# Mechanisms of Action of Probiotics: Recent Advances

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**Abstract:** The intestinal microbiota plays a fundamental role in maintaining immune homeostasis. In controlled clinical trials probiotic bacteria have demonstrated a benefit in treating gastrointestinal diseases, including infectious diarrhea in children, recurrent *Clostridium difficile*-induced infection, and some inflammatory bowel diseases. This evidence has led to the proof of principle that probiotic bacteria can be used as a therapeutic strategy to ameliorate human diseases. The precise mechanisms influencing the crosstalk between the microbe and the host remain unclear but there is growing evidence to suggest that the functioning of the immune system at both a systemic and a mucosal level can be modulated by bacteria in the gut. Recent compelling evidence has demonstrated that manipulating the microbiota can influence the host. Several new mechanisms by which probiotics exert their beneficial effects have been identified and it is now clear that significant differences exist between different probiotic bacterial species and strains; organisms need to be selected in a more rational manner to treat disease. Mechanisms contributing to altered immune function in vivo induced by probiotic bacteria may include modulation of the microbiota itself, improved barrier function with consequent reduction in immune exposure to microbiota, and direct effects of bacteria on different epithelial and immune cell types. These effects are discussed with an emphasis on those organisms that have been used to treat human inflammatory bowel diseases in controlled clinical trials.

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**Key Words:** probiotics, mechanisms of action, immunomodulation, barrier function, antimicrobial activity, dendritic cells

## RATIONALE FOR THE USE OF PROBIOTIC BACTERIA IN INTESTINAL INFLAMMATION

Experiments in murine models and clinical observations in man suggest that luminal contents provide the constant antigenic stimulus for intestinal inflammation.[1,2] The rationale for using probiotics in inflammatory bowel disease (IBD) stems from studies of dysbiosis in the intestinal microbiota in ulcerative colitis (UC), Crohn's disease (CD), and pouchitis, detected using conventional anaerobic culture or molecular probes.[3,4] The dysbiosis theory, reviewed by Tamboli et al,[5] suggested a breakdown in the balance between putative species of "protective" versus "harmful" intestinal bacteria, which leads to chronic intestinal inflammation. The number of different commensal bacteria is altered in IBD patients with increased bacteroides, adherent or invasive *Escherichia coli,* and enterococci, and reduced bifidobacterium and lactobacillus species.[3,6]

Illuminating work from Gordon's laboratory provides evidence that manipulating the microbiota with probiotics can influence the host. Germ-free mice were colonized with *Bifidobacteria thetaiotaomicron*, a prominent component of the adult human gut microbiota, and *Bifidobacterium longum*, a commonly used probiotic. *B. longum* repressed *B thetaiotaomicron* expression of antibacterial proteins that may promote its own survival in the gut, as well as influence the composition, structure, and function of its microbial community.[7] A single microbial molecule from *Bacterial fragillis* has also been shown to protect its host from inflammatory disease caused by *Helicobacter hepaticus* in an animal model of experimental colitis, suggesting that natural antiinflammatory molecules from the bacteria microbiota can actively promote human health, and may potentially be therapies for human inflammatory disorders.[8] Metabonomic studies have demonstrated that probiotics can modulate the gut microbiome and the metabolism of short chain fatty acids, amino acids, bile acids, and plasma lipoproteins, demonstrating the diversity of symbiotic co-metabolic connections between the gut microbial content and the host.[9]

## CONTROLLED CLINICAL TRIALS OF PROBIOTICS IN INFLAMMATORY BOWEL DISEASE

Evidence that probiotic bacteria provide therapeutic benefits in IBD is growing. Several recent articles have comprehensively reviewed controlled clinical trials using

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**TABLE 1.** Mechanisms of Action of Probiotics

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| **Antimicrobial Activity** |
| Decrease luminal pH |
| Secrete antimicrobial peptides |
| Inhibit bacterial invasion |
| Block bacterial adhesion to epithelial cells |
| **Enhancement of Barrier Function** |
| Increase mucus production |
| Enhance barrier integrity |
| **Immunomodulation** |
| Effects on epithelial cells |
| Effects on dendritic cells |
| Effects on monocytes/macrophage |
| Effects on lymphocytes |
| - B lymphocytes |
| - NK cells |
| - T cells |
| - T cell redistribution |

probiotic bacteria to treat IBD.[10–13] In summary, positive controlled clinical trials have demonstrated that the probiotic mixture, VSL#3, which consists of 4 strains of *Lactobacillus (L. acidophilus, L. casei, L. plantarum, L. delbrueckii),* 3strains of *Bifidobacterium (B. infantis, B. longum, B. breve),* and 1 strain of *Streptococcus salivarius* subsp. *thermophilus*, is beneficial in maintaining remission in pouchitis[14,15] and preventing the development of pouchitis in patients following ileo-anal pouch formation.[16] VSL#3 has also recently been shown to be effective in the treatment of acute mild to moderately active UC.[17] *E. coli* Nissle 1917 is effective, and equivalent to mesalazine, in maintaining remission in UC.[18] Bifidobacteria-fermented milk is superior to placebo in maintaining remission in UC.[19] *Saccharomyces bourladii*[20] appears useful in maintaining remission in CD but *Lactobacillus* GG[21] and *L. johnsonii* LA1[22,23] are not beneficial in preventing postoperative recurrence in CD.

