Microbiota-mediated colonization resistance against intestinal pathogens

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Abstract

Commensal bacteria inhabit mucosal and epidermal surfaces in mice and humans, and have effects on metabolic and immune pathways in their hosts. Recent studies indicate that the commensal microbiota can be manipulated to prevent and even to cure infections that are caused by pathogenic bacteria, particularly pathogens that are broadly resistant to antibiotics, such as vancomycin-resistant *Enterococcus faecium*, Gram-negative Enterobacteriaceae and *Clostridium difficile*. In this Review, we discuss how immune- mediated colonization resistance against antibiotic-resistant intestinal pathogens is influenced by the composition of the commensal microbiota. We also review recent advances characterizing the ability of different commensal bacterial families, genera and species to restore colonization resistance to intestinal pathogens in antibiotic-treated hosts.

Antibiotic therapy has decreased the mortality rate caused by infection and, in conjunction with improved sanitation and vaccine administration, has markedly increased human longevity. However, in recent decades, many microbial pathogens have acquired resistance genes that render antibiotics ineffective. The acquisition of resistance has been partly driven by the overuse of antimicrobials in clinical and agricultural settings[1], which results in a paucity of effective preventative or curative treatment options. Infections that are caused by antibiotic-resistant bacteria are particularly problematic in hospitalized patients, partly because hospitals have many resistant bacterial strains, which leads to their transmission to patients, and partly because many hospitalized patients have reduced immune defences.

The gastrointestinal tract is a reservoir for antibiotic-resistant pathogens that cause disease by a variety of mechanisms. Some pathogens, such as *Clostridium difficile*, destroy cellular barriers through the toxin-mediated destruction of epithelial cells, whereas other pathogens, such as *Enterococcus faecium*, traverse the epithelium and enter deeper tissues and the bloodstream. Antibiotic-resistant bacteria are not eliminated by antibiotic treatment, so they can proliferate and reach high densities in the intestinal lumen, from where they are excreted. This makes it very challenging to prevent their spread to other patients.

Protection of the host intestines from exogenous pathogens by commensal bacteria — a phenomenon termed colonization resistance — was described more than five decades ago (BOX 1) and was thought to result from microorganism-mediated direct inhibition. However, recent work has shown that commensal bacteria can also indirectly control invading pathogens by enhancing host immunity in the intestines (known as immune-mediated colonization resistance) (FIG. 1). Microbial colonization of the intestines fosters the development of immune cell populations that are involved in innate[2–7] and adaptive[3,8–10] immune processes, and stimulates the production of antimicrobial and pro-inflammatory factors. Antibiotic treatment, by killing commensal micro-organisms, decreases both direct inhibition and these microbiota-mediated innate immune defences and so enables residual, antibiotic-resistant species to proliferate and often to dominate mucosal surfaces.

The contributions of specific microbiota-derived molecules and microbial populations to immune defence against bacterial pathogens are beginning to be deciphered, but few cohesive mechanisms of commensal bacteria-mediated immune protection have been determined. In this Review, we discuss how the intestinal microbiota influences immune defence against infection by some of the major antibiotic-resistant intestinal pathogens. We also review how specific microbial populations, together and individually, can be therapeutically used to enhance resistance to antibiotic-resistant bacterial pathogens and we suggest approaches to limit these infections in an era of increasing antibiotic resistance.

Antibiotic-resistant intestinal pathogens

Commensal bacteria and the molecules they release into their surroundings stimulate mucosal immunity in the intestines through various mechanisms (BOX 2). Antibiotic therapies markedly decrease the intestinal microbiota and create an immunodeficient environment that can be exploited by the antibiotic-resistant pathogenic and opportunistic bacteria that are frequently encountered in hospital settings. The most clinically important antibiotic-resistant intestinal pathogens include Gram-positive *C. difficile* and vancomycin-resistant *E. faecium*, and Gram-negative bacilli belonging to the Enterobacteriaceae family[11,12].

C. difficile *infection*

C. difficile is a Gram-positive rod-shaped bacterium capable of forming endospores that are resistant to environmental stresses and sterilization measures; this facilitates its transmission between patients in hospital settings. *C. difficile* is resistant to many classes of antimicrobials[13–15] that create broad and lasting deficits in the composition and diversity of the intestinal microbiota[16,17]. In patients receiving antibiotics, ingested *C. difficile* spores can germinate, grow as vegetative bacteria and produce toxins that target the intestinal epithelium, which results in the development of disease that can range from mild diarrhea to colitis and toxic megacolon[18]. *C. difficile*-associated disease has been widely studied using a mouse model of infection in which mice are treated with antibiotics before oral challenge with *C. difficile*[17,19–21]. Germination of spores, growth of vegetative forms and toxin production and activity are likely to be influenced by microbiota-derived cues and host

responses, but the precise factors that are crucial at each stage of infection are currently poorly understood.

Innate and adaptive immune responses mitigate acute *C. difficile*-associated inflammation and the recurrence of disease. During acute *C. difficile* infection, nucleotide-binding oligomerization domain 1 (NOD1)[22], myeloid differentiation primary-response protein 88 (MYD88)[23] and interleukin-1β (IL-1β)[24] signalling enhance the expression of CXC-chemokine ligand 1 (CXCL1) by colonic lamina propria cells and increase the recruitment of neutrophils, in a process that is at least partly dependent on commensal bacteria. This limits *C. difficile*-induced mucosal damage, inflammation and mortality. Toll-like receptor 5 (TLR5) stimulation induces protection against *C. difficile* infection, as the administration of flagellin (the ligand for TLR5) to antibiotic-treated *C. difficile*-susceptible animals reduces bacterial growth and toxin production, ameliorates *C. difficile*-induced intestinal inflammation and reduces mortality[25]. Asymptomatic human carriers of *C. difficile* have higher levels of toxin A-specific serum IgG than symptomatic *C. difficile*-infected patients[26], and the addition of toxin A-specific monoclonal antibodies to conventional antibiotic treatment significantly reduces the recurrence of *C. difficile* infection[27], which indicates the importance of adaptive immune responses to clinical outcomes of infection with this pathogen.

Vancomycin-resistant enterococci

Enterococcus spp. are non-pathogenic commensal bacteria when they are confined to the intestinal lumen but, if they proliferate to a high density in the intestines, antibiotic-resistant strains can cause disease by translocating to deeper tissues and to the bloodstream. *E. faecium* has become a frequent cause of a bloodstream infection that is difficult to treat because most strains are resistant to broad-spectrum antibiotics, in particular the glycopeptide vancomycin[28,29]. *Enterococcus* spp. are normally minor components of the commensal microbiota in the intestines. Treatment with antibiotics that inhibit anaerobic bacteria, such as metro-nidazole, can lead to marked proliferation of vancomycin-resistant *Enterococcus* spp. (VRE) in the intestines and can result in infection of the bloodstream[30–32].

