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No Vacancy: How Beneficial Microbes Cooperate with Immunity To Provide Colonization Resistance to Pathogens

Martina Sassone-Corsi and Manuela Raffatellu

The mammalian intestine harbors a community of trillions of microbes, collectively known as the gut microbiota, which coevolved with the host in a mutually beneficial relationship. Among the numerous gut microbial species, certain commensal bacteria are known to provide health benefits to the host when administered in adequate amounts and, as such, are labeled “probiotics.” We review some of the mechanisms by which probiotics and other beneficial commensals provide colonization resistance to pathogens. The battle for similar nutrients and the bacterial secretion of antimicrobials provide a direct means of competition between beneficial and harmful microbes. Beneficial microbes can also indirectly diminish pathogen colonization by stimulating the development of innate and adaptive immunity, as well as the function of the mucosal barrier. Altogether, we gather and present evidence that beneficial microbes cooperate with host immunity in an effort to shut out pathogens. The Journal of Immunology, 2015, 194: 4081–4087.

The mammalian gastrointestinal (GI) tract is home to a community of trillions of microorganisms commonly known as the microbiota. The long coexistence of the microbiota and the host intestinal mucosa has established a mutual beneficial relationship. On one hand, the microbiota protect the host from infection with pathogenic microorganisms and contributes to both nutrient metabolism and to the development and function of the GI immune system; on the other hand, the host provides nutrient-rich niches to ensure the survival of its resident bacterial communities (1).

In the past decade, studies in germ-free (GF) mice and the advent of metagenomics have contributed tremendously to the elucidation of the complexity of the intestinal microbiota and its contribution to health and disease (2, 3). In healthy subjects, ≥1000 bacterial species contribute to intestinal homeostasis, with Firmicutes and Bacteroidetes representing the most common intestinal phyla, followed by Actinobacteria and Proteobacteria (4, 5). Within these phyla, some bacterial species, including Gram-positive *Lactobacillus* spp. (phylum Firmicutes) and *Bifidobacterium* spp. (phylum Actinobacteria), as well as certain Gram-negative bacteria, such as *Escherichia coli* Nissle 1917 (hereafter referred to as *E. coli* Nissle; phylum Proteobacteria), were shown to benefit the host by blocking harmful microorganisms; for this reason, they are referred to as “probiotics.”

The first observation that certain commensal bacteria have beneficial properties dates back to 1907, when Elie Mechnikoff proposed that lactic acid–producing strains are beneficial to the host by inhibiting the growth of other species within the colon (6). Today, probiotics are defined by the World Health Organization as “live bacterial species that confer a health benefit when administered in adequate amounts” (7). In addition to the few known probiotics, the microbiota, in general, have beneficial effects on the host (e.g., the absence of the microbiota renders GF mice more susceptible to infection in comparison with conventionally raised mice) (1, 8). Moreover, the use of antibiotics has been shown to enhance intestinal colonization of enteric pathogens; for example, altering the composition of the gut flora can increase susceptibility to infection with pathogens, such as *Salmonella enterica* serovar Typhimurium (*Salmonella Typhimurium*) and *Clostridium difficile* (9–12).

More recently, several studies have begun to elucidate the molecular mechanisms behind the beneficial role of commensal and probiotic strains. It is becoming clear that beneficial bacteria provide colonization resistance to pathogens by two major mechanisms (13, 14). The first mechanism involves the direct competition between certain commensals and pathogens for nutrients or niche establishment. The second mechanism includes indirect effects on pathogen colonization, deriving from the stimulation of the innate and adaptive immune system by commensal bacteria. In this review, we summarize some of the mechanisms by which commensal bacteria, including certain probiotic species, contribute to colonization resistance against pathogens, both by direct competition with pathogenic bacteria and by stimulation of host immunity.

