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Roles for Intestinal Bacteria, Viruses, and Fungi in Pathogenesis of Inflammatory Bowel Diseases and Therapeutic Approaches

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Abstract

Intestinal microbiota are involved in the pathogenesis of Crohn's disease, ulcerative colitis, and pouchitis. We review the mechanisms by which these gut bacteria, fungi, and viruses mediate mucosal homeostasis, via their composite genes (metagenome) and metabolic products (metabolome). We explain how alterations to their profiles and functions under conditions of dysbiosis contribute to inflammation and effector immune responses that mediate inflammatory bowel diseases (IBD) in humans and enterocolitis in mice. It could be possible to engineer the intestinal environment by modifying the microbiota community structure or function to treat patients with IBD— either with individual agents, via dietary management, or as adjuncts to immunosuppressive drugs. We summarize the latest information on therapeutic use of fecal microbial transplantation and propose improved strategies to selectively normalize the dysbiotic microbiome in personalized approaches to treatment.

Crohn's disease and ulcerative colitis (UC) appear to result from overly aggressive T cell-mediated immune responses to specific components of the intestinal microbiota in genetically susceptible hosts, with disease initiated and reactivated by environmental triggers.[1–3] This hypothesis implicates reciprocal interactions between host genetics, environmental factors, resident microbiota, and immune responses that normally mediate mucosal homeostasis, but when dysregulated induce and perpetuate chronic immune-mediated inflammation (Fig. 1). We review our rapidly developing understanding of how microbial composition, gene expression patterns, and metabolism regulate mucosal and immune homeostasis vs chronic inflammation and how host genetic and environmental factors influence intestinal microbial function. For additional reviews of dietary effects on inflammatory bowel diseases (IBD) see refs [4–13].

We emphasize major advances in understanding intestinal microbial composition, emphasizing not only enteric bacteria but also fungi and viruses, and extend horizons

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beyond microbial community structure to function, as measured by microbial metagenomic, transcriptomic, proteomic and metabolomics profiles.

Composition and Function of Intestinal Microbiota

Intestinal bacteria

Deep sequencing of variable regions of bacterial 16S ribosomal RNA genes have revolutionized understanding of complex intestinal bacterial ecology, beyond the previous limitations of culturing only the minority of strict anaerobes. Recent advances in improved, more cost-effective sequencing, expanded reference databases and analytic techniques have provided new insights into bacterial/host interrelationships.

Mammalian intestinal bacterial communities are characterized by axial (mucosal to luminal) and longitudinal (proximal to distal) gradients, with large variations between individual subjects[14]. These variations are determined in part by host genetics, diet, antibiotic exposure and maternal colonization at birth. The concept of 2–3 mutually exclusive enterotypes defining human bacterial community structure[15], in part defined by long-term dietary intake[16], has evolved to overlapping dominant assemblages[17]. In the human intestine, the most abundant bacteria are Firmicutes and Bacteroidetes; less-abundant species vary greatly among individuals[18, 19]. Most humans share a core set of resident bacteria and, to an even greater extent, a core set of microbiome-encoded genes and metabolic functions[19, 20].

The intestinal bacterial community evolves from low complexity at birth into a highly diverse network, following weaning and introduction of increasingly complex diets by 9–12 months of age.[21,22] This network becomes stable and resilient to environmental perturbations such as short-term antibiotic exposure or dietary changes[16, 23]. With aging, however, lower diversity and decreased resiliency of the enteric microbiome may contribute to infection and deteriorating mucosal barrier function[24].

These characteristics of stability and resiliency are relevant to therapeutic strategies. Many studies, for example, collect only a single sample for analysis, despite uncertainties about the temporal stability of the intestinal microbiome. There is considerable variation in bacterial community structure among serial fecal samples collected from single subjects, and even more variation in stability among individuals[25, 26]. In contrast to results of Wu et al[16], David et al found rapid alteration of bacterial communities with an extreme short-term dietary intervention[27]. Expression of bacterial genes changes rapidly with dietary changes[27, 28]. Therefore, serial samples should be collected, with optimal intervals to be defined, for studies of the effects of dietary factors and inflammation on the intestinal microbiome.

New imaging technologies can assess interactions among different types of bacteria and between bacteria and the intestinal mucosa [29]. Earle et al[30] developed a computer algorithm (BacSpace) that integrates data from multiple microscopic sections labeled with fluorescent DNA probes bound to specific bacterial species to provide a 3-dimensional image of bacterial interactions. The authors observed clustering of Bacteroidales and Firmicutes in

intestinal sections from mice fed high-fiber diets; these interactions were lost and the mucous layer thinned when mice were placed on low-fiber diets.

Reciprocal interactions between bacteria and intestinal cells have co-evolved; enteric bacteria evolved to live in specialized ecologic niches in different regions of the gastrointestinal tract, and mammals evolved to survive constant exposure to harmful and helpful bacteria. The human microbiota are constantly exposed to human proteins, anti-microbial peptides, proteases, bile acids, antibodies, and mucus. Many of the more than 200 genetic variants associated with risk for IBD alter production or secretion of these factors, affecting barrier function or autophagic clearance of intracellular bacteria[31]. An individual's genetic profile can affect their intestinal bacterial composition and function. Studies of twins demonstrated associated different groups of intestinal bacteria with genes related to diet sensing, metabolism, and immune defense[32].

The relative contributions to an individual's microbiome of genetics and their mother's microbiome are difficult to separate[33], but mechanisms can be studied in mice by cross-fostering or colonizing germ-free mice with fecal transplants or defined bacterial species consortia. Studies using these techniques found that genetic factors strongly affect intestinal bacterial profile[34] and function, and that individual gene variants can have specific effects. Variants in genes that regulate the innate immune response and are associated with IBD affect interactions between immune cells and bacteria. For example, altered microbiota of CARD9-deficient mice do not metabolize tryptophan to protective aryl hydrocarbon receptor ligands. This could account for the observation that transfer of dysbiotic fecal contents from *Card9*−|− mice to germ-free *Card9* wild-type mice increases the severity of colitis that develops following administration of dextran sodium sulfate[35]. Chu et al[31] showed that the ability of outer membrane vesicles of *Bacteroides fragilis* to protect mice colitis and promote development of regulatory T cells requires autophagy, mediated by ATG16L1 and NOD2. However, previous studies in mice showed that the presence or absence NOD2 did not affect the composition of fecal bacteria[36], in contrast to several studies reporting the association of mucosal-associated Enterobacteriaceae with *NOD2* polymorphisms in patients with IBD[37, 38]. Research is needed to resolve the disparities between findings from mouse and human studies.