Expression of the peptidoglycan-binding antimicrobial protein regenerating islet-derived protein IIIγ (REGIIIγ) is associated with colonization resistance to VRE. Antibiotic-mediated depletion of commensal bacteria decreases intestinal REGIIIγ expression[33,34], as do deficits in MYD88 signalling[35]. Stimulation of TLR4 or TLR5 by administration of exogenous lipopolysaccharide (LPS) or flagellin, respectively, restores intestinal REGIIIγ levels as well as resistance to colonization with VRE[33,34].

Gram-negative Enterobacteriaceae

As in the case of VRE, the proliferation of antibiotic-resistant Gram-negative bacilli of the Enterobacteriaceae family, such as *Escherichia coli* and *Klebsiella pneumoniae*, occurs after treatment with certain antibiotics and can lead to bacteraemia[11,32]. Some Enterobacteriaceae alsoproduce virulence factors and intestinal pathology, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium, as well as enterohaemorrhagic *E. coli* and its mouse

equivalent, *Citrobacter rodentium*[36]. Although some pathogenic Enterobacteriaceae are antibiotic sensitive and can infect hosts in the absence of antibiotic treatment, commensal bacteria and their associated molecular products also enhance colonization resistance against these pathogens. The study of such antibiotic-sensitive Enterobacteriaceae, which in many cases are phylogenetically similar to clinical isolates of antibiotic-resistant Enterobacteriaceae pathogens, can therefore be used to elucidate the mechanisms of commensal- mediated colonization resistance that restrain the growth of such pathogens.

A range of antimicrobial immune responses control the proliferation and the invasion of antibiotic-resistant Enterobacteriaceae pathogens. Commensal bacteria-driven MYD88-dependent and NOD2-dependent signals induce intestinal Paneth cell expression of numerous antimicrobial peptides, such as cryptdins, that limit penetration of the epithelial barrier by *S.* Typhimurium[37–39]. TIR domain-containing adaptor protein inducing IFN-β (TRIF; also known as TICAM1)-mediated signals are also required for proper interferon (IFN)-mediated activation of natural killer cells and macrophages, and resistance to *S.* Typhimurium and *E. coli* is dependent on TRIF[40], which mediates signalling pathways downstream of TLR3 and TLR4. In the intestinal lamina propria, activated CX C-chemokine receptor 1 (CX$_3$CR1)$^+$ mono-nuclear phagocytes and CD103$^+$ dendritic cells (DCs) can capture *S.* Typhimurium from the intestinal lumen using extended transepithelial dendrites[5,41].

In summary, various commensal bacteria-derived signals augment resistance against colonization by antibiotic- resistant pathogens, including *C. difficile*, VRE and pathogenic Enterobacteriaceae. Antibiotic-resistant pathogens gain a competitive advantage during antibiotic treatment, as the commensal bacteria that normally suppress invading pathogens, both directly and indirectly by stimulating host immune defences, are depleted. The extent to which deficits in direct and indirect commensal bacteria-mediated colonization resistance contribute to antibiotic-associated susceptibility to infection remains incompletely defined, and the precise populations of commensal bacteria that provide protective immune stimuli are unknown. Nevertheless, numerous mechanisms of microbiota-enhanced host defence against antibiotic-resistant intestinal pathogens are becoming clear and have laid the foundations for further study in this area.

Bacterial role in colonization resistance

The discovery that commensal bacteria modulate immune and inflammatory responses has focused attention on bacterial species that, until recently, were unknown to the immunology community. It is anticipated that many additional bacterial species that enhance immune defence or that protect against harmful inflammatory responses (see below) will be discovered in the coming years. Some commensal bacteria also directly antagonize pathogens (BOX 3) and these contributions are distinct from the immunomodulatory functions that have been attributed to the microbiota. Commensal bacteria that modulate host immunity and that thereby confer resistance to antibiotic-resistant intestinal pathogens are just beginning to be identified across phyla that constitute the majority of the intestinal microbiota: the Bacteroidetes, the Firmicutes and the Actinobacteria.

Bacteroidetes

In addition to tolerance-inducing *Bacteroides fragilis*[42] and colitis-associated Prevotellaceae[43], the Bacteroidetes phylum contains various species that protect the intestines against infection by pathogens. The intestinal commensal bacterium *Bacteroides thetaiotaomicron* antagonizes intestinal pathogens through a range of mechanisms that include the activation of host immune defences and direct interactions with other intestinal bacteria. *B. thetaiotaomicron* can enhance immune-mediated host defences against pathogens in several ways. Colonization of germ-free mice with *B. thetaiotaomicron* and *Bifidobacterium longum* triggered the expression of type I IFN-induced GTPases that are associated with resistance to viral infections[44], which is consistent with reports that show that commensal bacterial colonization in the intestines enhances antiviral immunity[45]. The same study showed that, in contrast to *B. longum*, which closely associates with the intestinal epithelium and downregulates antimicrobial peptide expression, *B. thetaiotaomicron* colonization enhances the expression of the antimicrobial C-type lectins REGIIIγ and REGIIIβ in the large intestine[44]. Microbiota containing Porphyromonadaceae family members that are closely related to *Bacteroides* spp. are associated with protection against *S.* Typhimurium-induced colitis but not against colonization[46]. Furthermore, *B. thetaiotaomicron* secretes a soluble factor that represses toxin production by enter-ohaemorrhagic *E. coli*[47], which suggests that these commensal bacteria may regulate immune defence against intestinal pathogens as well as the pathological inflammation that accompanies them. In addition, *B. thetaiotaomicron* competitively excludes *C. rodentium* from the intestinal lumen by consuming the plant-derived monosaccharides that are required for the pathogen's growth[48]. Co-colonization of *B. thetaiotaomicron* with *B. longum* upregulates the expression of enzymes that are involved in the metabolism of such carbohydrates by *B. thetaiotaomicron*[44], which shows a form of synergy that is probably quite common between intestinal commensal bacteria.

Barnesiella intestihominis is an abundant colonic anaerobe that is readily depleted by antibiotic treatment. Antibiotic-induced losses of microbiota components enable VRE to colonize the intestines and to proliferate to a high level, reaching approximately 10^9 bacteria per gram of luminal content[31]. Intestinal VRE domination increases the risk of dissemination and bacteraemia in humans[32], probably because of the increased bacterial translocation that results from markedly increased VRE density and decreased barrier function in the intestines following antibiotic therapy. Patients undergoing allogeneic haematopoietic stem cell transplantation that are colonized with *B. intestihominis* have decreased risk of VRE domination, and re-colonization of VRE-dominated mice with microbial consortia containing *B. intestihominis* eliminates VRE[49]. These findings indicate that an intestinal microbiota containing *B. intestihominis* confers resistance to VRE colonization, but it is unclear whether this antagonism is mediated by *B. intestihominis* itself or whether the presence of *B. intestihominis* simply reflects the re-establishment of a healthy, complex and protective microbiota. In addition, it is unclear whether *B. intestihominis* has a direct effect on VRE or whether it has an indirect effect that is mediated by host immunity.

