubacteria, whereas methanogens are the predominant intestinal

Correspondence to: Gabriel Núñez, gabriel.nunez@umich.edu.

archaea[2]. Many of these resident commensals are adapted to the intestinal environment and develop complex ecological networks with other bacteria to acquire nutrients. For example, Lactobacillus species and *Eubacterium dolichum* lack the ability to synthesize certain amino acids and therefore, acquire these critical molecules from their intestinal habitats [3, 4]. Methanogens obtain energy from hydrogen molecules, a waste product of other obligate anaerobes[2]. Consequently, host-microbial, microbial-microbial as well as microbial-environmental interactions dictate the distribution of individual commensals throughout the gastrointestinal tract.

A critical factor that defines the composition and distribution of the microbiota is the nutrient requirement of individual commensals (Figure 1). Microbes colonize mammalian hosts immediately after birth [5, 6]. In neonatal mice, the bacterial composition within the oral cavity and intestinal tract is similar and simply structured; however, after weaning as the diet changes from maternal milk to fiber-rich foods, the bacterial composition dramatically changes [5, 6]. The small intestine is rich in mono- and di-saccharides as well as amino acids, which support the growth of certain bacteria particularly Proteobacteria and Lactobacillales (Figure 1). In the distal small intestine including the terminal ileum, simple sugars are absorbed by host cells, so energy sources available for bacterial growth are significantly altered, resulting in changes in bacterial composition. Beyond the ileocecal valve, the vast majority of available carbohydrates are diet- (i.e., plant foods) or host-derived (e.g., mucin, cellular debris, etc.) complex carbohydrates (polysaccharides), which are indigestible by the host. Proteobacteria, such as *Escherichia coli*, are incapable of digesting polysaccharides, and therefore are unable to utilize complex carbohydrates as an energy source. In contrast, Bacteroides and Clostridiales harbor enzymes that can breakdown host-indigestible polysaccharides, including fibers and mucin, and use them as an energy source. Consequently, the abundance of proteobacteria and Lactobacillales is much lower in the colon, while Bacteroides and Clostridiales are dominant populations within the large intestine [7]. Notably, even among bacteria within the same phyla, the ability to scavenge polysaccharides is highly diverse[7], suggesting that the polysaccharide content of any diet can greatly affect the relative abundance of bacterial species within Bacteroidetes or Clostridiales. Thus, the distribution of nutrients within the intestine is a major driver of the microbial community structure in the gut. Many studies have shown differences in microbiota composition between healthy individuals and patients with intestinal diseases[8-10], which may reflect changes in the availability of host factors and nutrients in the disease state and/or be secondary to the inflammatory response. However, the diversity of microbiota among individuals cannot be simply explained by diet alone. For example, Bifidobacteria, which affect host responses to pathogens, colonize the gut of humans, but not SPF mice. Further ecological analysis of intra-commensal interactions and better characterization of the metabolic activities of individual bacteria are required to understand the diversity of bacteria within the intestine.

Protective effect of the microbiota against pathogen infection

Mounting evidence demonstrates that the gut microbiota play a crucial role in host resistance against invading pathogens within the intestine. Consequently, germ-free (GF) and antibiotic-treated mice are more susceptible to various enteric pathogens infection [11-16].

How resident microbes prevent pathogen colonization has been studied for many years, and the mechanisms involved largely fall into two categories : 1) Direct interactions between commensals and pathogens, such as competition for shared nutrients and niches, and 2) Commensal-mediated enhancement of host defense mechanisms.

Colonization resistance via direct commensal-pathogen interaction

Both pathogens and commensal bacteria require similar ecological niches to colonize and proliferate in the intestine, and mechanisms to out-compete each other have evolved. Commensal bacteria produce bacteriocins, proteinaceous toxins, that specifically inhibit members of the same or similar bacterial species [17]. For example, *E. coli* produces bacteriocin that directly inhibits the growth of the related pathogen enterohaemorrhagic *E. coli* (EHEC) [18](Figure 2). Commensals also prevent pathogen infection by altering host environmental conditions (e.g., pH) that are prohibitive for pathogen colonization[17]. A healthy vaginal flora reduces the pH in the vagina, thereby preventing the colonization of urinary tract pathogens whose optimal pH for growth is neutral [19]. Likewise, in the intestine, certain commensal bacteria generate short chain fatty acids (SFCAs), which can alter the local pH to inhibit the growth of certain intestinal pathogens [20, 21](Figure 2). Bifidobacterium blocks colonization of pathogenic *E. coli* through acidification of its environment[12]. Similarly, the pH is critical for *Bacillus cereus* growth and enterotoxin secretion[22].

