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Probiotic bacteria and intestinal epithelial barrier function

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Ohland CL, MacNaughton WK. Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* 298: G807–G819, 2010. First published March 18, 2010; doi:10.1152/ajpgi.00243.2009.—The intestinal tract is a diverse microenvironment where more than 500 species of bacteria thrive. A single layer of epithelium is all that separates these commensal microorganisms and pathogens from the underlying immune cells, and thus epithelial barrier function is a key component in the arsenal of defense mechanisms required to prevent infection and inflammation. The epithelial barrier consists of a dense mucous layer containing secretory IgA and antimicrobial peptides as well as dynamic junctional complexes that regulate permeability between cells. Probiotics are live microorganisms that confer benefit to the host and that have been suggested to ameliorate or prevent diseases including antibiotic-associated diarrhea, irritable bowel syndrome, and inflammatory bowel disease. Probiotics likely function through enhancement of barrier function, immunomodulation, and competitive adherence to the mucus and epithelium. This review summarizes the evidence about effects of the many available probiotics with an emphasis on intestinal barrier function and the mechanisms affected by probiotics.

mucin; tight junction; antimicrobial peptide; defensin; adherence

THE INTESTINAL EPITHELIUM is in constant contact with luminal contents and the varied, dynamic enteric microflora. The distal small bowel, cecum, and colon have increasing bacterial colonization relative to more proximal regions, with up to 10^{10} to 10^{12} colony-forming units per gram of intestinal content in the colon. In fact, 60% of the fecal matter mass in humans is due to bacteria (143). The small intestine supports lower numbers of commensal bacteria due to the low pH from stomach acid, as well as pancreatic enzymes and motility patterns that hamper colonization. More than 500 species of predominantly anaerobic bacteria are estimated to comprise the intestinal microflora, and these prokaryotes outnumber eukaryotic cells by a factor of ten in the human body. Since the vast majority of enteric bacteria cannot be cultured ex vivo by standard techniques, several groups are currently working on identifying the diverse species by PCR of 16S ribosomal DNA and examining the dynamic interplay between genera during health and disease (107, 112).

To protect itself from uncontrolled inflammatory responses, the epithelium has developed mechanisms to restrain bacterial growth, limit direct contact with the bacteria, and prevent bacterial dissemination into underlying tissue. Disruption of this barrier can lead to loss of immune tolerance to the microflora and an inappropriate inflammatory response, as is thought to occur in the inflammatory bowel diseases (IBD) ulcerative colitis and Crohn's disease (21, 68, 159). The intestinal barrier defenses consist of the mucous layer, antimicrobial peptides, secretory IgA, and the epithelial junctional adhesion complex (93). Consumption of nonpathogenic bacterial species can contribute to barrier function by decreasing paracellular permeability, providing innate defense against pathogens and enhancing the physical impediment of the mucous layer, which may help protect against infections, prevent chronic inflammation, and maintain mucosal integrity (Table 1) (13).

Probiotics and Disease

By definition, probiotics are viable, nonpathogenic microorganisms (bacteria or yeast) that are able to reach the intestines in sufficient numbers to confer benefit to the host (13, 29). Prebiotics are indigestible food ingredients that selectively promote the growth or activity of beneficial enteric bacteria, thereby benefiting the host (49). Synbiotics are combinations of probiotics and prebiotics designed to improve the survival of ingested microorganisms and their colonization of the intestinal tract (29). Commonly used bacterial probiotics include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli*, and *Streptococcus* species. *Lactococcus lactis* and some *Enterococcus* species have also been used. Most probiotic bacteria were originally isolated from healthy humans, since these were considered to be safe for human consumption (29). This means that probiotics have virtually no distinguishing characteristics from commensal organisms, except their beneficial effects when consumed. Currently, the only probiotic yeast used is the nonpathogenic *Saccharomyces boulardii*.

Clinically, the probiotic formula VSL#3 is often used and contains a mixture of *S. thermophilus*, four *Lactobacillus* species, and three *Bifidobacterium* species (16). In Central Europe, *E. coli* Nissle 1917 (EcN) has been sold as Mutaflor since 1917 to prevent infectious diarrhea and to treat functional bowel disorders (167). Although the long-term use of Mutaflor

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Table 1. *Summary of probiotics effects on epithelial barrier function in vitro and in vivo*