One possible explanation is indicated by a study showing that the caecal contents of antibiotic-treated mice depleted of anaerobic commensal bacteria sustain VRE replication *in vivo* and *ex vivo*, whereas caecal contents from untreated animals (with an unperturbed 'normal' microbiota) do not. Suppression of VRE growth by the caecal contents of untreated mice is abolished by filtration or autoclaving and cannot be rescued with either nutrient broth or short-chain fatty acid supplementation[50]. This indicates that insoluble, heat-sensitive host-derived or microbiota-derived factors found in the large intestine inhibit VRE growth. Microbial products activate TLR signalling and thereby restore intestinal antimicrobial peptide expression and VRE protection in antibiotic- treated mice[33,34,51], but it remains unclear whether *B. intestihominis* or other anaerobic commensal bacteria are sources of these molecular signals in untreated mice.

Firmicutes

Analysis of the microbiota in the mouse model of *C. difficile* infection showed that a decreased abundance of Firmicutes populations and an increased number of Proteobacteria populations correlate with more severe disease[21]. Colonization of germ-free mice with bacteria belonging to the Lachnospiraceae family of Firmicutes, but not with *E. coli*, significantly reduced *C. difficile* colonization and toxin production[52]. However, the mechanism by which Lachnospiraceae provide protection against *C. difficile* infection remains undefined.

Lactobacillus spp. of the Firmicutes phylum express mucus-binding pili that enable persistent intestinal colonization[53,54]; for example, *Lactobacillus reuteri* binds to the host mucosa via a novel mucus-binding protein[53,54] and secretes two proteins that activate AKT and decrease the apoptosis of colonic epithelial cells during inflammation[55]. Other reports indicate that cell-free supernatants from *Lactobacillus delbrueckii* limit the adherence of *C. difficile* to epithelial cells and decrease the cytotoxicity of *C. difficile in vitro*[56], which indicates that the secretion of factors by *L. delbrueckii* can reduce inflammatory and *C. difficile*-associated disease. Several *Bacillus* spp. also produce factors that directly inhibit *C. difficile* (BOX 3). *Lactobacillus* spp. strains also trigger TLR2 signalling and pro-inflammatory gene expression in monocytes[57,58]; they might thereby enhance the host immune response to pathogens. A clinical trial has shown that supplementation of vancomycin therapy with *Lactobacillus plantarum* can decrease the rate of recurrence of *C. difficile* infection compared with vancomycin treatment alone[59].

Segmented filamentous bacteria (SFB), provisionally named *Candidatus* Savagella (members of the Clostridiaceae family)[60], are commensal Firmicutes that adhere to the intestinal epithelium. They have been shown to have functional homology to the Clostridia genera on the basis of metagenomic analyses[61]. In contrast to other Clostridia[62] and epithelium-associated commensal bacteria[53,54,63,64] that enhance regulatory T (T_{Reg}) cell differentiation or anti-inflammatory cytokine expression, colonization of germ-free mice with SFB activates a range of antimicrobial defences, including the production of antimicrobial peptides and pro-inflammatory cytokines, IgA secretion by B cells and the development of CD4+ T helper 17 (T_H17) cells in the lamina propria of the terminal ileum[65–68]. Although the precise SFB-derived molecular signals that drive immune

activation remain unclear, colonization with SFB increases the expression of serum amyloid A in the terminal ileum, which is an acute-phase response protein that induces the DC-dependent differentiation of CD4^{+} T cells to T_H 17 cells *in vitro*[67]. Recent work indicates that commensal bacteria-induced IL-1β secretion by intestinal macrophages also drives T_H17 cell development in the small intestine[69], but the precise molecular signal and its bacterial source that induces IL-1β production remains undefined. Pre-colonization of mice with SFB ameliorates infection with *C. rodentium* and the associated colonic inflammation[67], but it can also induce colitis in genetically susceptible animals[70]. This indicates that commensal bacteria-induced immune activation in the ileum can enhance immune control of infection but it can also result in inflammation.

Actinobacteria

The *Bifidobacterium* spp. belong to the phylum Actinobacteria and are commensal bacteria that associate with intestinal tissues and that synthesize compounds that influence host immunity[63]. Like the *Lactobacillus* spp., *Bifidobacterium* spp. are well-adapted to the mammalian gastrointestinal tract as a result of their proteinaceous pili that facilitate adherence to mucosal surfaces and their transporter proteins that confer resistance to bile acids[71,72]. *B. longum* expresses a novel serine protease inhibitor that has putative immunomodula-tory functions[73]. However, *in vitro* studies indicate that many *Bifidobacterium* species confer direct resistance to intestinal pathogens by secreting antimicrobial substances; for example, *Bifidobacterium* spp. express enzymes that catabolize plant-derived and host-derived carbo hydrates[73], which enables them to synthesize antimicrobial organic acids and peptides. Experiments carried out *in vitro* indicate that these metabolites impair the adhesion of *C. difficile* to enterocytes[74] and provide direct, low pH-dependent antimicrobial activity against *C. difficile*[71,75,76]. In addition, *Bifidobacterium* spp.-secreted factors enhance protection against enterohaemorrhagic *E. coli* and *C. rodentium*. *Bifidobacterium* spp. that have been isolated from human faeces adhere to enterocytes and inhibit the adhesion and the growth of *E. coli* O157:H7 in a dose-dependent manner[77]. Certain strains of *B. longum* produce acetate in the colon, which thereby enhances the expression of anti-inflammatory and anti-apoptotic genes in the intestinal epithelium. This change in expression affected neither the growth of *E. coli* nor the toxin production by *E. coli*, but it improved the integrity of the epithelial barrier, decreased translocation of *E. coli* toxins and ultimately enhanced host survival[78,79]. Similarly, *Bifidobacterium breve* expresses a surface-associated exopolysaccharide (EPS) that insulates it from intestinal acid stress and immune detection, allowing the bacterium to evade B cell responses and to persist in the intestines. Colonization with EPS-positive *B. breve* also increased resistance to intestinal infection by *C. rodentium*, but it is unclear whether this enhanced defence can be attributed to direct antagonism by *B. breve* or to the immunomodulatory effects induced by EPS that facilitate persistent colonization of the commensal bacteria[80].

In summary, the dominant phyla of the intestinal microbiota contribute to colonization resistance against a range of intestinal pathogens. Commensal bacterial species that are associated with both direct and immune-mediated colonization resistance against intestinal pathogens are broadly distributed across the major bacterial phyla of the intestines (FIG. 2). Consequently, bacterial species that modulate antimicrobial responses are difficult to

distinguish from those that directly antagonize pathogens and, in fact, they may be one and the same. This complicates the interpretation of resistance- associated commensal bacteria that have been identified in the context of host biology, as their possible contributions to host defence encompass both direct and immune-mediated mechanisms. In addition, the interplay between distinct microorganism-mediated immuno modulatory effects has not been fully elucidated, but a balance between these effects is probably required for optimal host defence and health.