**Direct competition with pathogens**

One of the mechanisms by which commensal and probiotic bacteria provide colonization resistance to pathogens is by directly competing for the same niche. Some beneficial...
microbes acquire similar nutrients as pathogens, often more efficiently, thus hindering the replication and colonization of infectious agents. In addition, some microbes produce antimicrobial proteins that can target pathogens. Below, we discuss these two aspects of direct competition between beneficial and harmful bacteria (Fig. 1).

**Competition for nutrients.** Numerous studies on the battle between commensals and pathogens belonging to the Enterobacteriaceae family put forward the idea that competition for nutrients in the gut appears to occur primarily between metabolically related bacteria. For example, in GF mice, certain commensal *E. coli* strains reduce cecal colonization of the enteric pathogen enterohemorrhagic *E. coli* (EHEC), a leading cause of bloody diarrhea in humans, by competing for the amino acid proline (15). Similarly, precolonization of streptomycin-treated mice with specific human commensal *E. coli* strains prevented the growth of EHEC (16). This latter effect was later reported to be linked to the capacity of the human commensal *E. coli* strain HS and the probiotic strain *E. coli* Nissle (discussed below) to occupy a unique nutritional niche in the mouse gut. Indeed, use of multiple sugar molecules (six by *E. coli* HS and seven by *E. coli* Nissle) limited the nutrient availability to pathogenic strains of *E. coli*, thereby impeding their successful colonization of the gut (17).

It is worth noting that the competition for nutrients is not limited to bacteria within the same species. For instance, a commensal strain of *E. coli* was able to delay the intestinal colonization and translocation of the pathogen *Salmonella Typhimurium* in GF mice, possibly as a result of competition for nutrients (18). More recently, it was shown that the intestinal microbiota provide colonization resistance to infection with *Citrobacter rodentium*, a mouse pathogen used to model infection with diarrheagenic *E. coli* strains, including enteropathogenic *E. coli* and EHEC, by competing for similar carbohydrates (19). Similarly, consumption of fucose during infection supports the growth of the microbiota and protects mice from infection with *C. rodentium* (20).

Because competition for nutrients is one of the mechanisms by which commensals provide colonization resistance, it is not surprising that certain pathogens have evolved to evade this mechanism. This is the case, for instance, for certain pathogenic *E. coli* strains that can use sugars that are not used by commensal *E. coli* (21). Moreover, pathogens like EHEC, *Salmonella Typhimurium*, and *C. difficile* can catabolize sugars liberated by the microbiota (22, 23). Additionally, the types of nutrient sources available to both commensals and pathogens can vary if the intestinal environment is altered (e.g., because of inflammation or antibiotic treatment) (12, 22, 24). In such conditions, changes in nutrient type and availability enhance the growth of specific strains capable of

**FIGURE 1.** Direct mechanisms of colonization resistance against enteropathogens. The microbiota provide a barrier against incoming enteric pathogens via multiple mechanisms. (1) Adhesion exclusion: certain commensals reduce pathogen adherence to the intestinal mucosa. (2) Carbon source limitation: human commensal *E. coli* strain HS and probiotic strain *E. coli* Nissle both metabolize multiple sugar molecules, which limits the availability of nutrients in the gut to certain pathogens. (3) Micronutrient limitation: the probiotic strain *E. coli* Nissle can uptake iron via several mechanisms, limiting its availability to pathogens, such as *Salmonella Typhimurium*. (4) Secretion of antimicrobials: commensals act against pathogens by limiting nutrients, as well as by the production of antimicrobial compounds, such as bacteriocins and microcins. (5) Direct delivery of toxins: although not yet demonstrated in vivo, commensals can express T6SSs and contact-dependent inhibition systems, means by which to deliver growth-inhibiting toxins to close competitors. (6) Circumventing colonization resistance: pathogens use a variety of virulence factors to colonize the host and cause disease.
exploiting the new nutrient sources (e.g., the pathogen *Salmonella Typhimurium*, but not the microbiota, can use ethanolamine and fructose-asparagine in the inflamed intestine) (25). Similarly, antibiotic treatment of mice induces substantial changes in the gut metabolome, resulting in an increase in specific carbon sources that support the germination and growth of *C. difficile* (12). Taken together, these observations indicate that commensal bacteria are best equipped to provide colonization resistance to pathogens in the healthy, unperturbed intestine. Nevertheless, it may be feasible to design probiotics by engineering commensal strains to better compete with pathogens for nutrients in more hostile environments, like the inflamed gut. Of note, there is at least one example of an *E. coli* strain that is able to compete with pathogenic Enterobacteriaceae in such conditions: the widely studied and used probiotic *E. coli* Nissle (26).