An alternative strategy utilized by the indigenous microbial community is the preferential consumption of nutrients required for the growth of competing pathogenic bacteria. For example, commensal *E. coli* competes with EHEC for organic acids, amino acids, and other nutrients[23-26](Figure 2). By consuming common limited resources, the gut microbiota essentially causes the starvation of competing pathogens. Commensal bacteria, through production of specific metabolites, can also affect pathogen virulence to compromise their growth. The SCFA, butyrate, downregulates the expression of several virulence genes including those encoding the Type 3 secretion system (T3SS) proteins in *Salmonella enteria* Serovar Enteritidis and Typhimurium [27](Figure 2). Moreover, host mucin-derived fucose, which is generated by fucosidase-bearing commensal bacteria such as *Bacteroides thetaiotaomicron*, modulates the expression of the virulence factor *ler*, a master regulator of the locus of enterocyte effacement (LEE) genes, in EHEC[28](Figure 2). In addition to direct suppression of virulence genes by commensal metabolites, commensals also inhibit pathogen virulence by altering conditions required for virulence activity. For example, ambient oxygen tensions are required for competent secretion of virulence factors by Shigella flexneri [29], and therefore, the consumption of residual oxygen by commensal facultative anaerobes, such as Enterobacteriaceae, may lead to incomplete virulence expression in intestinal lumen (Figure 2).

Indirect mechanisms of colonization resistance by commensals via activation of host immunity

Commensal bacteria also prevent pathogen colonization and infection indirectly by enhancing host defense mechanisms such as functionally promoting mucosal barrier function and enhancing either innate immune responses. The first line of defense against any

pathogen invasion is the epithelial barrier. The promotion of epithelial barrier function by commensal bacteria is supported primarily by indirect evidence in which studies have demonstrated that germ free mice and mice deficient in proteins involved in microbial recognition such as Nod2 and the TLR signaling adaptor MyD88 have impaired production of antimicrobial peptides, particularly by Paneth cells of the small intestine [30, 31]. Consequently, MyD88-deficient or Paneth-cell deficient mice have impaired epithelial barrier function and increased bacterial translocation of pathogenic bacteria [31], associated with their inability to produce specific antimicrobial peptides (Figure 2). In addition, antimicrobial peptides not only limit enteric infection by their inherent bactericidal activity, but also restrict bacterial colonization. Specifically, mice deficient in MyD88 or RegIIIγ have higher mucosal bacterial loads compared to wildtype littermates with larger numbers of bacteria in direct contact with the surface epithelium within the small intestine [32]. Production of RegIIIγ is also upregulated by IL-22, which is induced in group 3 innate lymphoid cells, Th17 cells as well as a certain subset of DCs[33-35] by the gut microbiota. IL-22-mediated RegIIIγ production by intestinal epithelium is protective against enteric infection by *Citrobacter rodentium*, a mouse bacterium that models infection by enteropathogenic *E. coli* (EPEC) and EHEC[33, 36, 37] (Figure 2). In addition to antimicrobial peptide production, bacterial signaling likely through MyD88 also promotes barrier function through the production of secretory IgA that is released by intestinal epithelial cells and functions to bind to microbial antigens, neutralize pathogen activity and prevent infection [38-41]. However, since both antimicrobial peptides and IgA are also capable of shaping the gut microbiota [32, 42-44], it remains unclear whether the actual composition of the gut microbiota regulated by these molecules is the main determinant for pathogen resistance.

The gut microbiota, not only promotes mucosal barrier function, but also enhances host immunity to defend against enteric infection. IL-1β is a cytokine typically produced during active infection that is critical for neutrophil recruitment and pathogen eradication. The microbiota have an essential role in the production of homeostatic levels of pro-IL-1β in resident intestinal macrophages that is MyD88-dependent, thereby priming macrophages to respond rapidly to enteric infection by conversion of pro-IL-1β to mature active IL-1β[45] (Figure 2). The gut microbiota can also enhance host immunity through MyD88-independent mechanisms. Notably, colonization of GF mice by commensal bacteria induces development of Th17 cells in the intestine, which is important for protection against *C. rodentium* infection and is MyD88-, TRIF-, and Rip2-independent[46].