Immune defence and inflammatory disease

The human microbiome project has shown that 'normal' humans have distinct microbial communities and that there are significant differences in the representation of bacterial taxa among healthy individuals[81]. Although the effect of these differences in the microbiota on defence against infection is unclear, some differences in microbiota composition have been associated with obesity and with metabolic and inflammatory diseases. Colonization with immune-enhancing commensal bacterial species may augment resistance to microbial pathogens, but these bacterial strains may also promote inflammatory diseases. By contrast, other bacterial species decrease intestinal inflammation by promoting tolerogenic or regulatory immune responses. A balance of these intestinal commensal species is probably required to prevent infection with pathogens and pathological inflammation.

Commensal bacteria drive intestinal inflammation

Imbalances in the composition of the intestinal micro-biota (known as dysbioses) can be caused by antibiotics, immune deficits and dietary influences, and may induce inflammatory diseases, such as inflammatory bowel disease (IBD)[43,82–88]; for example, colonization with SFB can induce colitis in genetically susceptible severe combined immunodeficient (SCID) mice[70]. Mice that are deficient in T-bet (encoded by *Tbx21*) and V(D)J recombination-activating protein 2 (RAG2) develop a dysbiotic micro-biota and accompanying colitis (this is known as the TRUC model (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis model)), both of which are transferable to genetically normal mice[82]. Diets that are high in saturated fat can induce the proliferation of *Bilophila wadsworthia*, which is an intestinal pathobiont that is capable of inducing colitis in IL-10-deficient mice[88]. IBD-associated dysbioses can involve abnormal proportions of the major intestinal bacterial phyla, which span Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. Moreover, a recent metagenomic analysis of 231 patients with IBD and of healthy subjects indicated that IBD-associated changes in microbiota composition are accompanied by even greater changes in aggregate microbiome function, such as short-chain fatty acid production and metabolic pathways that influence oxidative stress in the intestines[89].

Although intestinal inflammation is associated with numerous changes in microbiota composition, cross-fostering experiments have identified Prevotellaceae (of the Bacteroidetes phylum) as key representatives of the IBD-sensitizing microbiota that is derived from NLRP6 (NOD-, LRR- and pyrin domain-containing protein 6)- deficient mice[43]. In addition, another study showed that the transfer of *Bacteroides* spp. (of the Bacteroidetes phylum) induced IBD in genetically susceptible mice. Although Enterobacteriaceae are abundant components of the microbiota of IBD-afflicted mice,

colonization with IBD-associated Enterobacteriaceae isolates did not result in inflammatory disease in genetically susceptible mice[86]. Taken together, these findings indicate that some components of IBD-associated dysbioses drive inflammation, whereas other alterations in microbial abundance are markers of IBD and are perhaps driven by the inflammatory changes induced by colitogenic bacteria.

Inflammation drives proliferation of intestinal pathogens

How might inflammation affect the composition of the intestinal microbiota? Chemical- and pathogen-induced intestinal inflammation results in the loss of microbial density and diversity and leads to the proliferation of Gram-negative bacteria that belong to the Enterobacteriaceae family, including commensal *E. coli* and the pathogen *C. rodentium*[90]. Intestinal inflammation is necessary and sufficient for the pathogen *S.* Typhimurium to outcompete the endogenous micro-biota[91]. Virulent *S.* Typhimurium invades the intestinal epithelium, which induces oxidative inflammatory processes that yield the sulphur-containing by-product tetrathionate, which the pathogen can use as a terminal electron acceptor for anaerobic respiration[92]. In addition, nitrate, which is generated by inducible nitric oxide synthase (iNOS) during intestinal inflammation, is used in a similar metabolic process by *E. coli*, which thereby promotes rapid outgrowth of this commensal bacterium[93]. As intestinal domination by Enterobacteriaceaein patients undergoing allogeneic stem cell transplantation markedly increases the risk of bacteraemia[32], it is possible that inflammation-driven changes to the microbiota can have clinically important sequelae.

Some microbiota components decrease inflammation

A subset of intestinal commensal bacteria can suppress host inflammatory responses; for example, *Bacteroides fragilis* associates with the intestinal epithelium through host mucins and influences lymphoid organogenesis and T cell differentiation. These immunomodulatory functions depend on the expression of polysaccha-ride A (PSA)[42], which can stimulate DCs through TLR2 (REF. 94). Forkhead box P3 (FOXP3)$^+$ CD4$^+$ T_{Reg} cells produce IL-10 and anti-inflammatory cytokines in response to PSA that is presented by DCs; this also occurs through TLR2 signalling[94]. Enhanced mucosal tolerance promotes intestinal colonization by *B. fragilis*[94] and can suppress pathogen-mediated colitis[95]. These observations are in contrast to the antimicrobial profile that is induced by the exogenous administration of TLR2 agonists[96], which suggests that the source, the localization and/or the inflammatory context of innate immune receptor agonists can alter the outcome of otherwise similar ligand–receptor interactions.

B. fragilis is not the only bacterium that is associated with the regulation of intestinal inflammation. The Gram-negative commensal bacterium *Helicobacter hepaticus* (of the Proteobacteria phylum) decreases the expression of inflammatory genes in intestinal epithelial cells and CD4$^+$ T cells, including those encoding TLR4 and nuclear factor-κB (NF-κB), and results in decreased IL-17 production. The induction of this anti-inflammatory gene profile by *H. hepaticus* depends on a bacterially encoded type VI secretion system (T6SS); by contrast, T6SS-deficient *H. hepaticus* strains promote experimental colitis in susceptible hosts[97]. The commensal bacterium *Faecalibacterium prausnitzii* (of the Firmicutes phylum) also secretes yet-to-be defined metabolites that block NF-κB activation

and pro-inflammatory cytokine production *in vitro*. Oral administration of *F. prausnitzii* attenuates chemically induced colitis in animal models, and a decreased abundance of this bacterium in the intestines of patients with IBD is associated with increased risk of disease recurrence[98].

Administration of a small consortium of probiotic bacteria, containing species of the *Lactobacillus*, *Streptococcus* and *Bifidobacterium* genera, upregulates IL-10, transforming growth factor-β (TGFβ) and cyclooxygenase 2 (COX2) expression in DCs. These regulatory DCs can drive the differentiation of FOXP3^{+} T_{Reg} cells, which in turn suppress a range of experimental inflammatory states[99]. Other commensal bacterial consortia indirectly function through intestinal epithelial cells to induce T_{Reg} cell-mediated mucosal immune tolerance; for example, colonization of germ-free mice with the spore-forming fraction of indigenous or human-derived microbiota, specifically 17 strains belonging to Clostridia clusters IV, XIVa and XVIII, induces TGFβ1 expression by intestinal epithelial cells. This TGFβ1-rich environment in turn increases the peripheral induction of, and IL-10 expression by, T cells, particularly in the colonic mucosa[62,100] Reg . Thus, a range of phylogenetically diverse commensal bacteria can suppress intestinal inflammation (FIG. 2), but the mechanisms remain mostly undefined.