Increasingly cost-effective and rapid deep sequencing technologies, relatively complete reference bases, free on-line bioinformatics platforms, and 16S rRNA sequencing techniques have revolutionized investigation of the intestinal microbiome. However, data collected from these approaches are restricted to broad taxonomic profiles—users cannot distinguish species or strain variations that define bacterial function—believed to be more important than microbial composition[14, 39]. Expanding reference bases now support shotgun metagenomic, transcriptomic, proteomic, and metabolomics profiles of bacterial function. (Fig. 2) Deep shotgun metagenomic sequencing expands resolution to the bacterial strain level, well beyond the genus level reached by 16S rRNA sequencing.[40, 41,42]. This more sensitive culture-independent sequencing technique will supplant current 16S surveys, particularly for serial community structure and stability studies, as newer software programs such as metaphlan2 and Constrains are widely used.[43]. Furthermore, metagenomic sequencing has additional benefits— it allows users to determine bacterial gene

representation and characterize the nonbacterial microbiota (fungi, archaea, and viruses). Genomic characterization, together with other more functional assays such as transcriptomics and metabolomics, can be used to evaluate rapid responses of resident enteric bacteria to environmental factors, such as diet, infection, and smoking[27, 28] that affect risk of IBD, as well as bacterial responses to the inflammatory milieu[44].

Current research into the gut microbiome focuses predominantly on taxa within the Kingdom Bacteria. Nevertheless, as DNA sequencing technologies, computational tools, and annotated databases continue to improve, growing attention is being paid to other intestinal microbes, such as fungi and viruses.

Fungi

Fungi are generally considered to be a relatively minor component of the gut microbiota, accounting for approximately 0.1% of the microbes[18]. However, this may be a significant underestimation due to challenges in the annotation of fungi in current genomic databases[45]. Mis-attribution of sequences and classification of sexual and asexual forms of the same fungus are some of the current limitations to characterizing fungal populations (the mycobiome) using next-generation DNA sequencing technology. Nevertheless, sequencing of marker genes such as 18S and its internal transcribed spacer with comparisons to annotated databases has led to a more complete understanding of the mycobiome in complex microbial communities such as the gut[46].

Fungi are ubiquitous, so we would expect that they would be found on every mucosal surface in the human body, varying in composition at each body site (similar to the bacterial microbiota). The urogenital tract, oral cavity, and gastrointestinal tract, where taxa of the *Candida* genus often predominate, contain approximately 160 species[47, 48]. There are species-specific colonization patterns of *Candida* in mammals; *Candida tropicalis* are common in mice whereas *C albicans*, *C blabrata*, and *C parapsilosis* predominate in humans[45]. In contrast to the relative stability of murine bacterial communities over time, cage effects have been observed in mouse gut mycobiota, which vary over time, indicating that the environment has strong effects on fungi in the gut[49].

Diet could affect the gut mycobiota. In humans, the presence of *Candida* was associated with diets high in carbohydrates, but not with diets high in amino acids, protein, and fatty acids[50]. These associations were also observed in the fecal mycobiota in human participants of a controlled feeding experiment; *Candida* species were increased in subjects consuming a plant-based diet and reduced in subjects on an animal-based diet[27].

Studies support the concept of a competitive relationship between bacteria and fungi in the gut. In humans, prolonged use of antibiotics promotes fungal infection and overgrowth, germ-free mice are susceptible to infection with Candida, and antibiotics lead to overgrowth of fungi in the gut [49, 51]. Consistent with these observations, antibiotic-induced fungal overgrowth in the gut primes the host for the development of allergic airway responses to an exposure to mold spores[51]. These observations provided evidence for the gut mycobiota in development of immune-mediated diseases in other parts of the body, away from the intestinal mucosal.

There is similar evidence to support a role for the intestinal mycobiota in the pathogenesis of IBD. Microbes can reduce immune tolerance, leading to inappropriate activation of pathways designed to protect against pathogen invasion. The complex and incompletely characterized innate immune response against molecules in fungal cell walls is reviewed in[52]. Glycoprotein cell wall components, beta-glucans, chitin, and mannans can activate components of the innate immune system such as toll-like receptors (TLRs 2 and 4 predominantly), dectin-1 (also known as CLEC7A, a C-type lectin receptor), members of the scavenger receptor family (CD5, CD 36, and SCARF1), and components of the complement system. Activation of these molecules leads to immune signaling via molecules such as CARD9, interleukin 17 (IL17), IL22, NF-κB, NFAT, and ITAM[45].

Viruses

Viruses are among the most diverse and abundant biological entities. A large proportion of viruses, bacteriophage, infect prokaryotic organisms (Bacteria and Archaea). Bacteriophage have a virulent (lytic) cycle, which leads to rapid viral replication and bacterial cell lysis, and a temperate (lysogenic) cycle, which leads to prophages that integrate their genetic material into prokaryotic genomes or reside as extra-chromosomal plasmids. The transmission of antibiotic resistance genes or virulence factors into the bacterial genome are 2 examples of ways in which lysogeny can alter the biological function of gut bacteria and health of the host; 1 example is the phage origins of cholera and shiga toxins.

The dynamics of bacteriophage interaction with its bacterial host can be described as a predator–prey relationship.[53] The abundance of bacteriophage (outnumbering bacteria by as much as 10 to 1) and their diversity have been proposed to affect the composition of bacteria communities in aquatic ecosystems,[54] and in the gut. There is remarkable inter-person and intra-person variation in the human gut prokaryotic virome, but significant stability over time [55]. The high inter-person variation in bacteriophage communities could be due to the persistence of a small portion of the global virome within the gut of each individual as well as the rapid evolution of some long-term members of the virome[56]. Additionally, the human prokaryotic gut virome may respond to diet, similar to the bacterial gut microbiota[16, 57]. As a defense mechanism against bacteriophage infection, bacteria and archaea have a mechanism, known as the clustered, regularly interspaced short palindromic repeat (CRISPR) system, that can lead to acquired phage resistance[58]. This prokaryotic mechanism has led to the development of the CRISPR interference technique for genome editing in both plants and animals.