Disruption of the commensal microbial community leads to outgrowth of pathogens and pathobionts

A consequence of disruption of commensal-mediated colonization resistance is that susceptibility to enteric infection will be markedly increased. *Salmonella enterica* Serovar Typhimurium (*Salmonella*) colonize poorly the mouse intestine in the presence of commensal microbiota [13]. However, it can proliferate and induce inflammation if the resident bacterial community is disrupted by treatment with antibiotics or if recipient mice have low-complexity microbiota [13, 47]. An altered bacterial community structure also may facilitate the overgrowth of potentially harmful subsets of indigenous bacteria within the intestine. In a mouse model, virulent *E. coli*, whose growth is normally suppressed by

commensals, accumulates after antibiotic treatment and can disseminate systemically when the intestinal epithelial barrier is breached by dextran sulphate sodium (DSS), thereby inducing lethal inflammasome activation [48]. Similarly, *Clostridium difficile*, a leading cause of healthcare-associated infectious diarrhea and colitis, [16] typically present at low abundance in the intestine of adult healthy individuals, but with disruption of indigenous bacteria after treatment with broad-spectrum antibiotics as in hospitalized patients, *C. difficile* can significantly increase in abundance followed by severe intestinal inflammation [16]. Like humans, in a mouse model of *C. difficile* infection, *C. difficile* can not colonize and induce inflammation in conventional mice, while antibiotic treatment increases the incidence of *C. difficile* infection. *C. difficile* does not invade the host systemically, but causes marked epithelial damage through the production of the toxins TcdA and TcdB[16, 49](Figure 2). The toxin-mediated epithelial damage can cause systemic dissemination of commensal bacteria which induce lethal septic shock [50](Figure 2). Vancomycin-resistant enterococcus (VRE), which can cause sepsis in immunocompromised patients, is also associated with antibiotic treatment[51]. Commensal microbiota-mediated induction of antimicrobial peptide RegIIIγ is particularly important for bacterial killing (Figure 2). A role for commensals to regulate RegIIIγ production is supported by the observation that bacterial signaling through MyD88, and more specifically, administration of the bacterial products lipopolysaccharide (LPS) or bacterial flagellin, upregulate RegIIIγ and increase VRE eradication[52, 53]. However, a recent study suggested that specific bacterial populations within the colon facilitate VRE clearance that occurred independently of host innate immune pathways including MyD88[54]. Although the mechanism is currently unknown, it may be related to a direct mechanism such as through competition for limited nutrients. It is also possible that both commensal-dependent indirect and direct mechanisms are essential for VRE clearance with antimicrobial production being the primary mechanism within the small intestine, where Paneth cells predominate, and direct inhibition by specific bacteria populations being a primary mechanism within the colon.

The gut microbiota as promoters of enteric infection

Although the commensal microbiota play crucial roles in resistance against enteric pathogen infections, certain pathogens can utilize the microbiota to facilitate their infection. For example, by-products derived from commensal bacteria such as bile salts promote the germination of *C. difficile* spores [55]. In addition to bacterial pathogens, certain enteric viruses can also take advantage of the gut microbiota to promote their replication and transmission. Offspring transmission of mouse mammary tumor virus (MMTV) and replication of poliovirus are significantly reduced in antibiotic-treated or GF mice [56, 57]. These enteric viruses directly bind and modify the stimulatory activity of LPS that is secreted by commensal bacteria. MMTV-bound LPS induces the production of the anti-inflammatory cytokine IL-10, which leads to depressed anti-viral immune response by the host and persistent infection of MMTV[56]. Thus, the microbiota can also potentiate pathogenic infection.

Strategies by which pathogens overcome the colonization resistance by the microbiota

As described above, the microbiota have developed multiple mechanisms to resist pathogen colonization. However, pathogens have evolved strategies to escape these mechanisms. For