*E. coli* Nissle was isolated in 1917 by Dr. Alfred Nissle from the stool sample of a soldier who did not develop diarrhea during an outbreak of shigellosis (27). Today, *E. coli* Nissle is the active component of a probiotic preparation used for the treatment of both infectious diarrheal diseases and inflammatory bowel disease (26). Although the mechanisms by which *E. coli* Nissle exerts its beneficial effects are not completely understood, new pieces of evidence suggest that this probiotic strain competes with related bacteria and modulates the immune system (reviewed in Ref. 28).

*E. coli* Nissle possesses multiple features that might contribute to its ability to colonize the intestine, including Curli, Type 1, and F1C fimbriae, which increase its adherence to the mucosa and can prevent the adhesion of other pathogens (29). In addition, the *E. coli* Nissle genome encodes several redundant mechanisms that might contribute to its fitness, one of which is a large number of iron-acquisition systems, including genes for the synthesis and uptake of small iron chelators known as siderophores (30–32). Such systems allow for growth in environments where the essential metal ion iron is limited, including the intestinal lumen during inflammation (33). As reported by our laboratory in 2013, the presence of multiple iron-acquisition systems allows *E. coli* Nissle to successfully compete with the pathogen *Salmonella Typhimurium* for colonization of the inflamed gut (34). Because other commensal *E. coli* strains were unable to reduce *Salmonella* Typhimurium gut colonization (34, 35), our work suggests that *E. coli* Nissle exhibits the uncommon ability to compete with *Salmonella Typhimurium*, and possibly other enteric pathogens, for iron. Nevertheless, it is feasible that other *E. coli* strains that colonize the gut but cause extra-intestinal infections, such as those that cause urinary tract infections, may also compete with *Salmonella Typhimurium* and/or with other bacteria via similar mechanisms. Overall, *E. coli* Nissle is a prime example of how a commensal strain can provide colonization resistance against pathogens by better competing for the limited nutritional resources available in the inflamed intestine.

**Production of antimicrobial peptides and toxins.** Another means of direct competition between commensals and pathogens within the gut involves the secretion of toxins and antimicrobial peptides. Although originally described as a virulence trait of pathogens to kill their commensal competitors (36), new evidence suggests that the secretion of antibacterial toxins via phage-like machinery, known as a “type VI secretion system” (T6SS), is also used by commensals to attack competitors vying for the same ecological niche (37). To this end, it was postulated that the abundance of Bacteroidetes in the intestine could be attributed to their T6SSs (38).

Other mechanisms used by bacteria to deliver toxins to their competitors [e.g., the contact-dependent growth inhibition system (39)] also could promote inter- and intraspecies competition, as well as colonization resistance to pathogens. Moreover, some commensal Enterobacteriaceae, including the probiotic *E. coli* Nissle, secrete small antimicrobial peptides called bacteriocins or microcins, which specifically target and kill related competitors, including pathogenic organisms (40, 41). Some microcins target competitors expressing the same nutrient receptors as the microcin producers and are internalized by hijacking these transporters via a so-called “Trojan horse” mechanism (41, 42). Other probiotic strains, such as *Bifidobacterium* spp., also secrete bacteriocins, which can exhibit either a narrow or broad spectrum of activity (43). Because most of the evidence for the activity of bacteriocins and toxins derives from in vitro studies, future studies should address the role of antimicrobial peptides and toxins secreted by commensal and probiotic bacteria to compete with pathogens in the host. Moreover, because most studies on human commensals and probiotics have analyzed their effects on the murine gut microbiota, their relevance in the human host is largely unknown. To this end, the broader use of humanized gnotobiotic mice [i.e., gnotobiotic mice colonized with human microbiota (3)] could begin to unravel the mechanisms by which human commensals and probiotics compete with the human microbiota.