The effects of bacteriophage on bacteria might be exploited therapeutically[59]. Interest in phage-based therapies was initially hampered by inadequately controlled trials and the discovery of antibiotics. However, the increase in multidrug-resistant bacteria has renewed interest in using phages as antimicrobial agents. Additionally, the prokaryotic virome might directly affect the mammalian immune system[60]. Additional technologies and resources are needed to address the myriad of challenges to studies of the prokaryotic virome and its effects on mammals, including better annotated databases of viral DNA sequences and techniques to deeply characterize RNA viruses.

Although humans carry a variety of enteric viruses, it has been hard to study viruses in fecal samples from healthy individuals or patients with Crohn's disease using shotgun metagenomic sequencing technologies[61]. Nevertheless, studies in mice indicated that the eukaryotic virome can affect mammalian health (recently reviewed in[62]). For example, norovirus supports intestinal homeostasis and shapes mucosal immunity in germ-free mice, similar to the beneficial function of commensal bacteria[63]. Interestingly, intestinal bacteria can promote viral infection. Transmission of mouse mammary tumor virus from mother to offspring via milk requires gut bacteria[64]. This virus binds to bacterial lipopolysaccharide (LPS) leading to an alteration in host immune tolerance thereby promoting virus replication and transmission[65].

Microbiota in Development and Progression of IBD

There is considerable clinical and experimental evidence that dysbiosis of the intestinal bacteria, with developing evidence for fungi and viruses, contributes to development of Crohn's disease, ulcerative colitis, pouchitis, and chronic experimental intestinal inflammation [1, 4–8, 10, 12, 31, 66–68]. Studies with new technologies, testing subsets of microbes with specific functions in gnotobiotic mice, have revealed important interactions between microbes and host cells that define mucosal homeostasis vs inflammation, and disease progression and resolution.

Bacteria in pathogenesis

Dysbiosis is a cause as well as an outcome of IBD. Characteristic compositional changes observed in patients with IBD include decreased bacterial diversity, with expansion of putative aggressive groups (such as Proteobacteria, Fusobacterium species, and *Ruminococcus gnavus*) combined with decreases in protective groups (such as Lachnospiraceae, Bifidobacterium species, Roseburia, and Sutterella) (Table 1)[4, 6, 10, 13, 69–73]. This dysbiosis is present at early stages of disease progression, before patients have been treated, but is affected by prior use of antibiotics[61, 69, 73].

It is important to determine whether these alterations are the cause or consequence of inflammation for development of therapeutics, diagnostic and prognostic tests, and strategies to monitor response to treatment. There is evidence for dysbiosis as a cause IBD and T cell-mediated chronic experimental colitis[74, 75]. For example, mice develop colitis following transfer of microbiota from feces of mice with colitis compared with mice without colitis (controls)[76], fecal transplants reduce symptoms in some patients with UC, and patients with pouchitis respond to antibiotics and certain probiotic combinations.[77]

In contrast, there is also compelling evidence that dybiosis is the response of a complex microbial community to inflammation, as well as antibiotics or diet[61]. Active IBD and experimental ileocolitis directly alters bacterial composition and gene expression[44, 78–81], luminal (fecal) and mucosal dysbiosis in various intestinal regions correlates with disease activity, being less abnormal in unaffected regions[69, 70, 79]. Proposed mechanisms for inflammation-induced reduction of strict anaerobes, such as Clostridium groups IV or XIVA, with parallel expansion of aerobic and facultative anaerobic taxa belonging to the Proteobacteria phylum such as Enterobacteriacea, include increased ambient oxygen

concentrations with hyperemia and increased vascular and mucosal permeability[82] disruption of physiologic epithelial NA+/H+ exchange causing dysregulated electrolyte concentrations[80], and the production of alternative electron acceptors that promote anaerobic respiration of facultative anerobes[83]. In mice, colitis alters enteric bacterial gene expression, whereas targeted disruption of *E coli* genes that are most highly induced by inflammation affects the severity of colitis[44, 84]. Furthermore, inflammation alters epithelial defenses, mucus thickness, and viscosity; this could account for increased association of bacteria such as *E coli* with the mucosa in patients with IBD[85]. Severe tissue damage with ulceration likely provides easy access for invasive, oxygen-tolerant bacteria[86].

Bacteria affect mucosal immune responses and bacterial function that determine mucosal homeostasis vs inflammation. Recent reviews highlight the effects of resident intestinal bacteria on differentiation and function of mucosal T cells, innate immune cells, innate lymphoid cells and IgA[87–91]. Many bacterial species that are selectively altered in the dysbiosis that accompanies active IBD have functional activities that mediate experimental intestinal homeostasis vs inflammation. This is particularly well documented for immune functions mediated by a Clostridium species subset, and *Faecalibacterium prausnitzii,* which might protect against IBD. These species are specifically decreased, whereas levels of aggressive *E coli* are increased, in the intestinal microbiota of patients with IBD (Fig. 2A). Seventeen strains of Clostridium have immune suppressive activities that inhibit acute and chronic experimental colitis[92]. These strains induce T-regulatory cells (Treg cells) mediated by IL10, ICOS,[92] and butyrate[93] and activate the epigenetic DNA methylation adapter UHR1, which affects differentiation and proliferation of Tregs[94]. These effects require a full T-cell receptor repertoire to maintain Treg cell-mediated immunologic tolerance to the intestinal microbiota[95]. Mice with restricted T cell receptor repertoires developed spontaneous colitis and IL17 production driven by intestinal microbiota, likely as a consequence of defective regulatory T cell function..

A single bacterial species might protect against disease pathogenesis where its abundance may have utility as a biomarker. *F prausnitzii* is decreased in patients with CD whereas low mucosal abundance predicts post-resection disease relapse[96]. From a functional standpoint, this species or its supernatant suppressed experimental colitis and the supernatant decreased NFκB activation and inflammatory cytokines while stimulating IL10 production[96]. Additionally, various *F prausnitzii* isolates have differential abilities to simulate IL10 secretion by dendritic cells[97].