## MECHANISMS OF ACTION OF PROBIOTICS

Probiotic bacteria have multiple and diverse influences on the host (Table 1). Different organisms can influence the intestinal luminal environment, epithelial and mucosal barrier function, and the mucosal immune system. They exert their effects on numerous cell types involved in the innate and adaptive immune responses, such as epithelial cells, dendritic cells, monocytes/macrophages, B cells, T cells, including T cells with regulatory properties, and NK cells. Figure 1 provides a simplified illustration of the main mechanisms of action of probiotics.[24,25]

## IN VITRO AND ANIMAL STUDIES

In animal models of IBD the requirement for bacterial colonization to induce an inflammatory phenotype is virtually

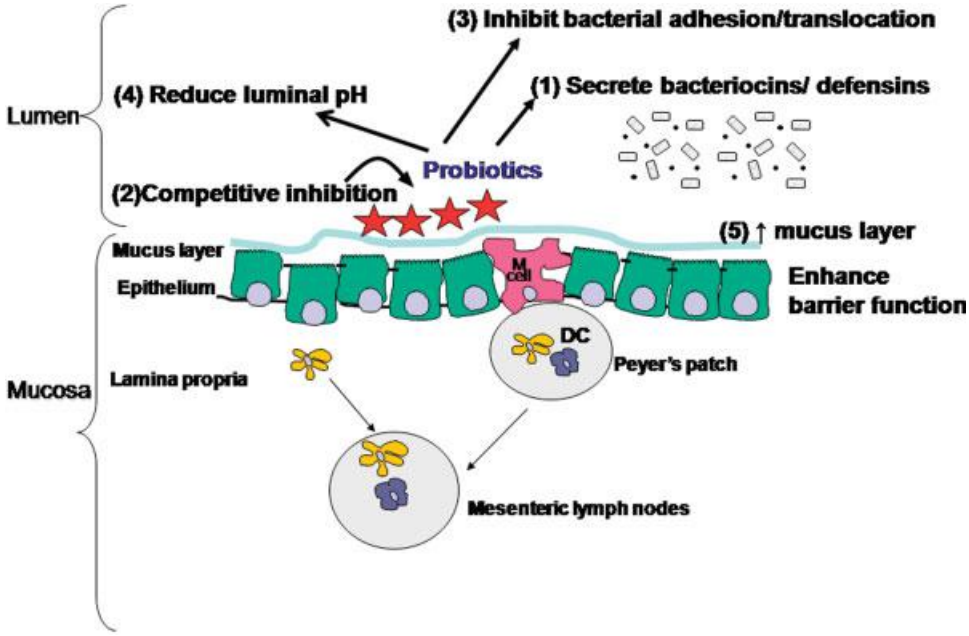


**FIGURE 1.** Inhibition of enteric bacteria and enhancement of barrier function by probiotic bacteria. Schematic representation of the crosstalk between probiotic bacteria and the intestinal mucosa. Antimicrobial activities of probiotics include the (1) production of bacteriocins/defensins, (2) competitive inhibition with pathogenic bacteria, (3) inhibition of bacterial adherence or translocation, and (4) reduction of luminal pH. Probiotic bacteria can also enhance intestinal barrier function by (5) increasing mucus production. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

universal.[26] Support for a favorable action of probiotics on intestinal inflammation comes mainly from animal models, including dextran sodium sulfate- and hapten-induced colitis, HLA-B27 transgenic rats, and interleukin (IL)-10- and IL-2-deficient mice.[27–30] Studies in animal models of colitis are useful in providing mechanistic and therapeutic proof of concept. However, not all commensal or probiotic bacteria have the same actions on gut immune function. For instance, in the IL-10 knockout mouse model of colitis, lactobacillus and bifidobacterium species are effective in reducing *established* intestinal inflammation,[29,31] whereas in HLA-B27 transgenic mice, *Lactobacillus* GG effectively *prevents* relapse of colitis after antibiotic treatment.[28]

## MODIFICATION OF THE INTESTINAL MICROBIOTA

Probiotic bacteria can antagonize pathogenic bacteria by reducing luminal pH, inhibiting bacterial adherence and translocation, or producing antibacterial substances and defensins. One of the mechanisms by which the gut flora resists colonization by pathogenic bacteria is by the production of a physiologically restrictive environment, with respect to pH, redox potential, and hydrogen sulfide production. Probiotic bacteria decrease the luminal pH, as has been demonstrated in patients with UC following ingestion of the probiotic preparation VSL#3.[32] In a fatal mouse Shiga toxin-producing *E. coli* O157:H7 infection model, the probiotic *B. breve* produced a high concentration of acetic acid, consequently lowering the luminal pH. This pH reduction was associated with increased animal survival.[33]