Furthermore, in some cases, commensal bacteria-derived molecular signals suppress intestinal inflammation but the microbial source of these molecular signals remains unknown; for example, microbiota-derived nucleic acids signal through IFN-regulatory factor 3 (IRF3) in enterocytes to induce the production of thymic stromal lymphopoietin, which is a cytokine that is crucial for protection and recovery from experimental colitis[101]. In addition, the presence of an intact, non-dysbiotic intestinal microbiota prevents the transport of intestinal bacteria to the mesenteric lymph nodes in a MYD88-dependent manner, which thereby limits inflammatory responses that might be induced by innocuous commensal bacteria[3].

As for microbiota-mediated colonization resistance, phylogenetically diverse commensal bacteria modulate inflammatory tone in the intestines, and species that promote inflammation are sometimes closely related to those that induce tolerance. The mechanisms by which many individual species and consortia regulate inflammatory tone, as well as the bacterial sources of some of the tolerogenic signals, remain undefined. Improved understanding of the effects of commensal bacteria on innate immune tone will facilitate the targeted manipulation of immune-enhancing microbiota while avoiding pathological inflammation.

Manipulation and reconstitution of the microbiota

Colonization resistance that is conferred by endogenous commensal bacteria can be therapeutically exploited, but the practice of adoptively transferring commensal bacteria to reconstitute a health-associated microbiota in individuals with disease carries risks as well as promise. Microbiota transplants may contain bacteria that have uncharacterized infectious and pathological properties, and the overall effect of transplantation on the native intestinal community can be dynamic and unpredictable. To avoid such risks and to maximize

therapeutic efficacy, various clinical and animal studies seek to define and to understand the bacterial populations that are necessary for treatment of or prophylaxis against infection by antibiotic-resistant intestinal pathogens.

Faecal microbiota transplantation

Antibiotic administration alters the microbiota and renders hosts more susceptible to infection with *C. difficile* and, in some cases, decreases the host's ability to clear the pathogen. Reconstitution of the commensal flora by adoptively transferring live bacterial populations has a long history. In China, administration of 'yellow soup', which is a dilute solution of human faeces, was used to treat patients with intestinal infections for hundreds of years[102]. More recently, faecal transplantation has been sporadically practised in health care settings in many countries in cases of *C. difficile* infection that do not respond to conventional antibiotic treatment. Transplantation of the faecal microbiota from a healthy individual to a patient with intractable recurrent *C. difficile* infection is remarkably effective. Compiled case reports show that faecal microbiota transplantation has been carried out using several techniques, ranging from instillation into the upper gastrointestinal tract to infusion into the rectum. These techniques have similar beneficial outcomes and an overall cure rate for recurrent *C. difficile* infection of approximately 90% (reviewed in REF. 103). Transfer of a standardized, previously frozen source of faecal material results in the efficient engraftment of donor microbial populations in recipients and similarly high therapeutic efficacy against *C. difficile* infection[104,105]. The first prospective randomized controlled trial evaluating duodenal infusion of donor faecal material was recently completed. The trial reported high success rates that greatly exceeded standard treatment with vancomycin. Patients receiving faecal microbiota transplantation were reconstituted with a broad range of intestinal microbial taxa, spanning the Bacteroidetes and Firmicutes phyla[106].

Identifying protective bacterial consortia

As faecal composition is difficult to completely define — which makes the potential transmission of unidentified infectious agents a concern that is difficult to eliminate — efforts are underway to identify specific bacterial species that can, following transfer, confer resistance to *C. difficile* infection. The first study to show that adoptive transfer of a defined consortium of 10 bacterial species can eliminate *C. difficile* infection correlated the reconstitution of *Bacteroides* species with cure of *C. difficile* infection[107]. In a more recent study, 33 taxonomically diverse bacterial isolates were cultured from healthy donor stool samples and administered to two patients with recurrent *C. difficile* infection[108]. Both patients experienced a resolution of symptoms and remained symptom free for at least 24 weeks. As has been observed using faecal microbiota transplantation, the relative abundances of Bacteroidetes and Actinobacteria increased, whereas Proteobacteria concentrations decreased, following reconstitution with this defined bacterial mixture. However, the infusion produced markedly different changes in microbiota composition at the family level between the two patients, and in both cases the fraction of faecal bacteria in recipients that matched the infused isolates decreased markedly over the 6-month period following treatment[108]. Thus, despite the efficacy of the infusion, the lack of consistency and poor durability of donor bacterial engraftment complicate interpretation of the roles of the infused bacteria in influencing host immunity and in clearing *C. difficile* infection.

Another recent study identified a minimal consortium of six taxonomically diverse bacterial species that cleared *C. difficile* infection in a mouse model[109]. In these experiments, transfer of six bacterial species resulted in marked increases in overall microbiota diversity, which suggests that the transferred bacterial population facilitated intestinal repopulation with a diverse range of bacterial taxa.

The therapeutic efficacy of the minimal bacterial consortia evaluated in these studies is promising, but fundamental questions about the interactions between components of the consortia, the necessity and the sufficiency of individual populations contained therein, and the specific roles of these populations in influencing host immunity and resistance to *C. difficile* infection remain unanswered. Moreover, additional questions remain concerning the host specificity and species specificity of immunity-enhancing commensal bacteria[2,9,100]; the relevance of the microbiota-mediated protection that has been identified in animal models must be carefully evaluated in the context of human disease.

Summary

C. difficile, VRE and numerous Enterobacteriaceae are important nosocomial intestinal infections that are increasingly difficult to treat. Components of the intestinal microbiota, including whole faecal transplants, multiphyla consortia, individual species and associated molecular products, have been shown to influence innate and adaptive host immune responses to these pathogens. However, the microbial source of many therapeutic molecules has yet to be identified and, conversely, the molecular mechanisms by which most probiotics restore immunity have yet to be elucidated. Closing these complementary knowledge gaps would facilitate approaches that target commensal bacterial populations or products to their appropriate intestinal niches, which would thereby activate precise immune responses to specific pathogens while avoiding the pathological inflammation that can be associated with dysbiosis. Although commensal bacteria that directly antagonize pathogens through competitive exclusion or the production of inhibitory factors may complicate the study of immune-mediated colonization resistance, understanding these contributions to pathogen resistance may yield additional alternatives to current antibiotics. In summary, a more cohesive understanding of how the microbiota increases immune-mediated antimicrobial resistance will open new therapeutic avenues for treating antibiotic-resistant intestinal pathogens.