Secretion of a 15 kDa anti-inflammatory molecule inhibits NFκB activation and prevents development of colitis in mice[98] and induces production of protective metabolites, including salicylic acid, which inhibits colitis[99]. Finally *F prausnitzii* can induce a unique CD4CD8αα Treg-cell subset that secrets IL10: these *F prausnitzii*-responsive regulatory cells are decreased in patients with IBD[100]. Likewise, a capsular polysaccharide from *Bacteroides fragilis* secreted within outer membrane vesicles activates Treg cells and reduces the severity of colitis in mice via TLR2 signaling[101–103]. These protective activities of *B fragilis* require proper ATG16L1- and NOD2-mediated autophagy[31], and provide mechanisms for defects in immune regulation in patients with CD. Together, commensal enteric bacterial species that are reduced in patients with IBD selectively induce well-defined immune regulatory

pathways; this should guide development of therapies. Multiple searches for additional immunoprotective commensal species are underway, including screening human strains for regulatory activity in gnotobiotic mice[104].

Alternatively, aggressive functionally altered resident strains (pathobionts) expand in the intestine of patients with IBD to promote pathogenic immune responses (Fig. 2B). Mechanisms by which adherent and invasive *E coli* (AIEC) and *Enterococcus faecalis* activate disease-promoting immune responses and epithelial damage have been particularly well characterized. AIEC are found in the mucosa of approximately 30%–40% of patients with ileal Crohn's disease[85, 105]. These functionally altered *E coli* adhere to and invade intestinal epithelial cells and persist and replicate within epithelial cells and macrophages. AIEC strains isolated from mice and a patient with Crohn's disease induced colitis in mono-associated gnotobiotic IL10-deficient mice and promoted development of *E coli* antigen-specific interferon gamma (IFNG)- and IL17-producing $CD4^+$ cells; lack of disease in mice with wild-type *Il10* indicates that this strain is not a pathogen[106].

When gnotobiotic *Il10*−\− mice were colonized with 8 bacterial species associated with IBD, AIEC and *Ruminococcus gnavus* antigens specifically elicited secretion of IFNG and IL17[78]. Mechanisms by which AIEC adhere to, invade, and persist within cells have been characterized[107, 108]. Unique AIEC gene products determine epithelial attachment via long polar fimbria and mutated FimH to human epithelial CEACAM 6, biofilm formation, mucus penetration, epithelial cell invasion and persistence within epithelial cells and macrophages[109–113]. Variants in genes associated with risk for Crohn's disease that regulate autophagy promote AIEC intracellular persistence; AIEC modulate autophagic clearance[114, 115]. These pathways provide opportunities for novel therapeutic approaches to block attachment or promote clearance[108, 116]. Unfortunately, current molecular signatures cannot distinguish AIEC from non-pathogenic *E coli* strains, although sequencing of broad panels of clinical isolates have identified enriched pathways, such as propanediol utilization and iron acquisition[117, 118]. These observations, along with identifying AIEC genes most highly expressed under inflammatory conditions[44] may help to identify specific inhibitors of AIEC pathogenicity.

E faecalis are also involved in IBD pathogenesis. These bacteria can activate bacterial antigen-specific T cells induce chronic colitis in mono-associated *Il10*−\− mice[106]. The co-colonization of *E faecalis* and AIEC strain dramatically accelerate and potentiate experimental colitis, indicating inflammatory bacterial reciprocal interactions[119]. Mechanisms by which *E faecalis* induce injury include metalloprotease damage of epithelial barrier integrity through protease-activated receptor 2[120, 121] and activation of innate immune pathways via TLR2 ligation by membrane lipoproteins[122]. One caveat is that *E faecalis* has not been shown to be consistently increased in IBD patients.

Metagenomic, transcriptomic, proteomic, and metabolomic profiles integrated from microbial and mammalian sources have considerable potential for providing insights into regulation of mucosal homeostasis vs inflammation, developing improved biomarkers of disease activity, and differentiating clinically important disease subsets (Fig. 3). However, these technologies have not yet been widely used in studies of IBD, so reference data sets

are incomplete[7, 123–126]. Metagenomic sequencing has distinct advantages over imputed (inferred) information from 16S sequences because actual gene content within the microbiota can be characterized. Analysis of the meta-transcriptome goes even further—this technique examines expression patterns of genes under conditions of inflammation[44].

Metabolomic data provide exceptionally important information regarding small molecules that are either produced or modified by the gut microbiota that affect mucosal responses. These molecules include short-chain fatty acids (SCFAs), which stimulate mucosal protection and immune regulatory functions, bile acids, and injurious hydrogen sulfide. Levels of volatile organic metabolites are increased in stools and exhaled breath samples from patients with active IBD, whereas fecal levels of SCFAs are decreased[72, 127–129]. Decreased SCFA is consistent with lower concentrations of butyrate-producing commensal bacterial species, such as Lachnospiraceae, Roseburia, and *F prausnitzii,*[72, 96, 130] and the poor outcomes of persons with low dietary fiber intake[131]. Likewise, fecal levels of medium-chain fatty acids were reported to be decreased in patients with UC, Crohn's disease, or pouchitis; fecal concentrations of hexanoate correlated inversely with disease activity[123]. Levels of trimethylamine-N-oxide, produced by bacterial metabolism of dietary carnitine and phosphatidylcholine followed by hepatic metabolism, are decreased in plasma samples from patients with IBD vs controls and correlate with activity of UC but not Crohn's disease[132].

Altered fecal bile acid profiles (such as increased conjugated and sulfated bile acids and decreased secondary bile acids)[133] can be used in diagnosis and assessment of patients with IBD. Secondary bile acids have anti-inflammatory properties, and bacterial release of sulfide from sulfated bile acids can induce colitis in mice[134]. Although bacterial transcriptome profiles are incompletely studied, ileal gene expression in untreated pediatric patients with Crohn's patients correlate with bacterial community structure[135]. Increases in antimicrobial dual oxidase were associated with increased Proteobacteria, whereas decreased expression of apolipoprotein A1 (APOA1) correlated with low levels of Firmicutes.

The non-bacterial gut microbiota in pathogenesis

Some evidence supports a functional role for fungi in the pathogenesis of IBD and a comprehensive review on this topic has recently been published[136]. CLEC7A knockout mice have increased susceptibility to chemically induced colitis due to their altered responses to indigenous fungi[137] (CLEC7A recognizes β-glucans in the fungal cell wall). Furthermore, a polymorphism in the gene encoding dectin-1 is associated with a severe form of UC in humans[137]. A recent report shows that prolonged treatment of mice with antifungal drugs worsen colitis as well as allergic airway disease and led to altered bacterial microbiota as well as mycobiota with reduced representation of *Candida* spp. and increased *Aspergillus*, *Wallemia*, and *Epicoccum* spp.[138] The administration of the latter three fungal organisms recapitulated the development of airway disease.