Production of antimicrobial compounds, termed bacteriocins, by probiotic bacteria is also likely to contribute to their beneficial activity. Several bacteriocins produced by different species from the genus *Lactobacillus* have been described.[34] The inhibitory activity of these bacteriocins varies; some inhibit other lactobacilli or taxonomically related Gram-positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria as well as yeasts and molds.[35] For example, the probiotic *L. salivarius* subsp. *salivarius* UCC118 produces a peptide that inhibits a broad range of pathogens such as *Bacillus, Staphylococcus, Enterococcus, Listeria*, and *Salmonella* species.[36] Lacticin 3147, a broad-spectrum bacteriocin produced by *Lactococcus lactis* subsp., inhibits a range of genetically distinct *C. difficile* isolates from healthy subjects and patients with IBD.[37] A further example is the antimicrobial effect of *Lactobacillus* species on *Helicobacter pylori* infection of gastric mucosa, achieved by the release of bacteriocins and the ability to decrease adherence of this pathogen to epithelial cells.[38] Probiotics can reduce the epithelial injury that follows exposure to *E. coli* O157:H7 and *E. coli* O127:H6. The pretreatment of intestinal (T84) cells with lactic acid-producing bacteria reduced the ability of pathogenic *E. coli* to inject virulence factors into the cells or to breach the intracellular tight junctions.[39] Adhesion and invasion of an intestinal epithelial cell line (Intestine 407) by adherent invasive *E. coli* isolated from patients with CD was substantially diminished by co- or preincubation with the probiotic strain *E. coli* Nissle 1917. These findings demonstrate that probiotics prevent epithelial injury induced by attaching-effacing bacteria.[40,41]

Defensins are antimicrobial peptides involved in innate defense mechanisms. The probiotic *E. coli* Nissle strain induced expression of human beta-defensin 2 (hBD-2) in Caco-2 intestinal epithelial cells[42]; this type of effect may contribute to an improved mucosal barrier and provide a means of limiting access of enteric pathogens. Induction of human beta-defensin 2 by *E. coli* Nissle 1917 is dependent on the nuclear factor-kappa B (NF-$\kappa$B) and AP-1-pathways, mediated through bacterial flagellin.[43]

## ENHANCEMENT OF BARRIER FUNCTION

In addition to the inhibition of growth of "conventional" organisms or potential pathogens, probiotics can influence mucosal cell–cell interactions and cellular "stability" by the enhancement of intestinal barrier function through modulation of cytoskeletal and tight junctional protein phosphorylation.

Intestinal barrier function is maintained by several interrelated systems including mucus secretion, chloride and water secretion, and binding together of epithelial cells at their apical junctions by tight junction proteins. Disruption of epithelial barrier function is seen in several conditions including IBD, both active and inactive, in the healthy relatives of patients with IBD,[44–49] enteric infections,[50] coeliac disease, and some autoimmune diseases such as Type 1 diabetes.[51] Enhancement of mucosal barrier function may be an important mechanism by which probiotic bacteria benefit the host in such diseases.[52]

Enhancement of barrier function by probiotic bacteria has been observed both in in vitro models and in vivo in the whole animal. The probiotic mixture VSL#3 normalized barrier integrity as assessed by short circuit currents, transepithelial potential differences, and mannitol fluxes in excised tissue from mice.[53] Furthermore, in an in vitro culture using T84 epithelial cells, VSL#3, but not the other probiotic bacteria, *L. reuteri, S. bovis,* and a nonpathogenic *E. coli* decreased monolayer permeability and conductance, indicating that the increase in resistance was specific to 1 or more of the bacteria in VSL#3. Increased barrier integrity in response to probiotic bacteria has been observed in healthy animals and in animal models of colitis. For example, in healthy rats, *L. brevis* enhanced barrier function as assessed by permeability to mannitol in excluded colonic loops.[54] In IL-10-deficient mice with chronic colitis lactobacillus improved barrier function in vivo.[29] In a methotrexate-induced model of colitis, *L. plantarum* and *L. reuteri* enhanced barrier function.[55] However, enhancement of barrier function was not observed in all

models of colitis studied. *L. plantarum* did not enhance the barrier function in the context of TNBS colitis.[56] The mechanisms by which probiotics bacteria enhance gut mucosal barrier function are unclear, but may relate to alterations in mucus or chloride secretion or changes in tight junction protein expression by epithelial cells. Some probiotic bacteria modify MUC gene expression and mucus secretion. For example, *L. plantarum* 299v increased MUC2 and MUC3 mRNA expression when incubated with the epithelial cell line HT-29.[57] VSL#3 and *E. coli* Nissle strain increased MUC2, MUC3, and MUC5AC gene and protein expression.[58] Some probiotic bacteria limit chloride and water secretion. For example, *S. thermophilus* and *L. acidophilus* reversed the increase in enteroinvasive *E. coli*-induced chloride secretion by an epithelial cell line.[59] Tight junction proteins are dynamic structures subject to structural changes that dictate their functional status. In epithelial cells the tight junction protein zonula occludens-1 (ZO-1) redistributes when exposed to pathogenic bacteria such as *S. dublin.*[58] However, coculture of epithelial cells with VSL#3 probiotic bacteria in addition to *S. dublin* prevented the redistribution of ZO-1 and stabilized the barrier function, suggesting that probiotic bacteria may be important in preservation of the cytoskeleton architecture. Other probiotic bacteria have altered other cytoskeleton structures; for example, *L. acidophilus* protected against F-actin rearrangement, which was induced in an epithelial cell line on exposure to a pathogenic *E. coli.*[60] *S. thermophilus* and *L. acidophilus* maintained (actin, ZO-1) or enhanced (actinin, occludin) cytoskeletal and tight junctional protein structures in epithelial cell lines.[59] *E. coli* Nissle 1917 can counteract the disruptive effects of enteropathic *E. coli* (EPEC) on T-84 epithelial cells monolayers. This effect is achieved by altering protein kinase C signaling and increasing the redistribution and expression of zonula occludens-2 (ZO-2), a crucial factor in maintaining epithelial tight junction function.[61]