example, to counteract nutritional competition by commensals, some pathogens have developed alternative nutrient utilization. The pathogen EHEC can utilize galactose, hexuronates, mannose, and ribose as a carbon source for its growth, while commensal *E. coli* cannot catabolize these sugars[25, 58](Figure 3). In addition to altered carbohydrate metabolism, EHEC harbors the EA utilization *eut* operon in its genome and is capable of utilizing pathogen-specific nutrients in the intestine, such as ethanolamine (EA), which is released into the intestine during epithelial cell turnover[25, 59, 60](Figure 3). In contrast, the *eut* operon is absent in the genome of commensal members of non-pathogenic *E. coli* thereby prohibiting them from using EA as a nutrient[60]. Although *C. rodentium* exhibits a similar carbohydrate metabolic profile as the commensal *E. coli*[61], *C. rodentium* resides in a distinct niche during replication to escape from the nutritional competition by commensals. Specifically, *C. rodentium* expresses intimin, a LEE-encoded adhesion molecule, that allows the bacterium to localize to the intestinal epithelial surface where commensal microbiota do not normally reside [61](Figure 3). In addition, some pathogens more efficiently utilize common resources. Iron is an essential resource for the growth of bacteria, and many bacteria produce iron-chelating small molecules, known as siderophores, to acquire ferric iron[62]. Host cells secrete lipocalin 2 (Lcn2), which blocks the 2,3 dihydroxy benzoate-based siderophore enterobactin (Ent) in *E. coli*, thereby preventing iron acquisition and proliferation of commensal *E. coli*. However, some enteric pathogens, such as *Salmonella*, pathogenic *E. coli* and *Klebsiella pneumoniae*, display modified forms of Ent, referred to as salmochelins (also: vary the positioning of hydroxyl groups to the 3,4 positions or use non-catecholate siderophores)[63]. Lcn2 does not inhibit salmochelin-mediated iron uptake, leading to a growth advantage of the pathogens over the commensals (Figure 3). Thus, enteric pathogens use different strategies to overcome colonization resistance by utilizing pathogen-specific nutritional resources and/or localizing to distinct niches separate from competing commensals.

Another important strategy utilized by pathogens to acquire a growth advantage over commensals is to promote host inflammation that impedes commensal survival. Most virulent microorganisms express virulence factors that cause intestinal inflammation. Inflammation caused by toxin- or pathogen-mediated diarrhea significantly decreases the number of commensal microbiota in the intestine, and, in turn, increases the chance of colonization/proliferation of incoming pathogens because of less competition[64]. DSS-induced intestinal inflammation markedly increases the proliferation of *C. rodentium* in the intestine; however, specific virulence factors are required for optimal colonization and proliferation as the *ler* mutant *C. rodentium*, which lacks expression of all LEE-encoded virulence genes is avirulent and fails to acquire a survival advantage from DSS-induced inflammation [61](Figure 3). Recent evidence also demonstrates that *Salmonella* benefits from intestinal inflammation triggered by the pathogen itself. In the intestine, commensals produce abundant hydrogen sulfide (H_2S), and the epithelium converts H_2S to thiosulphate ($S_2O_3^{2-}$) to avoid the H_2S-mediated toxic effects on host cells. Infection by *Salmonella* results in the recruitment of neutrophils that produce reactive oxygen species, resulting in the conversion of $S_2O_3^{2-}$ into tetrathionate ($S_4O_6^{2-}$)[65, 66]. *Salmonella*, but not commensals, contains a gene operon, *ttrSR ttrBCA*, which allows the utilization of the $S_4O_6^{2-}$. This tetrathionate respiration provides a growth advantage to *Salmonella* over commensal

microbes within an inflammatory environment[67](Figure 3). Moreover, tetrathionate supports anaerobic growth of *Salmonella* on ethanolamine[68](Figure 3). Like *Salmonella*, pathogenic *E. coli* including EPEC, EHEC, and *C. rodentium*, also may benefit from intestinal inflammation. In the inflamed intestine, intestinal epithelium and recruited neutrophils and macrophages that express inducible nitric oxide synthetase (iNOS), upregulate the production of nitrate (NO_3^-) [69, 70]. Obligate anaerobes, such as Bacteroidetes or Firmicutes that are the vast majority of healthy microbial community in the gut, cannot utilize nitrate as an electron acceptor [71]. Rather, nitrate reductase-harboring facultive anaerobes, such as *E. coli*, can utilize NO_3^- to generate energy for growth, leading to a growth advantage over obligate anaerobes in the inflamed intestine[71]. Although this mechanism of *E. coli* overgrowth within the inflamed gut involves commensal-commensal competition, pathogenic *E. coli* strains, which bear nitrate reductase genes such as *narZ* in their genome, may use a similar mechanism to acquire a growth advantage over the competitive commensal community (Figure 3). Furthermore, the host inflammatory environment can act as a signal to trigger and enhance virulence factor expression. The human opportunistic pathogen *Pseudomonous aeruginosa* can bind to interferon-γ through its outer membrane protein OprF, thereby expressing quorum-sensing dependent virulence determinant type I *P. aeruginosa* (PA-I) lectin[72]. Thus, pathogens can take advantage of the inflammatory response to promote their growth in host tissues.