**Indirect effects against pathogens**

Studies of GF mice unequivocally showed the importance of the microbiota for the development of a normal gut mucosa and GALT (44). Thus, it follows that the ability of the microbiota and of certain probiotics to provide colonization resistance to pathogens is also mediated by their enhancement of the gut mucosal barrier and of the innate and adaptive immune systems. Examples of such mechanisms are described in detail below (Fig. 2).

**Enhancement of epithelial barrier function.** An important first step for gut colonization by pathogens is the adherence to mucosal surfaces. However, this step is hindered by the thick mucus layer that covers the intestinal epithelium, providing a first layer of defense against bacterial colonization (45). The optimal development of the mucus layer is dependent on the microbiota; indeed, GF mice develop a much thinner epithelial mucus layer in comparison with conventionally raised mice. In line with this, the administration of bacterial components, such as LPS or peptidoglycan, to GF mice restores their mucus layer formation and reduces their susceptibility to bacterial infections (46). Of particular importance is the highly glycosylated mucin protein MUC2, which is densely packed and insoluble in the inner mucus layer but loose and soluble in the outer layer, thereby providing a barrier to the colonization and translocation of both commensal and pathogenic bacteria (47, 48).

Although the development of the mucosal barrier primarily results from the generally cooperative interactions between host and microbiota, some specific bacterial species likely contribute to this process. For instance, certain commensal...
bacteria can enhance the epithelial barrier function through the production of specific metabolites. One example is *Bifidobacterium longum* subspecies *Infantis* [a probiotic bacterium that constitutes up to 90% of the microbiota of healthy infants (49)], which secretes peptides that normalize intestinal permeability and reduce intestinal pathology in a mouse model of colitis (50). Additionally, production of the short-chain fatty acid (SCFA) acetate by *Bifidobacterium* improved intestinal defenses mediated by epithelial cells, as well as induced protection against lethal infection with EHEC (51).

Tissue culture studies also showed that certain probiotics modulate the immune response through a direct interaction with intestinal epithelial cells. One such example is *E. coli* Nissle, whose K5 capsule was shown to stimulate the production of chemokines from Caco-2 cells and from ex vivo mouse intestine (52), although it is not known whether the K5 capsule plays an immunomodulatory role in vivo.

**Enhancement of innate immunity.** Commensal organisms also use several mechanisms to boost the immune response against pathogens at epithelial mucosal surfaces. Although pathogens and commensals can exhibit similarity in terms of surface molecules and Ags, mucosal secretion of proinflammatory cytokines is typically dependent on whether the host encounters commensal or pathogenic microbes. In general, macrophages and dendritic cells in the lamina propria of the intestine are hyporesponsive to commensal microbial ligands, whereas their interaction with a pathogen like *Salmonella Typhimurium* results in the secretion of mature IL-1β and in the recruitment of neutrophils to the site of infection (53). Nevertheless, during infection with certain pathogens, the intestinal microbiota are also able to promote immune defenses by triggering specific responses, which ultimately lead to secretion of host proinflammatory and antimicrobial proteins. To this end, Hasegawa et al. (54) showed that commensal bacteria are able to stimulate the innate immune system and, thus, protect the host from infection. Mice lacking the adaptor protein ASC, an essential mediator of IL-1β and IL-18 processing, are highly susceptible to *C. difficile* infection, mostly because of impaired recruitment of neutrophils to the intestine (54). Surprisingly, translocation of commensal bacteria was essential to promote IL-1β secretion and protection against *C. difficile* intestinal colonization, indicating that stimulation of proinflammatory responses by commensal organisms can have a protective function (54). Nevertheless, the particular mechanisms by which commensals induce IL-1β processing and secretion remain to be elucidated.