## IMMUNOMODULATION

### Effects of Probiotic Bacteria on Epithelial Cells

There may be intrinsic differences in how epithelial cells sense commensal or probiotic bacteria versus pathogenic bacteria at the level of signal transduction pathways and cytokine production. This concept was demonstrated by Lammers et al[62] and Otte and Podolsky[58] who showed that probiotic bacteria in the VSL#3 combination did not induce IL-8 secretion by epithelial cells compared with intestinal pathogens such as enteropathogenic *E. coli, Salmonella dublin, Shigella dysenteriae,* and *Listeria monocytogenes,* all of which did induce secretion of IL-8. Furthermore, coculture of the pathogenic bacteria *S. dublin* with VSL#3 probiotic bacteria decreased IL-8 secretion seen with the pathogenic bacteria alone, indicating that probiotic bacteria can override the effects of pathogenic bacteria. However, the probiotic bacteria *E. coli* Nissle 1917 induced IL-8 secretion by intestinal epithelial cell lines in a dose-dependent manner, suggesting that the ability to prevent secretion of IL-8 from epithelial cells is not a feature of all probiotic bacteria. One other effect of probiotic bacteria on epithelial cells is the ability of commensal organisms to act through pattern recognition molecules or Toll-like receptors (TLR), such as TLR-2 and TLR-4, possibly on epithelial cells. Such interactions induce the production of protective cytokines that enhance epithelial cell regeneration and inhibit epithelial cell apoptosis.[63] *L. casei* prevents the development of acute dextran sodium sulfate (DSS)-induced colitis in TLR-4 mutant (lps–/lps–) mice by inhibiting myeloperoxidase activity and IL-12p40, and increasing TGF-beta and IL-10 mRNA. These effects suggest that the mechanism of action of *L. casei* depends largely on TLR-4 status.[64]

The signaling pathways that allow epithelial cells to distinguish commensal or probiotic organisms from pathogenic organisms appear to be different. Pathogenic bacteria induce proinflammatory responses in intestinal epithelial cells by activating the transcription factor NF-κB. In contrast, nonpathogenic species can attenuate proinflammatory responses by blocking the degradation of the counter-regulatory factor IκB. This method of blocking proinflammatory responses is shown by nonpathogenic *Salmonella pullorum,* which attenuates IL-8 secretion elicited by pathogenic *S. typhimurium.*[65] Another method of avoiding proinflammatory responses to commensal bacteria has been demonstrated for *Bacteroides thetaiotaomicron*, which induced an antiinflammatory response in epithelial cells by shuttling transcription factor NK-κB out of the nucleus by a pathway involving the nuclear hormone receptor PPARγ, resulting in attenuation of NF-κB-mediated inflammatory gene expression independent of the IκB pathway.[66] VSL#3 probiotic bacteria produced soluble factors that inhibited chymotrypsin-like activity of the proteosome in intestinal epithelial cells, thereby inhibiting the NF-κB pathway and inducing expression of cytoprotective heat shock proteins.[67] Furthermore, DNA derived from the probiotic mixture VSL#3 delayed NF-κB activation, stabilized levels of IκB, and inhibited proteasome function.[68]

Probiotic bacteria may also enable epithelial recovery or prevent apoptosis, as suggested by a study in which cytokine-induced apoptosis was prevented in intestinal epithelial cells in the presence of *Lactobacillus rhamnosus* GG.[69] Culture of probiotic bacteria with either mouse or human colon cells activated antiapoptotic Akt/protein kinase B and inhibited activation of the proapoptotic p38/mitogen-activated protein kinase by tumor necrosis factor-α (TNFα), IL-1α, or interferonγ (IFNγ). An inhibition of apoptosis may enhance survival of intestinal cells and promote proliferation during recovery from epithelial injury. In addition, a nonpathogenic commensal bacterium, *S. pullorum*, influenced epithelial cell proliferation by "injecting" factors into the gut epithelium