Microbiota-targeted therapies for disease treatment

The notion of harnessing the gut microbiota to prevent or fight microbial infection is not new. However, the lack of knowledge about the mechanisms by which commensals regulate colonization resistance against pathogens has hampered progress in the area. Recent mechanistic insight into pathogen-commensal interactions suggest ways to promote the eradication of pathogens. Diarrheagenic *Escherichia coli* strains, including EPEC and EHEC, cause substantial morbidity and mortality worldwide each year [73]. In the *C. rodentium* model, the capacity to metabolize simple sugars regulates the ability of commensals like *E. coli* to out-compete the pathogen for energy resources. Thus, administration of commensals metabolically related to EPEC or EHEC or treatment with prebiotics to boost the growth of natural “competitors” may prove effective in the treatment of these enteric diseases. Eradication of EPEC and EHEC by commensals could be further boosted by targeting pathogen LEE virulence at the early phase of infection[61]. This approach may be effective in eradicating not only enterovirulent *E. coli* infection, but also other intestinal infections by pathobionts such as *C. difficile* and VRE. Overgrowth of *C. difficile* and VRE are leading causes of healthcare-associated infectious diarrhea and colitis [16, 51]. There is also evidence that specific bacterial populations within the gut promote *C. difficile* and VRE clearance[54, 74]. Although the mechanism is currently unknown, it may be also mediated via a direct mechanism such as through competition between VRE and certain commensals for limited nutrients. Notably, intestinal microbiota transplantation, the infusion of stool microorganisms from a healthy donor, has proved effective in treating recurrent *C. difficile* infection that is refractory to antibiotic therapy [74-76]. However, the variability of the donor commensal populations and the potential presence of hazardous microbes may hamper the use of microbiota transplantation in the

clinic. Thus, identification and characterization of the gut commensals that restrain the growth of *C. difficile* and VRE may lead to the use of single species or defined combinations of protective commensals to treat infections. In addition, understanding the metabolic pathways that are used by gut commensals to prevent the growth of *C. difficile* and VRE may lead to the development of genetically engineered commensal species with enhanced capacity to limit pathogen colonization.

Conclusion

Recent studies are providing new insight into the mechanisms by which the microbiota regulates the colonization and eradication of pathogens. Particularly revealing have been studies that indicate that the ability of commensals to restrain pathogen growth is dictated by metabolic pathways that control the competition for limited nutrients in the intestine. Furthermore, inflammatory responses have profound effects on the growth of pathogens and certain commensal species. However, the relative contributions of each metabolic pathway and the commensal species involved remain poorly understood. In addition, little is known about how the inflammatory responses affect interactions between pathogens and commensals. There is a delicate balance in microbiota populations in the gut and disruption in this balance leads to dysbiosis and overgrowth of pathobionts leading to pathologic immune responses and disease. The identification and characterization of natural "competitors" that suppress the growth of pathogens and pathobionts may lead to the development of rational approaches to manage intestinal disease. There is also a clear role for host immunity in controlling microbiota populations. However, recent studies have challenged a critical role of innate recognition receptors in determining the composition of the gut microbiota[77]. Further studies are needed to clarify the mechanism by which the host regulates the microbiota.