Observations from mice parallel the role for fungi in the pathogenesis of IBD in humans. Antibodies against *Saccharomyces cerevisiae*, a marker of CD, react with mannan, a yeast cell wall polysaccharide[139]. Multiple studies have shown that patients with IBD have alterations to the gut mycobiota; increases in specific fungal taxa have been associated with

bacterial dysbiosis, increased human DNA in feces, and antibiotic use[61, 140, 141]. There is preliminary evidence that the anti-fungal agent fluconazole reduces inflammation mice with colitis and patients with IBD[142]. A recent study reported expansion of fecal *Candida tropicalis* levels in patients with CD, which correlated with fecal *E coli* and *Serratia marcescens* concentrations as well as serum anti-*S cerevisiae* titers. These 2 bacterial species and the fungus *C tropicalis* formed enhanced interkingdom biofilms in cultures.[143]

The composition of complex bacterial communities may be, in part, determined by the dynamics of relationships between bacteriophage and bacteria, so there is interest in characterizing the prokaryotic virome in patients with IBD. Recently, Norman et al described the enteric virome of 3 independent cohorts of patients with IBD individuals without IBD in the same households (controls)[144]. Shotgun metagenomic sequencing of fecal virus-like particles showed significant expansion of *Caudovirales* bacteriophage in patients with CD or UC, disease- and cohort-specific viromes, and increased virome diversity in patients with IBD, compared with controls; this is in contrast to the reduced diversity and richness of the bacterial microbiota in patients with IBD. However, the virome was not altered over time as disease activity changed. These results support a model in which bacteriophage contribute to development of bacterial dysbiosis associated with IBD. Additionally, the disease-specific nature of the enteric virome indicates the potential value of characterizing the composition of bacteriophage in patients with IBD, which could lead to new biomarkers.

Studies in mice have shown that eukaryotic viruses might be involved in IBD pathogenesis. Humans with a polymorphism in the CD susceptibility autophagy gene *ATG16L1*[145] have alterations in Paneth cell morphology that is phenocopied in mice with a hypomorphic allele *Atg16L1* but only in mice infected with murine norovirus[146]. Interestingly, although the Paneth cell phenotype in these mice depends on infection with murine norovirus, the development of colitis in this model can be ameliorated by antibiotic treatment showing that viral-induced pathology can be driven by transkingdom interactions with bacteria[62].

Finally, reduced immune surveillance associated with immune suppression in patients receiving treatment for IBD might lead to alterations in the eukaryotic virome that could be used as biomarkers. This concept has been demonstrated in solid organ transplant recipients. In these patients, the plasma viral load of anellovirus correlated inversely with graft rejection[147]; similar observations were made in recipients of lung transplants, based on analysis of broncoaveolar lavage fluid[148]. Further studies are needed to support this model for patients receiving immunosuppression for IBD, since no significant anellovirus signature was observed in the feces of pediatric patients receiving treatment with a tumor necrosis factor antagonist[61].

THERAPY

Differences in profiles of protective vs detrimental microbiota, and their genes and metabolic functions, could be used to discover therapeutic targets for IBD. Analyses of individual microbial profiles and activities could be used to develop personalized treatments.

Targeting dysbiosis

Antibiotic and probiotic therapies have only modest effects in patients with IBD; these are far less impressive than initially anticipated (see Fig. 4). The exception is pouchitis, for which antibiotics are the preferred approach—perhaps in combination with probiotic cocktails to maintain remission[149, 150]. Single antibiotics provide some benefit to patients with Crohn's colitis or septic complications, such as abscesses and fistulae; they might prevent post-resection recurrence, but have provided no demonstrable benefits to patients with UC. Broad antibiotic combinations might improve outcomes[151, 152], although long-term efficacy is likely limited by development of antibiotic resistance. Traditional probiotics have a limited role in treatment of UC, whereas *E coli* Nissle and the probiotic combination VSL#3 can maintain remission and possibly reduce active inflammation. However, these agents do not benefit patients with CD.

These modest results may be due to reliance on non-native probiotic species, which are unable to colonize the intestine and are rapidly cleared. Therapeutic strategies might be developed to restore levels of certain clostridium groups that are decreased in patients with IBD, including *F prausnitzii*, which protects the gut, as well as immunosuppressive and barrier-enhancing SCFAs, which stimulate Treg cells and production of IL10. *F prausnitzii*, 17 strains of clostridium, and *B fragilis* reduce the severity of colitis multiple mouse models [92, 96, 102]. Moreover, molecules secreted by these species can be screened for efficacy in vivo, and might provide new sources of therapeutic agents. Although prebiotic formulations have not been well studied, the concept of providing dietary substrates such as fiber and prebiotic oligosaccharides to selectively increase the abundance of SCFA-producing commensal species seems attractive.

Alternatively, recently discovered pathogenic mechanisms of putative aggressive bacterial species that expand during inflammation provide highly selective therapeutic targets. For example, blocking AIEC epithelial adherence by glycopolymers or FimH antagonists, or inducing blocking antibodies to flagellin might inhibit AIEC epithelial invasion and translocation[116, 153]. Likewise, blocking the protease activity of *E faecalis*, or protease receptor binding, might inhibit the mucosal permeability mediated by these molecules[120, 121]. Similarly, specifically blocking expression of virulence gene products or their activity could diminish the pathogenic activity of aggressive bacterial populations that expand in the dysbiotic inflammatory environment.

Management of IBD is limited by our inability to predict its aggressiveness or response to therapeutic agents, leading clinicians to adopt either bottom-up or top-down treatment approaches. Either approach is inefficient, with under treatment of some patients leading to disease complications or, conversely, overaggressive treatment resulting in unnecessary medication side effects and expense. Rapidly improving 16S deep sequencing, metagenomic profiling, and metabolomic databases should lead to rational microbial profile analyses. Knowledge of which protective microbes are absent, decreased, or expanded will guide selection of therapy to optimize an individual's microbial balance and function. This selective approach will depend on the availability of highly individualized mixtures of various protective species[154] and effective ways to target expanded aggressive species.