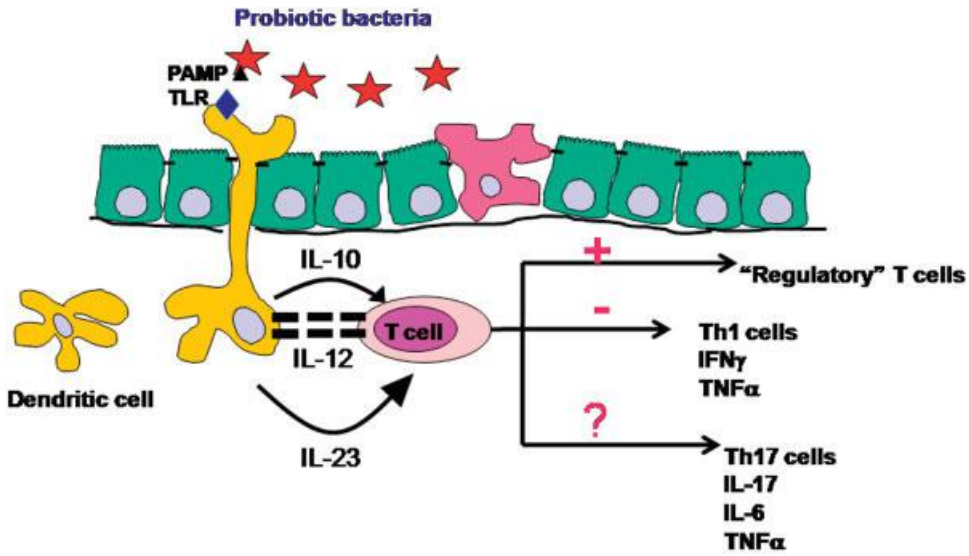


**FIGURE 2.** Modulation of mucosal immune response by probiotic bacteria. Pathogen-associated molecular patterns (PAMPs) derived from probiotic bacteria are recognized by pattern recognition receptors, such as Toll-like receptors (TLRs) on DC in the epithelium or lamina propria. Probiotic bacteria can shape the mucosal immune system toward a noninflammatory, tolerogenic pattern through the induction of T cells with regulatory properties. Probiotics can also downregulate Th1 response and inhibit the production of proinflammatory cytokines, IL-12, TNF-$\alpha$, and IFN-$\gamma$ by DC. The predominant cytokine profile depends on the nature of the stimulus and the types of probiotic bacteria. The IL-23/IL-17-mediated inflammatory axis has recently been implicated in the pathogenesis of IBD but there remain gaps in our knowledge on how probiotics influence the differentiation of Th17 cells. This diagram is a simplified synthesis of data derived in vitro and in vivo. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that blocked $\beta$-catenin degradation, a substance that has been implicated in epithelial growth control.[65]

## Effects of Probiotic Bacteria on Dendritic Cells

Dendritic cells (DCs) are antigen-presenting cells that are important in earliest bacterial recognition and in shaping the subsequent T-cell responses. In the intestine DC have specialized functions, contributing to oral tolerance induction by generating regulatory T cells and IgA-producing B cells through production of cytokines such as IL-10 and TGF$\beta$.[70–72] Intestinal DC interact directly with luminal bacteria by passing their dendrites between epithelial tight junctions into the gut lumen[73] and indirectly with bacteria that have gained access via M-cells.[74] The pivotal position of DC at the intersection of innate and adaptive immunity with their ability to recognize and respond to bacterial components, to initiate primary immune responses, and to direct developing T- and B-cell responses underlines the importance of understanding the functional effects of different bacteria on DC. The effects of different probiotic bacteria on DC have been studied in different experimental systems (whole blood DC, freshly isolated lamina propria DC, monocyte-derived DC, and bone-marrow derived DC) and in different species (human and mouse). Figure 2 summarizes the interactions between DC and probiotics. In humans the probiotic combination VSL#3 was a potent inducer of IL-10 by both blood and lamina propria DC in vitro.[75] We have also shown that these results can be extrapolated to the in vivo situations. Patients with UC treated with VSL#3 have increased IL-10 and reduced IL-12p40 production by colonic DC; these effects were not seen in placebo-treated patients.[76] DC were defined as a population of cells that were HLA-DR+ and negative for a set of lineage markers (CD3, CD14, CD16, CD19, CD34), thereby excluding T cells, B cells, macrophages, NK cells, and myeloid progenitor cells. These cells have functional properties of DC in that, on maturation, they stimulate a primary allogeneic mixed leukocyte reaction and display endocytic activity intermediate between lymphocytes, which are nonendocytic, and monocytes, which are highly endocytic.[77,78] In patients with pouchitis treated with the probiotic combination VSL#3 there were enhanced levels of IL-10 in their mucosa and decreased levels of TNF-$\alpha$, IL-1, inducible nitric oxide synthase, and matrix metalloproteinase,[79] suggesting that the in vitro findings correlate with those in vivo, although the cellular source of the IL-10 in vivo was not known. In agreement with the observation that VSL#3 was a potent inducer of IL-10 by DC, Drakes et al[80] showed that murine bone marrow-derived DC incubated with VSL#3 increased IL-10 detected by enzyme-linked immunosorbent assay (ELISA). Individual strains within VSL#3 displayed distinct immunomodulatory effects on DC; the most marked antiinflammatory effects were produced by bifidobacteria