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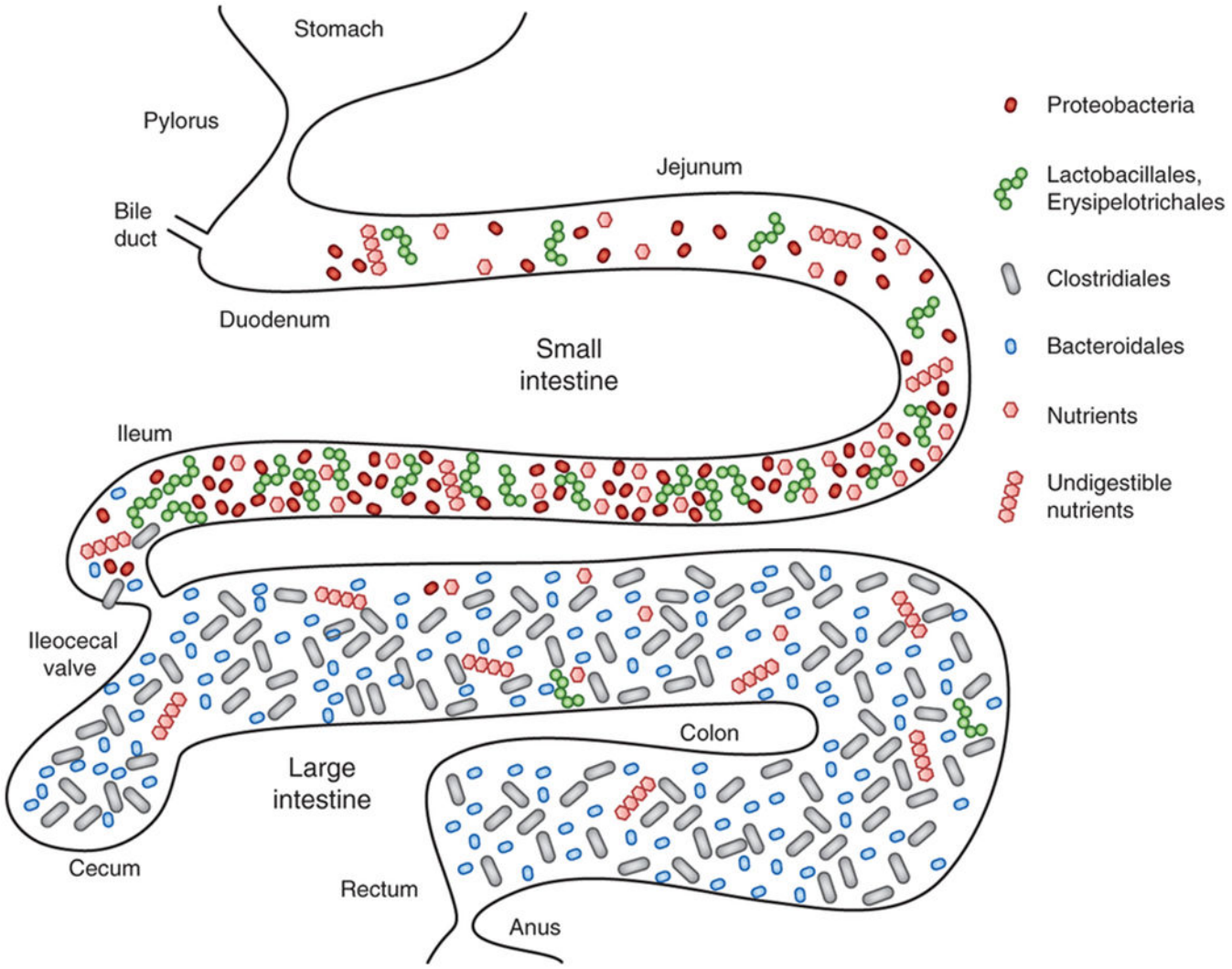

Figure 1. Localization of dominant bacterial groups within the intestine

The small intestine is rich in nutrients utilized by both the host and microbe for growth. Proteobacteria (mainly enterobacteria), Lactobacillales and Erysipelotrichales (especially Turicibacter) are dominant in the small intestine. In contrast, the large intestine is poor in such nutrients and therefore harbor much fewer numbers of these bacteria, while Bacteroidetes and Clostridia which can utilize host indigestible fibers as energy sources are enriched.