Acknowledgments

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Box 1 Origins of the concept of colonization resistance

Antibiotic-associated susceptibility to secondary intestinal infections has been recognized for nearly as long as the therapeutic benefits of antibiotics. In the 1960s, it was shown that the minimum infective oral dose of *Salmonella enterica* subsp. *enteritidis* decreased 10,000-fold in mice following streptomycin therapy[110]. Several groups at that time investigated the microbial populations and the mechanisms that are crucial for protection against enteric pathogens — a concept that was later described as colonization resistance[111]. Continuous flow systems that reproduced aspects of gastrointestinal physiology *in vitro*[112] were used to show how components of the caecal microbiota could outcompete *Shigella flexneri* for carbon sources[113]. Similar methods were used to identify antibiotic-sensitive commensal *Bacteroides* species as sources of metabolites, including acetic and butyric acids, that could inhibit the growth of *Salmonella* spp.[114–116]. As work during this period was mainly confined to *in vitro* culture-based methods, direct mechanisms of colonization resistance by commensal bacteria (such as competitive exclusion and the secretion of soluble inhibitory factors) were the focus of study and discovery. This work uncovered pathways that mediate the direct antagonism of pathogens by antibiotic-sensitive microbiota in the intestines, and it laid the foundations for contemporary study of both direct and immune-mediated colonization resistance.

Endospores

Metabolically inactive bacterial forms that are resistant to chemical and physical stresses and that reactivate under specific environmental conditions.

Vegetative bacteria

Metabolically active bacterial forms.

Toxic megacolon

A potentially lethal complication of infectious colitis or inflammatory bowel disease that is characterized by mucosal inflammation, dilatation of the colon and systemic toxicity.

Box 2 Microbiota-derived molecules enhance mucosal immunity

Commensal bacteria-derived molecules provide signals that sustain the innate immune tone of the intestines; for example, nucleotide-binding oligomerization domain 2 (NOD2)-dependent responses to bacterial peptidoglycan fragments enhance the expression of antimicrobial cryptdin peptides by Paneth cells of the intestinal epithelium[117]. Commensal bacteria also induce Paneth and epithelial cells to express regenerating islet-derived protein IIIγ (REGIIIγ), which is a C-type lectin that binds peptidoglycan and that mainly kills Gram-positive bacteria[118,119]. REGIIIγ expression is driven, at least partly, by myeloid differentiation primary-response protein 88 (MYD88)-dependent Toll-like receptor (TLR) stimulation[35,38]. REGIIIγ expression is decreased by the antibiotic-mediated depletion of commensal bacteria, but can be restored by the stimulation of TLR4 on intestinal epithelial cells[33,38] or TLR5 on CD103^{+} dendritic cells (DCs) in a process that is dependent on interleukin-23 (IL-23) and IL-22 (REF. 51).

TLR signalling in intestinal epithelial cells[120] also induces lamina propria-resident CX_3C-chemokine receptor 1 (CX_3CR1)[+] mononuclear phagocytes to extend dendrites between epithelial cells to contact and engulf bacteria in the intestinal lumen[41,120,121]. Furthermore, TLR-dependent cues induce the migration of CD103^{+} DCs from the lamina propria into the intestinal epithelium, from where they too extend dendrites into the intestinal lumen to capture commensal and pathogenic bacteria[5]. These DCs sample and transport commensal bacteria from the intestines to the mesenteric lymph nodes to selectively induce IgA production by B cells[10] at mucosal surfaces, which limits the penetration of commensal[10,122] and pathogenic[122–124] intestinal bacteria and prevents the associated inflammation.

Sampling of luminal bacteria by CX_3CR1^{+} mononuclear phagocytes and by CD103^{+} DCs can also direct the differentiation of lamina propria-resident CD4^{+} T helper 17 (T_H17) cells[125] that control attaching and effacing intestinal pathogens[36,123,126]. Under non-inflammatory conditions, CD103^{+} DCs restrain T_H17 cell development and promote the differentiation of CD4^{+} forkhead box P3 (FOXP3)[+] regulatory T (T_{Reg}) cells in a transforming growth factor-β- and retinoic acid-dependent manner[127–130]. T_{Reg} cells restrain inflammation that is mainly independent of the intestinal microbiota[131], but colonic commensal bacterial antigens do influence local T_{Reg} cell development in the periphery[9], and tolerance to commensal bacteria can be lost following gastrointestinal infection[8].

The transcription factors T-bet[132], GATA-binding factor 3 (GATA3) and retinoic acid receptor-related orphan receptor-γt (RORγt)[133] drive the differentiation of three lineages of innate lymphocytes, which resemble adaptive T_H1 cells (in the case of T-bet), T_H2 cells (in the case of GATA3) and T_H17 cells (in the case of RORγt) in function[134]. Similarly to T_H17 cells, type 3 innate lymphocytes restrain intestinal bacteria[135] and promote mucosal immune defence through the production of IL-22 (REF.136) in a process that at least partly depends on the microbiota[137,138]; however, the strict requirement of an intact microbiota for the development and the function of type 3 innate lymphoid cells remains under investigation (as reviewed in REF. 139).

Innate lymphocytes

Lymphoid cells that are dependent on signalling through the common cytokine receptor γ-chain but that lack recombined antigen receptors. They have important roles in mucosal defence, epithelial homeostasis and lymphoid tissue development.

Paneth cell

Specialized epithelial cell that is found at the base of crypts in the small intestine and that expresses various antimicrobial proteins.

Cryptdins

Microbicidal peptides that are expressed in granules of phagocytic leukocytes and in secretory granules of Paneth cells.

Bacteroidetes

A major bacterial phylum of the intestinal microbiota that comprises physiologically diverse aerobic and anaerobic Gram-negative bacteria commonly associated with the degradation of complex carbohydrates.

Firmicutes

A major bacterial phylum of the intestinal microbiota that primarily comprises Gram-positive bacteria that have low guanine and cytosine DNA content. They are phenotypically diverse, commonly polyphyletic and are often distinguished by their ability to form endospores.

Actinobacteria

A bacterial phylum that is abundant in the intestinal microbiota and that is primarily composed of Gram-positive bacteria that have high guanine and cytosine content in their DNA and that are commonly associated with secondary metabolite production.

Box 3 Direct antagonism of enteropathogens by commensal bacteria

Phylogenetically diverse bacteria directly target and kill intestinal pathogens using antimicrobial substances and contact-dependent mechanisms (FIG. 1). Bacilli (of the Firmicutes phylum) are Gram-positive spore-forming bacteria that are abundant in the intestinal microbiota. They have been of interest to researchers since the 1970s because of their ability to produce antimicrobial substances that inhibit enteric pathogens *in vitro*[140]. Studies using continuous culture conditions that emulate intestinal physiology have more recently shown direct Bacilli-mediated growth inhibition of *Clostridium difficile*. This inhibition was specific to *C. difficile* and was not active against other enteropathogens, such as *Escherichia coli* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium[141]. A single *Bacillus thuringiensis* isolate from the human faecal microbiota has been identified that produces the bacteriocin thuricin CD. Thuricin CD has potent antimicrobial activity against *C. difficile* as well as *Listeria monocytogenes*[142]. Notably, thuricin CD has little effect on other components of the intestinal microbiota[143], in contrast to the bacteriocins that are produced by *Lactobacillus* spp.[144] and the conventional antibiotic treatments that are currently used to treat *C. difficile* infection.