strains (*B. longum, B. infantis* and *B. breve*), which upregulated IL-10 production by DC. Antiinflammatory effects of bifidobacteria strains have been described in other studies. Using DC derived from human cord blood monocytes, Young et al[81] demonstrated that *B. longum, B. bifidum,* and *B. pseudocatenulatum*, but not *B. infantis*, induced high levels of IL-10. Rigby et al[82] showed that murine freshly isolated lamina propria DC incubated with *B. longum* secreted IL-10 and IL-12, but a greater proportion of DC secreted IL-10 than IL-12. In a different experiment, purified human monocytes and monocyte-derived DC were stimulated with ultraviolet-inactivated Gram-positive (*L. plantarum* and *B. adolescentis)* and Gram-negative (*E. coli* and *Veillonella parvula)* bacterial strains. *B. adolescentis* induces low amounts of IL-12, TNF$\alpha$, IL-6, and IL-8; *L. reuteri* and *L. casei*, but not *L. plantarum*, primed monocyte-derived DC to drive the development of T cells with regulatory properties.[83,84] These "T reg" cells produced increased levels of IL-10 and were able to inhibit the proliferation of bystander T cells in an IL-10-dependent fashion. Strikingly, both *L. reuteri* and *L. casei*, but not *L. plantarum*, bind the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Blocking antibodies to DC-SIGN inhibited the induction of the "T reg" cells by these probiotic bacteria, implying that ligation of DC-SIGN can actively prime DC to induce "T reg" cells. Thus, the targeting of DC-SIGN by certain probiotic bacteria might explain their beneficial effect in the treatment of a number of inflammatory diseases, including atopic dermatitis and CD.[85] In contrast to the antiinflammatory activity of some strains of bifidobacteria and lactobacilli, other lactobacilli strains have proinflammatory activity. When cultured with human monocyte-derived DC, *L. reuteri, L. gasseri* and *L. johnsonii* induced activation and maturation of DC, enhanced IL-12 production, and induced allogeneic T-cell priming.[86] However, Christensen et al demonstrated that *L. reuteri* induced little or no IL-12 production by DC in an experimental system using murine bone marrow-derived DC and inhibited proinflammatory cytokine production (IL-12, IL-6, and TNF-$\alpha$) by *L. casei,* which was a potent inducer of IL-12.[83,84,87,88] These varying effects of probiotic bacteria highlight differences that arise when different experimental systems in different animals are used. However, there appear to be different responses of different bacterial strains even within a genus. In human monocyte-derived DC, *L. rhamnosus* and *L. plantarum* induced no or low levels of IL-12, in contrast to the high levels of IL-12 noted with *L. gasseri* and *L. johnsonii.*[86] Braat et al[84] demonstrated that *L. rhamnosus* may "educate" DC to stimulate proliferation of peripheral CD4+T cells and reduce CD3/CD28-stimulated cytokine production in vitro. *L rhamnosus* resulted in a reduction of IL-4 from peripheral CD4+ T cells of normal individuals, and a decrease in IFN-$\gamma$ and IL-2 production by CD4+ T cells from patients with CD. This observation suggests that a comparable mechanism may exist in humans. It supports an antiinflammatory mechanism of action for probiotics that, apart from influencing both Th1 and Th2 immune responses, also has an indirect effect via antigen presenting cells in the gut.

## Effects of Probiotic Bacteria on Monocytes and Macrophages

Blood monocytes and tissue macrophages are effective secondary presenters of antigens to memory T cells. *L. plantarum* increased IL-10 synthesis and secretion in macrophages derived from the inflamed colon.[89] In contrast, *L. rhamnosus* GG promoted the production of IFN$\gamma$, IL-12, and IL-18,[90] and induced NF-$\kappa$B and STAT DNA-binding in primary human macrophages. He et al[91] showed that *B. bifidum, B. breve*, and *B. infantis* stimulated more IL-10 and less IL-12 and TNF$\alpha$ from a murine macrophage-like cell line than *B. adolescentis,* again suggesting strain differences within a genus. DNA derived from the probiotic mixture VSL#3 activated NF-$\kappa$B and induced low levels of IL-6 and IL-12 by bone marrow-derived macrophages compared with immunostimulatory oligonucleotides.[92]

## Effects of Probiotic Bacteria on Lymphocytes

Probiotic bacteria may affect lymphocytes directly or secondarily via changes in stimulation induced by alterations in antigen-presenting DC or macrophages. These effects have been demonstrated for different types of lymphocytes.

### *B Lymphocytes*

Probiotic bacteria may exert beneficial effects and modulate the immune response to potentially harmful antigens via B lymphocytes and antibody production. For example, *L. rhamnosus* GG administered to children with acute gastroenteritis enhanced a nonspecific humoral immune response, reflected by an increase in IgG, IgA, and IgM secretion from circulating lymphocytes.[93] *B. bifidum* enhanced the antibody response to ovalbumin, and yogurts containing *L. acidophilus, L. bulgaricus, S. thermophilus, B. bifidum*, and *B. infantis* stimulated the IgA response to cholera toxin in mice.[94]

The effects of probiotic bacteria on B lymphocytes and antibody production have also been evaluated in vaccination trials. The immunogenicity of rotavirus vaccination was enhanced in children who received *L. casei* GG compared with those who received placebo.[95] Similarly, increased *Salmonella*-specific IgA levels were found in subjects who received a combination of *L. rhamnosus* GG and *Salmonella* vaccination.[96]

### *Natural Killer (NK) Cells*

The symbiotic *L. casei ssp. casei* with dextran (prebiotic) significantly elevated the NK cell activities in spleen mononuclear cells from BALB/c mice, and oral administration of this symbiotic to healthy volunteers induced NK cell

activities and increased production of IL-12 in human peripheral blood mononuclear cells.[97] Takeda et al[98] showed that *L. casei* Shirota can enhance NK cell activity in vivo and in vitro in humans; this effect may be dependent on IL-12.