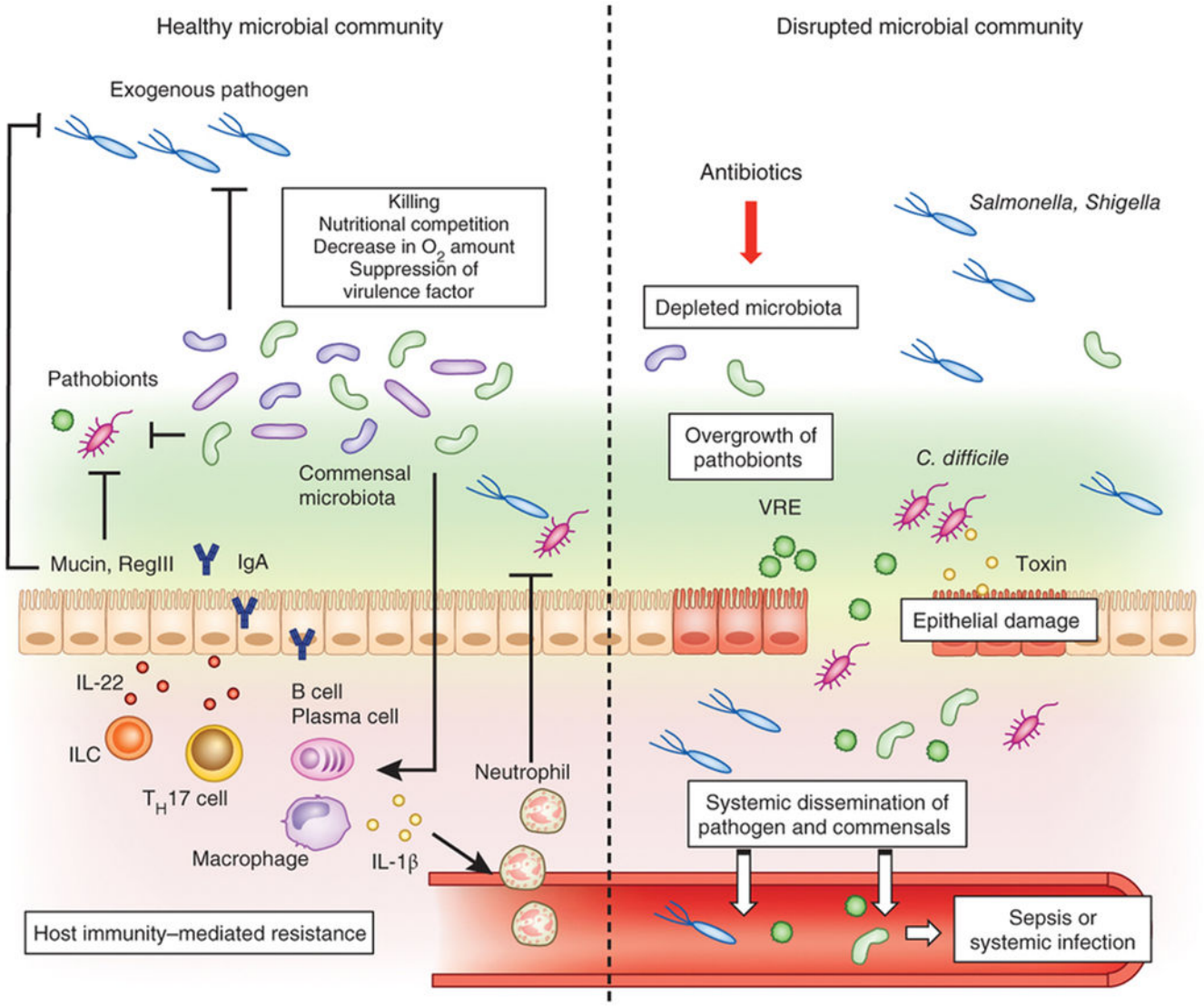

Figure 2. Commensal microbiota prevents colonization by exogenous pathogens and pathobionts

In the healthy gut, the resident bacteria occupy intestinal colonization niches. Commensal microbiota suppresses the proliferation and colonization of incoming enteric pathogens as well as opportunistic pathobionts through multiple mechanisms. Microbiota produces bacteriocins and short-chain fatty acids, which directly inhibit the growth of pathogen and pathobiont. Commensals can also modify virulence factor expression in pathogens by consuming residual oxygen or suppressing growth by their metabolites. Commensal microbiota facilitates host barrier function through up-regulation of the mucus layer, induction of antimicrobial molecules, such as RegIIIβ and γ, and regulating secretion of IgA. Commensal bacteria also prime intestinal macrophages by upregulating pro-IL-1β. Pathogen infection results in the conversion of pro-IL-1β into the enzymatically active mature form of IL-1β, which promotes neutrophil recruitment and pathogen eradication. Antibiotic treatment or other environmental factors that disrupt the commensal microbial community results in diminished colonization resistance against pathogens (e.g. *Salmonella, Shigella*) and allow the outgrowth of indigenous pathobionts (e.g. *Clostridium difficile*, vancomycin-

resistant *Enterococcus*) that have the potential to disseminate systemically and induce septic shock and/or systemic organ infection.