Various Gram-negative bacteria commonly express type VI secretion systems (T6SSs) that mediate contact-dependent killing of other bacteria and eukaryotic host cells by translocating toxic effector proteins into their targets; for example, *Vibrio cholerae* targets *E. coli* and *Pseudomonas aeruginosa* (all of which belong to the Proteobacteria phylum) for T6SS-dependent killing. A T6SS-mediated attack is lethal to *E. coli*[145]; however, *P. aeruginosa* also expresses a T6SS and can survive attack and mount a lethal T6SS-mediated counterattack on *V. cholerae*[146]. This shows that there is a dynamic interaction between these bacteria. This is probably reflected in the relationships between related commensal bacteria and pathogens in the more complex microbial ecosystem of the intestines.

Proteobacteria

A bacterial phylum, which is abundant in the intestinal microbiota, that is composed of Gram-negative bacteria that can be distinguished by their collective morphological and metabolic diversity.

Segmented filamentous bacteria

(SFB). Gram-positive, spore-forming, non-culturable, Clostridia-related bacteria, provisionally named *Candidatus* Savagella (of the Clostridiaceae family), that closely adhere to the small intestinal epithelium in various vertebrates and that stimulate immune responses.

TRUC model

(*Tbx21⁻/⁻Rag2⁻/⁻* ulcerative colitis model). A mouse model of inflammatory bowel disease that resembles human ulcerative colitis, wherein conventionally-raised mice that lack T-bet and V(D)J recombination-activating protein 2 (RAG2) spontaneously develop an aggressive, highly penetrant, communicable form of colitis.

Type VI secretion system

(T6SS). A protein structure that is used by Gram-negative bacteria to translocate effector proteins that are commonly involved in virulence and bacterial competition into other prokaryotic and eukaryotic cells.
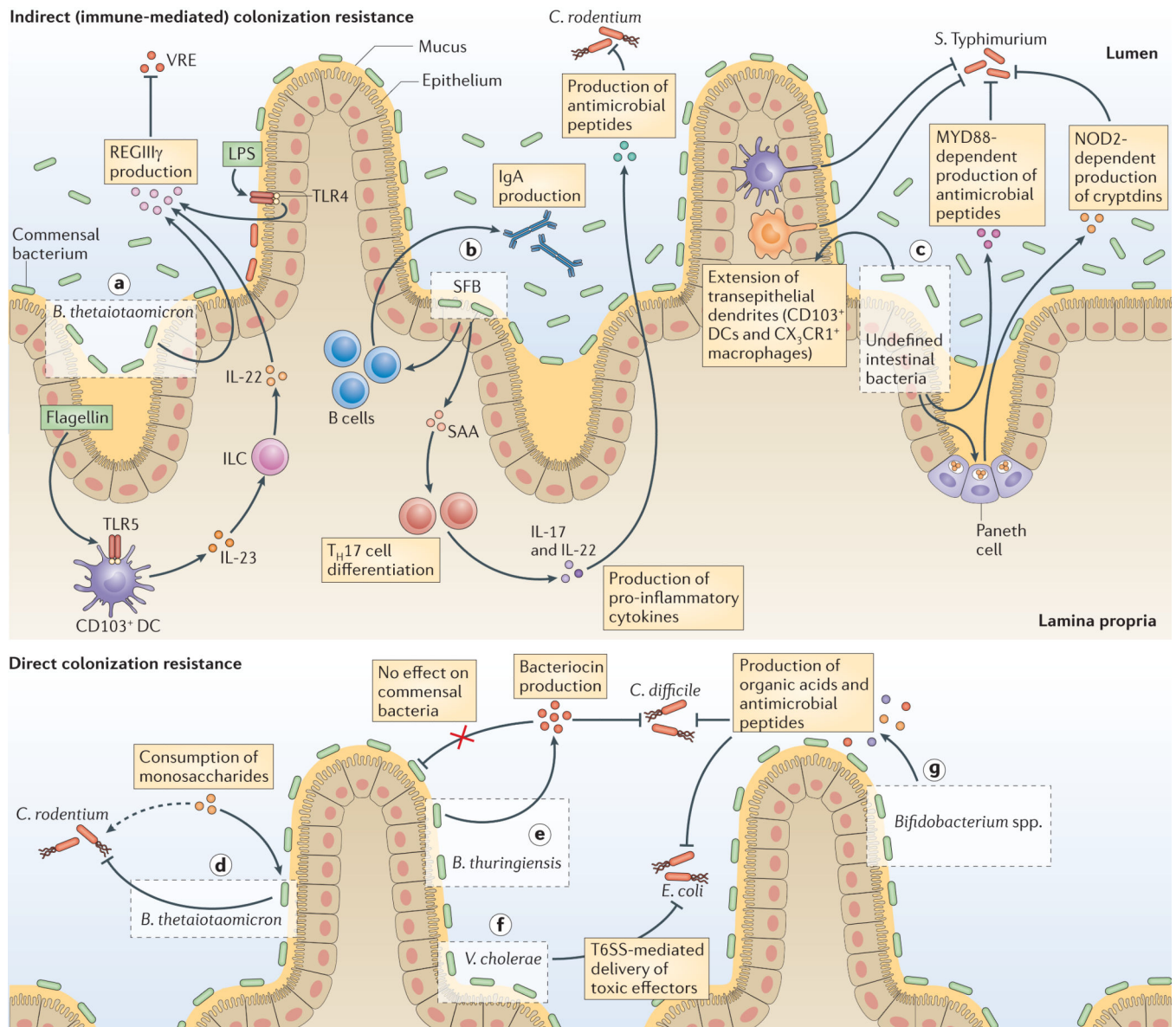
Figure 1. Intestinal bacteria confer indirect (immune-mediated) and direct colonization resistance against enteric pathogens