This ambitious therapeutic approach requires clinically available, cost-effective diagnostic tests to rapidly determine a patient's microbial structure and function. We also need markers that can predict which patients are likely to have a benign vs aggressive course, to optimize use of available therapies. There is evidence that microbial profile analyses could provide these types of prognostic markers. For example, level of APOA1, combined with data on microbial structure, can predict steroid-free remission in children newly diagnosed with Crohn's disease[135]. Pre-operative ileal concentrations of *F prausnitzii* can determine risk for post-operative recurrence of Crohn's disease[96]. Bacterial profiles can determine risk of pouchitis after colectomy in patients with UC[155]. Microbiome signatures have been associated with response to therapy[156] and dysbiosis has been associated with relapse in patients who stopped taking infliximab[157].

Fecal microbiota transplantation (FMT)

It is ironic that during a time of rapid technological advances in DNA sequencing and computational tools, which have increased our understanding of the gut microbiome, there has been renewed interest[158] in FMT—a highly effective therapy for recurrent *Clostridium difficile* infection (CDI)[159]. From a scientific standpoint, the success of FMT in treating CDI provides important proof for the concept that dysbiosis can be modified to treat a human disease. This approach has been extended to studies of other disorders, such as IBD[160] and metabolic syndrome[161]. There are currently 68 clinical studies involving FMT for indications other than CDI, including 5 for patients with Crohn's disease and 15 for UC (Clinicaltrials.gov 06/02/16).

Evidence for the efficacy of FMT in patients with IBD is equivocal; initial evidence has been based on small case reports or studies that were under-powered, open-label, lacked uniformity in treatment protocols and delivery approaches, or did not include control groups (reviewed in[162]). A systematic review and meta-analysis of 18 studies that included 122 patients with IBD found that 45% of patients achieved clinical remission, but only 36.2% of patients in cohort studies[163]. Results have been reported from 2 placebo-controlled trials of FMT for patients with UC; only 1 achieved its primary endpoint for clinical effficacy[164,165]. Although there were significant differences in the design of these 2 studies that may have contributed to the divergent outcomes[166], together with the lack of consistent efficacy in uncontrolled studies, it is clear that additional studies are needed before FMT can be recommended as a treatment for IBD. Caution is required because there is evidence that some patients with UC developed fevers and increased levels of c-reactive protein after FMT [167]. Furthermore, disease flares in patients with UC or Crohn's disease after FMT for the treatment of CDI have been described [168–170], raising important questions around the potential to worsen disease in some patients.

There is no strong evidence for the efficacy of FMT for diseases other than CDI, so FMT should be considered an experimental procedure for non-CDI indications. The effects of FMT in patients with IBD should be studied only in well-designed clinical studies, with approval from the Food and Drug Administration via submission of an investigational new drug application. Findings from these studies can be used to determine efficacy, based on rigorous evidence, and to document adverse outcomes[171]. Even for patients with CDI, in

whom FMT has been shown to be efficacious with little evidence for significant short-term adverse outcomes, there are unclear long-term consequences of transferring an uncharacterized complex and dynamic consortium of bacteria, fungi, arachaea, and viruses from another person. Thirty percent to 50% of the donor's bacterial microbiota persists in the recipient after FMT[42] and bacteriophage transfer from donor to recipient as also been documented[172]. The American Gastroenterological Association, together with the Infectious Diseases Society of America, the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, and the Crohn's and Colitis Foundation of America, will be developing a National FMT Registry, with funding from the National Institutes of Health. This registry can be used to determine the short- and long-term safety of FMT, gather information on practice in the United States, and assess its effectiveness; it should promote further studies of microbial transfer.

Ultimately, it is likely that FMT will be replaced by the use of defined microbial consortia prepared under laboratory conditions. Defined combinations of microbes would have more predictable responses and reduce adverse outcomes, including the risk for pathogen transmission. Results from studies of mice with colitis indicate that such a targeted approach might be feasible for treatment of IBD[92].

FUTURE DIRECTIONS

The incidence of IBD is increasing globally, associated with industrialization. Changing environmental factors are likely to affect the human intestinal microbiota and contribute to the pathogenesis of IBD. The composition and function of luminal and mucosal bacterial, fungal, and viral communities are reproducibly altered in IBD and models of ileocolitis; this dysbiosis promotes aggressive mucosal immune responses and injury that perpetuate disease. It is not clear whether dysbiosis is a primary initiator of IBD, but genetic variants associated with IBD affect bacterial composition and alter immune responses to resident microbiota. The interactions between the dysbiotic microbiota and a dysregulated immune response as fundamental pathogenic elements strongly supports selective targeting of the gut microbiota as a therapeutic approach for IBD. This would be most effective if it is selected based on an individual's microbial, genetic, and immunologic factors. Although traditional antibiotics, probiotics, and prebiotics have limited efficacy, findings that gut bacteria affect chronic inflammation in animal models provide a rationale to discover agents that alter the intestinal microbiota in patients with IBD and microbial markers that can predict patient outcome. These can be identified using available genomic, transcriptomic, and metabolomic technologies and rapidly developing computational and biostatistics tools.

Agents or dietary changes that alter the intestinal microbiota might be effective alone as treatments of IBD, or as adjuncts to current immunosuppressive drugs. Profiles of a patient's microbiome and metabolome could be used to determine the optimal composition and diet for treatment. Whether this personalized normalization of disrupted microbiomes will effectively treat active inflammation, or more likely maintain remission induced by traditional anti-inflammatory or immunosuppressant therapies, remains to be determined. The ambitious goal of using personalized targeted therapy to optimize microbial function will require improved diagnostic techniques, improved metabolomic databases, and large

investments in prospective serial profiling techniques to define individual microbiota community structure and function during various phases of IBD disease activity.