An additional mechanism by which commensals enhance the host immune response to pathogens is by triggering the secretion of host antimicrobial peptides. In the small intestine, Paneth cells are the main source of these peptides (predomi-
antly C-type lectins and β-defensins), whose primary function is to protect the host against enteric pathogens (55). Paneth cell secretion of the C-type lectins REG3γ and REG3β requires the stimulation of the MyD88 pathway, underlining the role of TLR-mediated recognition of the microbiota for this process (56, 57). The importance of REG3γ and REG3β is highlighted by studies showing that these two antimicrobial peptides provide protection to infection with some bacterial pathogens, including Enterococcus faecalis, Yersinia pseudotuberculosis, and Listeria monocytogenes (58–60).

The β-defensins are also key components for the control of enteric pathogens (61). Similar to C-type lectins, the microbiota play an important role in the induction of β-defensin expression, which, in turn, controls pathogen growth and contains commensals within the intestine (62–65). In particular, both mice lacking the MHC class I–related protein CD1d and Crohn’s disease patients with mutations in the peptidoglycan sensor NOD2 show a reduced secretion of β-defensins, suggesting that recognition of commensal ligands enhances β-defensin expression (66, 67). However, Nod2 mutations in mice do not affect the expression of β-defensins or the composition of the microbiota, which could possibly reflect differences between humans and mice (68, 69). With regard to β-defensins, some bacteria, such as the probiotic strain E. coli Nissle, were shown to induce β-defensin expression in cell culture through TLR5-mediated recognition of flagellin (70). Still, in vivo studies are required to determine whether the ability of E. coli Nissle to induce β-defensins subsequently reduces the colonization of enteropathogens and perhaps controls gut homeostasis by inhibiting bacterial translocation.

Another example of commensal-mediated gut immunity enhancement is the induction of IL-22, a cytokine that enhances the mucosal barrier against pathogens by inducing the secretion of chemokines and antimicrobials by epithelial cells (71). Mice that lack IL-22 were shown to be more susceptible to gut infection with C. rodentium (72, 73). Secretors of IL-22 include innate lymphoid cells (ILCs), in which the production of this cytokine is enhanced by the aryl hydrocarbon receptor (AhR) via specific bacterially derived molecules (74, 75). Recently, Zelante et al. (76) showed that a subset of Lactobacillus species (specifically, L. reuteri in the GI tract) uses tryptophan as an energy source and produces a metabolite, indole-3-aldehyde, which, in turn, activates AhR on ILCs. Once activated, ILCs secrete IL-22, which protects the host against the opportunistic pathogen Candida albicans by reducing its colonization. This host–commensal interplay is a prime example of how beneficial bacteria might enhance the immune response to protect the host from pathogens. In addition, the observation that only a subset of lactobacilli produces indole-3-aldehyde and activates AhR-mediated secretion of IL-22 highlights the importance of how differences in genomic content and gene expression, even within the same genus, might account for significant differences in host immune regulation.

Enhancement of adaptive immunity. Commensal and probiotic bacteria are implicated in the activation of innate immune responses, and they are able to promote adaptive immune responses. In recent years, a few studies provided evidence that specific commensal bacteria are able to preferentially drive the differentiation of individual T cell subsets. A seminal study by Ivanov et al. (77) demonstrated that bacteria of the Clostridiales family, segmented filamentous bacteria (SFB), specifically induce the differentiation of mucosal Th17 cells in the lamina propria of the intestine, which, in turn, secrete the proinflammatory cytokines IL-17 and IL-22. Because IL-22 induces the production of antimicrobial proteins, mice administered SFB were more resistant to infection with C. rodentium (77).