### T Cells

The type of T cell response, whether it be a Th1, Th2, or Th3/Tr1 response, is controlled predominantly by interactions between DC and T cells. In humans, VSL#3 potently induced IL-10 by DC and coculture of naïve T cells with probiotic-treated DC led to a decrease in Th1 polarized cells.[75] In a different experimental system in which monocyte-derived DC were cultured with the probiotic *L. rhamnosus* and the subsequent effect on T cells was assessed, decreased T-cell proliferation and T-cell cytokine production, particularly IL-2, IL-4, and IL-10, was demonstrated.[84] This in vitro effect of *L. rhamnosus* on DC and subsequent T-cell hyporesponsiveness was reflected in in vivo studies in which healthy controls and patients with CD were fed *L. rhamnosus* for 2 weeks. Ingestion of *L. rhamnosus* reduced IFN$\gamma$ and IL-2 production by peripheral T cells in CD patients and also reduced IL-4 production in healthy controls. Probiotic bacteria influence the generation of regulatory T cells in a murine model of contact dermatitis. Daily oral administration of fermented milk containing the probiotic *L. casei* DN-114 001 reduced antigen-specific skin inflammation by controlling the antigen-specific T cell response in hapten 2,4-dinitrofluorobenzene, a model of allergic contact dermatitis mediated by CD8+ CTL and controlled by CD4+ regulatory T cells. The alleviation of contact hypersensitivity by prior feeding with *L. casei* was due to downregulation of the hapten-specific CD8+ T-cell response as indicated by a decrease in expansion of hapten-specific IFN$\gamma$-producing CD8+ effectors. Furthermore, experiments in mice deficient in CD4+ cells indicated that CD4+ cells are mandatory for the effect of *L. casei* on contact hypersensitivity. It was proposed that *L. casei* reduced contact hypersensitivity by direct or indirect activation of regulatory CD4+ T cells.[99] Von der Weid et al[100] have also reported that in vitro a different probiotic organism, *L. paracasei* NCC2461, induced the development of a population of CD4+ T cells with regulatory properties in that they had low proliferative capacity and produced TGF$\beta$ and IL-10. Probiotic bacteria also induce regulatory T cells in the context of intestinal inflammation. In TNBS colitis, the probiotic combination VSL#3 ameliorated intestinal inflammation; the beneficial effect of VSL#3 was attributable to IL-10-dependent-regulatory CD4+ T cells bearing surface TGF$\beta$.[101] These cells appear to be similar to CD25+ regulatory T cells that inhibit cell-transfer colitis by a TGF$\beta$-dependent mechanism.[102,103]

### Effects of Probiotic Bacteria on T-Cell Redistribution

Dalmasso et al[104] reported a novel biological property of probiotics: the capacity to affect immune cell redistribution by improving the competence of lymphatic endothelial cells to trap T lymphocytes. In *S. boulardii*-fed mice, IFN-$\gamma$ production by CD4+ T cells was reduced in the colon but increased in the mesenteric lymph nodes. *S boulardii* has a unique action on inflammation by a specific alteration of the migratory behavior of T cells causing accumulation of these cells in mesenteric lymph nodes.

## MECHANISMS OF PROBIOTICS IN CLINICAL DISEASES

A number of studies in IBD have shown that probiotics can induce regulatory cytokines, including IL-10 and TGF-$\beta$, and suppress proinflammatory cytokines, such as TNF, in the mucosa of patients with CD and pouchitis.[79,89,105] VSL#3 induces IL-10 and downregulates IL-12p40 production by lamina propria DC in patients with UC; similar cytokine changes were seen in patients who were treated with corticosteroids.[76,106]

In a study aimed at assessing the antiinflammatory effects of probiotics, healthy controls and IBD patients consumed *L. rhamnosus GR-1* and *L. reuteri RC-14* supplemented yogurt for 30 days. The proportion of putative regulatory CD4(+) CD25(high) T cells in peripheral blood increased significantly in IBD patients after treatment, but not in controls. The basal proportion of TNF-alpha(+)/interleukin (IL)-12(+) monocytes and myeloid DC decreased in both subject groups, but of stimulated cells only in IBD patients. In addition, serum IL-12 concentrations and the proportion of IL-2(+) and CD69(+) T cells from stimulated cells decreased in IBD patients. The increase in CD4(+) CD25(high) T cells correlated with the decrease in the percentage of TNF-$\alpha$- or IL-12-producing monocytes and DC. Thus, probiotic yogurt intake was associated with significant antiinflammatory effects that paralleled the expansion of the peripheral pool of putative "T reg" cells in IBD patients, with few effects in controls.[107]

Adhesive *E. coli* have been implicated in the pathogenesis of UC. Studies using a 16S rRNA technique have shown reductions in bifidobacteria[108,109] and lactobacilli in patients with UC.[110] Using a short-term synbiotic treatment, there was an increased bifidobacterial colonization of the rectal mucosa. In a separate experimental system, colonic biopsies from UC were cocultured for 24 hours with *B. longum.* The concentrations of TNF and IL-8 in supernatants of inflamed UC tissue cocultured with probiotics were lower than those cultured alone. The number of lamina propria mononuclear cells (LMC) with NK-$\kappa$B p65 positive in cocultured tissues was also reduced.[111] Release of TNF-$\alpha$ by inflamed CD mucosa was significantly reduced by coculture of *L. casei* or *L. bulgaris,* but not *L. crispatus* and *E. coli.* No change in TNF-$\alpha$ production was seen in experiments with noninflamed Crohn's mucosa and control mucosa. Certain probiotic bacteria may therefore interact with immunocompetent cells at

the mucosal interface and thus modulate local production of proinflammatory cytokines by inflamed tissue.[105]

## SYSTEMIC ANTIINFLAMMATORY ACTIVITIES OF PROBIOTICS

Recent findings have suggested that the antiinflammatory effects of probiotic bacteria could be systemic, at least in part, rather than localized. Beneficial effects were observed after parenteral administration of inactivated and fractionated bacteria.[92,112] In 1 study, coculture of *L. casei* or *L. bulgaris* with mucosal explants from affected intestinal mucosa of CD reduced the inflammatory response induced by coculture of bacteria. This was associated with a significant reduction in proinflammatory cytokines including TNF-$\alpha$, a reduction in the number of CD4 cells, as well as TNF$\alpha$ expression among intraepithelial lymphocytes, suggesting that the antiinflammatory effect might be systemic.[105]