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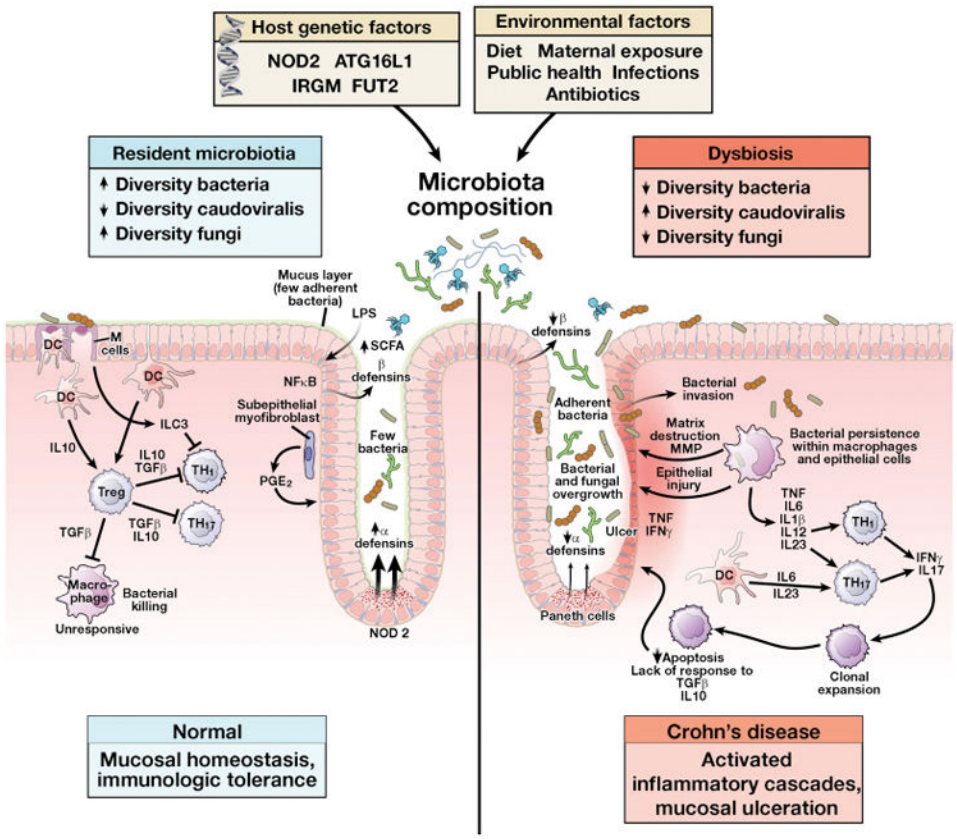

Figure 1. Genetic and Environmental Factors that affect the Intestinal Microbiota

Key commensal microbes regulate activity of the immune response, including Treg cells, and mucosal homeostasis. Antigens from certain dysbiotic microbes activate T-helper 1 (TH1) and TH17 cells, leading to tissue injury. This mucosal injury leads to further uptake of microbial antigens, toll-like receptor (TCR) ligands and viable organisms that perpetuate immune responses.

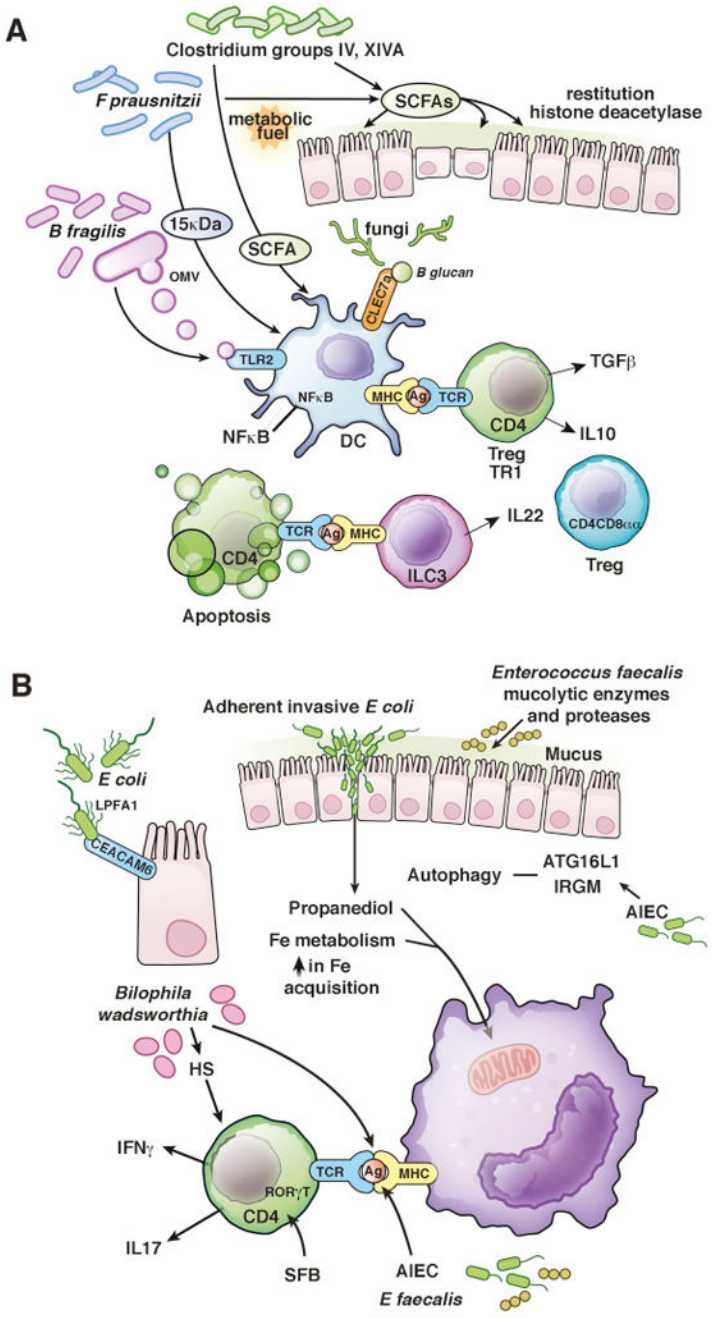

Figure 2. Mechanisms of Barrier Function and Immune Regulation by the Intestinal Microbiota

A. Commensal bacterial and fungal species produce SCFAs and TLR ligands that activate protective epithelial and lamina propria innate cells, while microbial antigens, immunoregulatory proteins and secreted SFCAs stimulate adaptive regulatory cells. B. Products of microbial species expanded during dysbiosis injure epithelial cells and activate effector cells. Adherent and invasive *E coli* penetrate epithelial cells, proliferate within epithelial and antigen-presenting cells and produce antigens that stimulate TH1 and TH17 cells. These bacteria proliferate in the presence of ethanolamine, propanediol and iron liberated by the inflammatory process. Production of hydrogen sulfide by *Bilophilia*

wadsworthia stimulate TH1 cells and segmented filamentous bacteria (SFB) specifically activate TH17 cells. Mucolytic enzymes and proteases produced by *E. faecalis* injure the mucosal barrier, which promotes uptake of injurious microbial products and viable organisms.