In addition to Th17 cells, the differentiation of the regulatory T cell (Treg) lineage is impacted by the microbiota. Atarashi et al. (78) demonstrated that colonization of GF mice with Clostridioides spp. generates an environment rich in TGF-β and colonic Tregs. Moreover, oral administration of a mixture of 17 Clostridia strains attenuated the pathogenesis of colitis by shaping an anti-inflammatory environment rich in Tregs and IL-10 (79). In some cases, a single component of a commensal bacterium is sufficient to induce a specific cell subset. For example, the protective effect of Bacteroides fragilis against experimental colitis induced by Helicobacter hepaticus was shown to be dependent on the expression of a specific capsule, termed polysaccharide A. In this model, administration of polysaccharide A alone was able to protect mice from colitis by inducing anti-inflammatory, IL-10–producing CD4+ T cells (80). In other cases, certain metabolites produced by the microbiota can impact Treg lineage homeostasis. For instance, SCFAs generated by the intestinal microbiota contribute to the regulation of Treg cell size and function by interacting with the G protein–coupled free fatty acid receptor 43 (81).

Another important arm of the adaptive immune response modulated by the microbiota involves the differentiation and activation of B cells, along with mucosal secretory IgA (slgA) (82, 83). Because slgA plays an essential role in promoting intestinal barrier function and in determining the composition of the gut microbiota, the presence of certain bacterial species is essential to regulate this important aspect of gut homeostasis (84). In particular, dendritic cells that phagocytize small numbers of commensal microbes in the intestine can selectively induce slgA and protect the host from its own commensal bacteria (85, 86). Akin to the development of T cell subsets, specific commensal microbes can contribute to the development of B cells. For instance, SFB, but not commensal E. coli, were shown to contribute to the development of intestinal lymphoid tissue, including Peyer’s patches, and to the development of intestinal slgA (87). Some human in vivo studies also reported the importance of the microbiota for B cell maturation, such as with early life colonization by E. coli and B. longum subspecies Infantis being associated with higher numbers of mature CD20+ B cells (88). Because most of these studies were aimed at investigating the development of the immune response, rather than how the microbiota contribute to colonization resistance to pathogens, future studies should address whether the development of specific immune responses are linked to colonization resistance.

Conclusions

Beneficial microbes provide colonization resistance against harmful microorganisms by stimulating the immune response and by directly inhibiting pathogen growth. Although some
such mechanisms have now been described, future studies are needed to identify and/or characterize probiotics and their modes of action against specific pathogens. For instance, IL-22–mediated induction of antimicrobial responses, stimulated by L. reuteri, could be advantageous against pathogens that are susceptible to these effects, such as C. albicans (76). However, the same probiotic bacterium would likely be ineffective against pathogens like Salmonella Typhimurium, which evades and exploits IL-22–mediated antimicrobial responses (35). In contrast, because Salmonella requires iron to grow in the inflamed intestine, administration of E. coli Nissle, which can compete with the pathogen for iron, would be a better therapeutic and preventive strategy (34). In light of this, the identification of vulnerabilities within target pathogens is an essential first step to discover and design probiotics.

Another promising strategy is to screen the intestinal microbiota for natural competitors to specific pathogens. This approach proved successful in a recent seminal study by Buffie et al. (89), who discovered that a specific intestinal commensal bacterium, Clostridium scindens, provides colonization resistance to C. difficile infection by producing inhibitory metabolites derived from bile salts. With the constant emergence of antibiotic resistance, as well as new knowledge concerning the detrimental effects that antibiotics have on the microbiota, the discovery of new probiotics and their mechanisms of action could provide a strong foundation for developing novel therapeutics against infection.

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