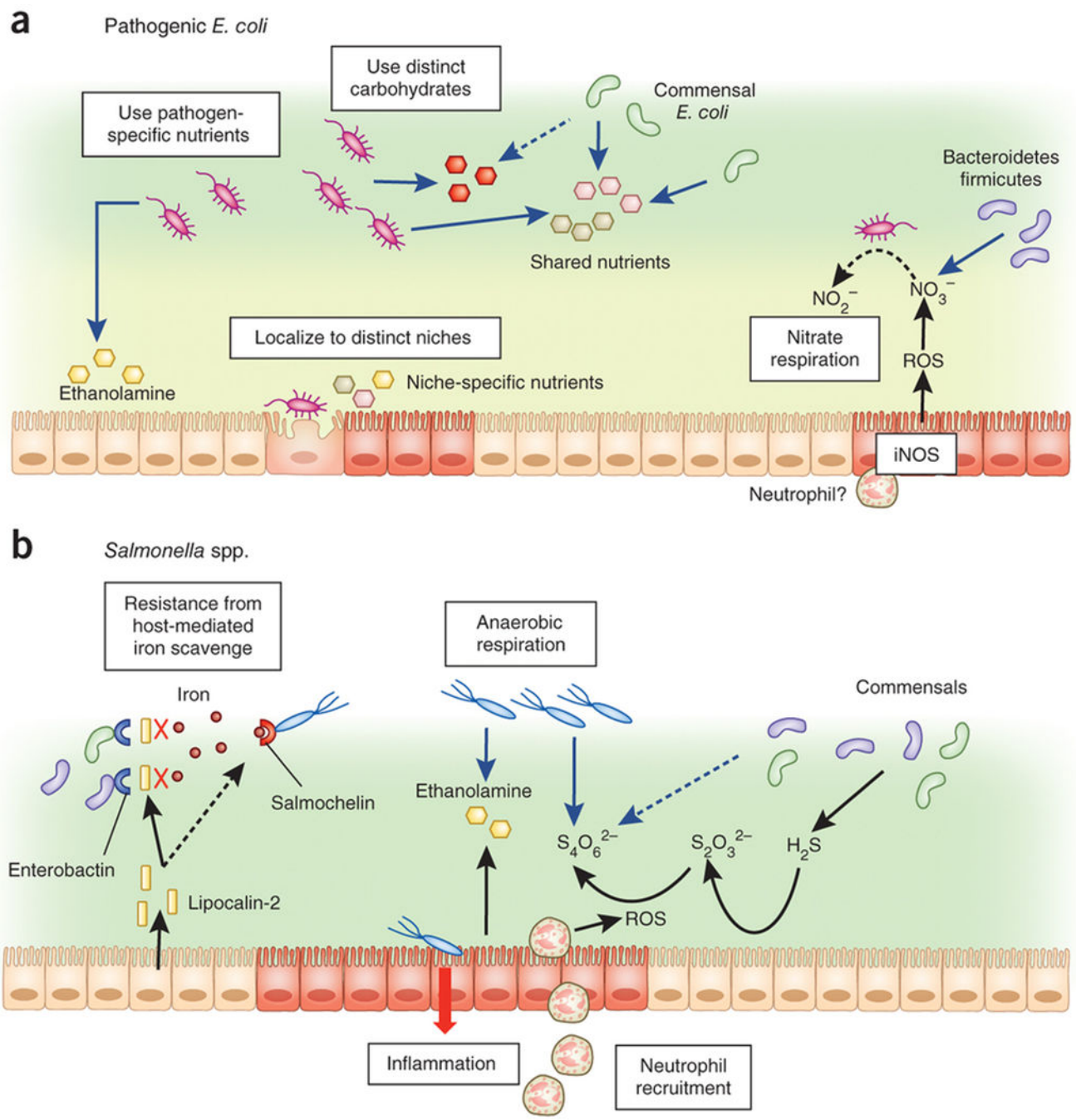

Figure 3. Pathogen overcome commensal-mediated resistance through multiple strategies

a| Pathogenic *Escherichia coli*: Pathogenic *E. coli* is capable of utilizing carbohydrates and other resources, such as ethanolamine distinct from that scavenged by commensals. Pathogenic *E. coli* can also localize to the intestinal epithelial surface that is devoid of commensal microbiota through expression of adhesion molecules, such as intimin. Pathogen-induced gut inflammation confers a growth advantage to the pathogen through the generation of molecules such as inducible nitric oxide synthase (iNOS) expression by host innate immune cells leading to the release of nitrate (NO_3-) that can be utilized as an

electron acceptor by E. coli to generate energy through nitrate respiration. Commensal obligate anaerobes, such as Bacteroidetes or Firmicutes lack this ability.

b| *Salmonella* spp.: Lipocalin-2 derived from host cells block commensal bacterial iron uptake by binding to the bacterial siderophore, enterobactin. Salmonella, however, has a distinct siderophore, salmochelin, for iron uptake, which is not blocked by lipocalin-2. *Salmonella*-induced gut inflammation also promotes migration of neutrophils that produce reactive oxygen species (ROS), which facilitate conversion of thiosulphate ($S_2O_3^{2-}$), generated by commensal bacteria, into tetrathionate ($S_4O_6^{2-}$). *Salmonella*, but not commensals, is capable of utilizing tetrathionate as an electron acceptor for anaerobic respiration.