The intestinal microbiota enhances colonization resistance to intestinal pathogens by both direct and indirect (immune-mediated) mechanisms of action. Commensal bacterial species and microbial products (green) protect against infection indirectly by activating immune responses that in turn target pathogenic bacteria (red) (parts **a**–**c**); for example, *Bacteroides thetaiotaomicron* enhances expression of the peptidoglycan-binding C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ), which is an antimicrobial peptide that primarily targets and kills Gram-positive bacteria. Microbial products such as lipopolysaccharide (LPS) and flagellin stimulate Toll-like receptor 4 (TLR4)+ stromal cells and TLR5+CD103+ dendritic cells (DCs) to enhance epithelial expression of REGIIIγ, which impairs colonization by Gram-positive vancomycin-resistant *Enterococcus* spp. (VRE) (part **a**). Segmented filamentous bacteria (SFB) closely associate with the intestinal

epithelium and enhance IgA production by B cells, serum amyloid A (SAA)-dependent T helper 17 (T_H17) cell differentiation, pro-inflammatory cytokine production and epithelial production of antimicrobial peptides. These processes confer protection against *Citrobacter rodentium* (part **b**). Undefined microbial populations and products activate immune defences, including nucleotide-binding oligomerization domain 2 (NOD2)- dependent cryptdin expression by Paneth cells' myeloid differentiation primary-response protein 88 (MYD88)-dependent production of antimicrobial peptides and extension of transepithelial dendrites by phagocytic DC populations in the lamina propria. These processes enhance resistance to *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection (part **c**). Other bacteria directly inhibit intestinal pathogens by competing for nutrients or by inducing the production of inhibitory substances (parts **d**–**g**); for example*, B. thetaiotaomicron* consumes carbohydrates used by *C. rodentium*, which contributes to the competitive exclusion of the pathogen from the intestinal lumen (part **d**). *Bacteroides thuringiensis* secretes a bacteriocin that directly targets spore-forming Bacilli and Clostridia, including *Clostridium difficile*, through an unknown mechanism of action (part **e**). Gram-negative bacteria, such as *Vibrio cholerae*, deliver toxic effector proteins directly to *Escherichia coli* through type VI secretion systems (part **f**). A variety of *Bifidobacterium* spp. produce organic acids and peptides that impair growth and adhesion of pathogenic *E. coli* to enterocytes (part **g**). Some bacterial populations may inhibit colonization by pathogens through a combination of direct and indirect mechanisms (for example, *B. thetaiotaomicron* (parts **a** and **d**)), or may require other bacteria to carry out antagonistic effects, which complicates the identification and the interpretation of bacterial species that enhance colonization resistance. CX_3CR1, CX_3C-chemokine receptor 1; IL, interleukin; ILC, innate lymphoid cell.
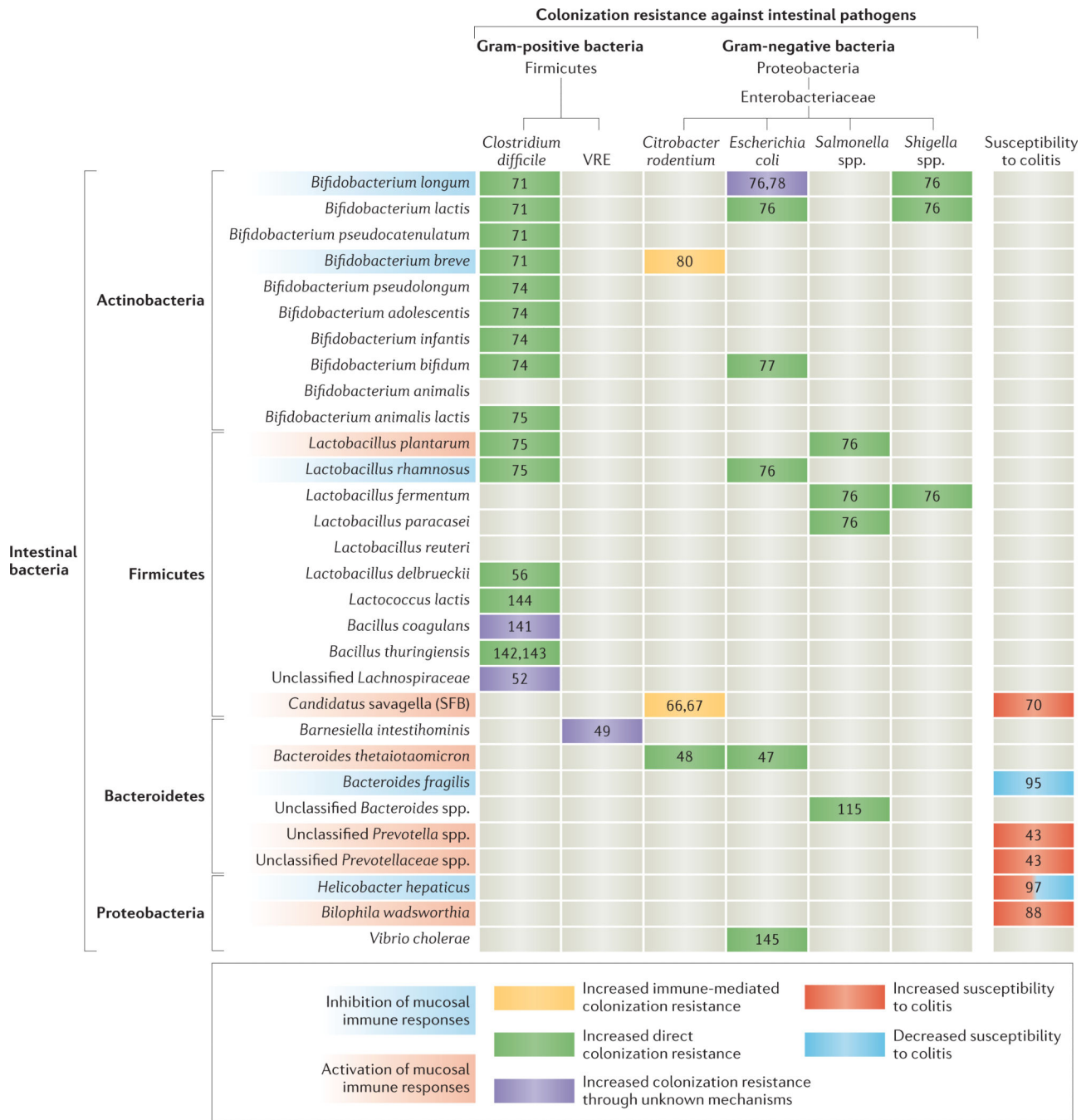
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Figure 2. Phylogenetic relationships of intestinal bacteria that influence host immunity and colonization resistance to pathogens

Individual bacterial species that influence host immune-mediated and/or direct colonization resistance against antibiotic-resistant intestinal pathogens have been identified among the four phyla that comprise the majority of the intestinal microbiota (that is, Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria). A phylogenetically diverse group of commensal bacteria can restrain immune responses (blue), which often facilitates the colonization of intestinal niches and sometimes mitigates inflammatory disease such as colitis (blue cells). A distinct but phylogenetically related group of commensal bacteria

activate mucosal immune responses (red). A subset of these pro-inflammatory bacteria are associated with the development of colitis (red cells), whereas others enhance immunity to intestinal pathogens (yellow cells). Other commensal bacteria secrete antimicrobial factors or otherwise impair the growth and pathogenesis of intestinal pathogens *in vitro*, which suggests that these commensal bacteria directly antagonize pathogens (green cells). Additional commensal bacteria inhibit infection with pathogens *in vivo*, but the dependence of this inhibition on immunity or other host factors remains unclear (purple cells). Reference numbers are indicated in table cells. SFB, segmented filamentous bacteria; VRE, vancomycin-resistant *Enterococcus* spp.