Investigators from Cork, Ireland, administered *L. salivarius* subcutaneously to IL-10 knockout (KO) mice and reported that the antiinflammatory effect of subcutaneous injection was not specific, and was also seen in a murine model of arthritis. The fecal microflora remained unchanged following subcutaneous administration, but TNF and IL-12 levels from splenocytes stimulated by *S. typhimurium* decreased and TGF$\beta$ levels were maintained, suggesting a mechanism of action distinct from colonic flora modulation.[112]

Probiotics may not need to encounter the mucosal immune system directly to exert an effect. Parenteral administration of *L. salivarius* in IL-10 KO mice ameliorates the severity of colitis, a beneficial effect similar to that demonstrated by the same group previously for oral administration. They also showed that the probiotic effect is not disease-specific, with a similar beneficial effect demonstrated in collagen-induced murine arthritis.[112]

## PROBIOTIC EFFECTS: LIVE BACTERIA, DEAD BACTERIA, OR BACTERIAL DNA

One of the tenets of bacteriotherapy is that viable bacteria are required to have a beneficial effect. Recent studies suggest that bacterial DNA sequences may provide the same effects as live bacteria.[27,68] Bacterial DNA contains nonmethylated CpG motifs that bind to TLR-9. TLR-9 signaling is dependent on the adaptor protein MyD88. In the presence of both TLR-9 and MyD88, nonviable bacteria may have the ability to signal and elicit beneficial effects. In an experiment that used methylated and nonmethylated genomic DNA isolated from the probiotic preparation VSL#3, DNAse-treated probiotics, and *E. coli* genomic DNA, the authors demonstrated that genomic DNA (but not methylated DNA, calf thymus DNA, or DNAse-treated probiotics) ameliorated the severity of colitis in DSS-induced, TNBS-induced, and spontaneous colitis in IL-10 KO mice. In the same study, intragastric and subcutaneous administration of gamma-irradiated nonviable bacteria and live bacteria had similar beneficial effects.[92] Lammers et al[113] demonstrated that bifidobacterium genomic DNA induced the secretion of IL-10 by peripheral blood mononuclear cells from healthy donors, demonstrating the immunomodulatory effects of bacteria DNA. By using a VSL#3-conditioned medium, Petrof et al[67] showed that early proteasome inhibition may account for NF-$\kappa$B inhibition and heat shock protein induction in a cell line from the intestinal epithelium of mice. The use of VSL#3-conditioned medium further challenges the concept that to exert beneficial effects probiotics must be live bacteria.

## QUESTIONS THAT REMAIN UNANSWERED

Studies in animal models of colitis and experimental studies with probiotics are characterized by a high level of heterogeneity due to the wide variety of animal models used. Furthermore, in most currently available animal models disease mostly occurs as a result of experimental manipulation. Such models only present limited specific pathogenic features of the complex, multifaceted nature of IBD in humans, and may not accurately reflect human disease. Nonetheless, it is increasingly apparent that different probiotic bacteria act through multiple and contemporaneous pathways rather than by a single common mechanism.

Ibnou-Zekri et al[114] highlighted that the activity of probiotic strains in vitro may not parallel similar in vivo behavior. In their study, 2 strains of *Lactobacillus* that exhibited very similar in vitro growth, survival, and adherence properties showed distinct differences in colonization patterns and resultant host immune responses at both the mucosal and systemic levels. In contrast, we have demonstrated consistent in vitro and in vivo disease effects, with VSL#3 inducing IL-10 and downregulating IL-12p40 production by colonic DC, both in vitro and in vivo. Even such consistent in vitro and in vivo mechanistic data, however, only correlate with the clinical outcome in some patients.[76]

To determine further the role of probiotics in the IBD treatment armamentarium, large, well-designed, multicenter controlled clinical trials are needed. Not all lactobacillus and bifidobacterium species are equally beneficial; each may have individual mechanisms of action that are dependent on host characteristics. Different bacteria may have dominant effects in different genetic backgrounds and in diseases that vary in their pathogenesis. Thus, optimal use of various probiotics may depend on identifying patient subsets by genetic, phenotypic, stool microbiologic, serologic, or T-cells immune response criteria. The concept of synbiotics (simultaneous use of prebiotics and probiotics) may reduce the dose, frequency, or duration of each treatment. Recent interesting work has focused on the bacteria–host interaction showing that the host, a component of its microbiota, and probiotic

bacteria can adapt their substrate utilization in response to one another.

## CONCLUSION

The therapeutic potential of probiotic bacteria is vast but is just beginning to be tapped due to the huge diversity of the commensal enteric microenvironment. The intestinal bacteria flora contributes significantly to the pathogenesis of IBD, along with genetic susceptibility and mucosal immune dysregulation. Probiotics are likely to become an integral component of the therapeutic armamentarium of IBD in combination with traditional antiinflammatory and immunosuppressive agents.

Understanding probiotic action may permit modulation of the immune system, both locally and systemically. Knowledge of probiotics on the host immune system has entered a new and fascinating phase of research and progression in this field is likely to offer novel and useful means to modulate host immunity for protection from, or treatment of, a wide variety of human disorders, including IBD.

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