Ag; Antigen, MHC; major histocompatibility complex, LPFA1; long polar fimbria A1, Fe; iron.

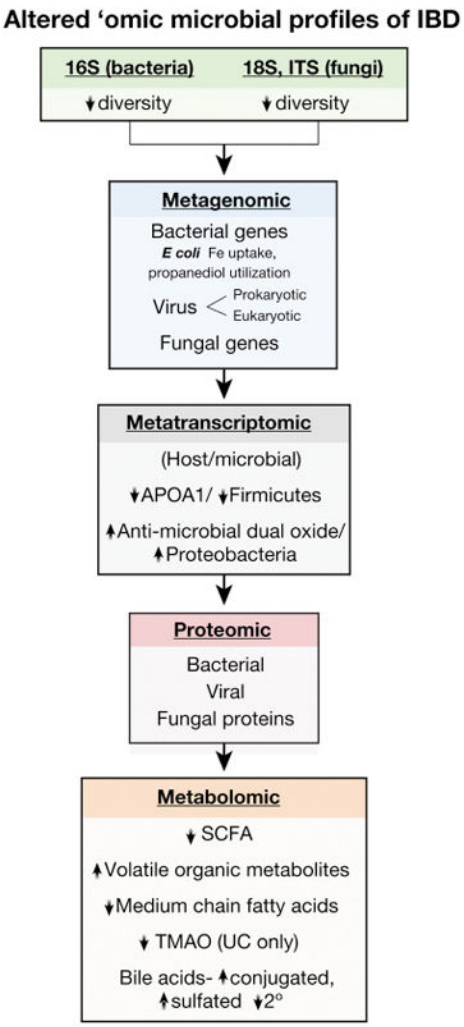

Figure 3. Microbial Profile, Genome, Transcriptome, Proteome, and Metabolome Features of Individuals With vs Without IBD

Arrows indicate whether these features are increased or reduced in patients with IBD compared to persons without IBD. Extensive data are available for bacterial 16S rRNA profiles of normal subjects and patients with IBD, however fungal 18S or ITS sequencing, shotgun metagenomic sequencing and metabolomic studies are only now beginning to be performed. Fecal metatranscriptome and proteomic studies of microbes and IBD are rudimentary. The sequence of 16S and ITS profiles leading to metabolomic studies is depicted because of timelines of applications of these –omic technique to intestinal bacteria and because microbial composition determines the genes present (metagenomic results). The available microbial genetic pattern, along with the environment and diet, help to determine which microbial genes are transcribed (metatranscriptomic profiles), the proteins produced and metabolites secreted under homeostatic vs. inflammatory conditions.

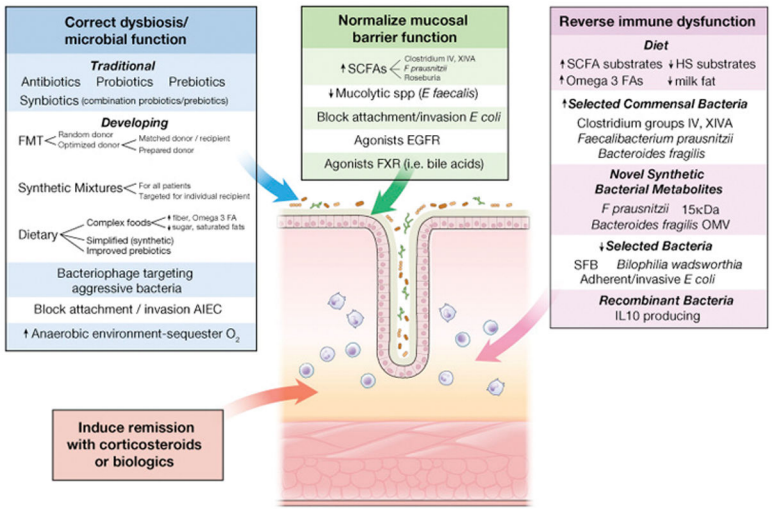

Figure 4. Treatment of IBD by Altering Microbial Composition or Function

A sustained remission of IBD might be achieved by sequential induction of remission using traditional corticosteroids and/or biologic therapies, followed by less toxic and more physiologic therapies that specifically target the microbiota. Alternatively, it may be possible to primarily treat inflammation by targeting the microbiota. The goal of this microbiota-centric therapy is to correct dysbiosis and restore normal microbial function, normalize the immune dysfunction and repair barrier defects. These goals could be accomplished by using traditional approaches (probiotics, antibiotics, diets, combinations of the above), developing methods (fecal microbial transplants; synthetic mixtures of defined microbes, perhaps personalized for an individual's specific microbiota profile; highly selective antibiotics targeting key aggressive microbial species; and personalized diets), and still hypothetical novel approaches (bacteriophages targeting key aggressive bacteria; inhibiting bacterial attachment, promoting a more anaerobic environment; blocking bacterial receptors; stimulating protective mammalian pathways; using synthetic microbial metabolites or recombinant bacterial species).

Table 1

Protective and Aggressive Bacteria in Patients with IBD and Mice with Colitis

Expanded in IBDPotentially inflammatory	**Contracted in IBDPotentially protective**
Proteobacteria *	Bifidobacterium sp.
Escherichia coli – adherent/invasive *	Groups IV & XIVA Clostridium **
Fusobacterium species	*Faecalibacterium prausnitzii* **
Ruminococcus gnavus *	Roseburia species
Pasteurellaceae	Suterella species
Veillonellaceae	Bacteroides ***
Caudovirales	*Saccharomyces cerevisiae*
Clavispora lusitaniae	
Kluyveromyces marxianus	
Candida albicans, Candida tropicalis	
Cyberlindnera jadinii	

* Documented ability to induce experimental colitis

** Documented ability to ameliorate experimental colitis

*** A Bacteroides species, *B. fragilis,* has protective ability in experimental colitis.

Resident bacteria, fungi and viruses that are altered in IBD patients or mice with colitis. This balance is unique in each individual host and each individual responds differently to various bacterial species.

Those with aggressive functions in experimental models are indicated with an asterisk; those with protective functions are indicated with double asterisks. One Bacteroides species, *B. fragilis,* has protective ability in experimental colitis.