Barrier Function and Probiotic	Effect	Model	Reference
Mucous layer			
Lactobacillus	↑ MUC2 and/or 3 expression	Caco-2, HT29	67, 78, 87
VSL#3	↑ MUC2, 3, and 5AC expression (no effect on MUC1)	HT29	105
EcN	No change	HT29	105
VSL#3	No change	mouse	45
VSL#3	↑ MUC1, 2, and 3 expression and secretion	rat	16
Host cell antimicrobial peptides			
Lactobacillus, EcN, VSL#3, Symbioflor 2	↑ hBD-2 expression and/or secretion	Caco-2	97, 132, 155
Symbioflor 2	↑ hBD-2 protein in feces	human	97
Probiotic antimicrobial factors			
L. delbrueckii	Inhibit growth of other *Lactobacillus* strains and *Streptococcus thermophilus*	in vitro	153
Bifidobacterium (2 strains from human)	Kill *Escherichia coli, Klebsiella pneumoniae, Yersinia pseudotuberculosis, Staphylococcus aureus and Salmonella typhimurium*	in vitro	72
	Prevent invasion and kill intracellular *S. typhimurium*	Caco-2	72
	Prevent *S. typhimurium* illness	monoassociated mouse	72
L. acidophilus	Kill *S. aureus, L. monocytogenes, S. typhimurium, Shigella flexneri, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Enterobacter cloacae*, but not *Lactobacillus* or *Bifidobacterium*	in vitro	11
	Prevent *S. typhimurium* illness	monoassociated mouse	11
L. salivarius	Bacteriocin ABP-118 inhibits growth of *Bacillus, Listeria, Enterococcus* and *Staphylococcus* species, but not other *Lactobacillus*	in vitro	40
Lactococcus lactis	Produces broad-spectrum bacteriocin, lacticin 3147	in vitro	117, 127
Epithelial adherence			
L. rhamnosus, L. acidophilus, L. helveticus	Prevent EPEC and EHEC binding	Hep-2, T84	63, 139
L. gasseri, L. casei, L. plantarum	No change	Hep-2, T84	139
L. rhamnosus, L. casei	Prevent *Salmonella* species binding and displace attached bacteria	Caco-2, human mucins	71
L. rhamnosus, L. helveticus	Reduce commensal adherence and invasion	rat chronic stress	164
L. rhamnosus	Reduce bacterial translocation	rat hemorrhagic shock	76
L. fermentum	No change	rat hemorrhagic shock	76
S. boulardii	No change in EPEC adherence; ↓ invasion by prevention of ERK1/2 activation	T84	25
S. boulardii	No change in EHEC adherence	T84	26
S. boulardii	No change in *S. flexneri* adherence or invasion	T84	99
S. boulardii	Citrobacter rodentium adherence due to ↓ EspB and Tir secretion	mouse	157
Secretory IgA			
L. casei	↑ levels of IgA+ and IL-6-producing cells in the lamina propria	mouse	44
L. casei	No change	monoassociated mouse	84
B. animalis, E coli EMO	↑ total sIgA	monoassociated mouse	84
B. bifidum and *B. infantis*	↑ levels of rotavirus-specific sIgA	mouse	115
B. lactis	↑ levels of EHEC-specific sIgA	mouse	140
L. casei	↑ levels of EHEC- or Shiga toxin-specific sIgA leading to increased survival	rabbit	103
L. helveticus	↑ lamina propria IgA+ B cells and sIgA	rat	70
L. rhamnosus and *B. lactis* combination	No change	rat	124
S. boulardii	↑ total and anti-*S. boulardii* sIgA	monoassociated mouse	122
S. boulardii	↑ total sIgA	conventional and monoassociated mouse	84, 114
Tight junctions			
S. thermophilus, L. acidophilus	↑ TER, ↓ permeability; activation of occludins, ZO-1, ERK 1/2	HT29, Caco-2	119
S. thermophilus, L. acidophilus	Prevent ↓ ion secretion, ↓ TER, ↑ permeability by IFN-γ and TNF-α	HT29, Caco-2	120
B. infantis	↑ TER, ↓ permeability; ↑ ZO-1, occludin, ↓ claudin-2 expression; prevent IFN-γ and TNF-α effects	T84	32
EcN	↑ TER; ↑ expression and TJ localization of ZO-2; ↑ PKC-ζ expression, but not TJ localization	T84	167
EcN	↑ ZO-1 (not ZO-2) expression	monoassociated mouse	152
EcN	↑ ZO-1 expression; prevent DSS-induced decrease in permeability and illness	DSS-treated mouse	152
L. rhamnosus and *L. helveticus* combination	No change in ileal or colonic permeability	rat	164

Continued

Table 1.—*Continued*

Barrier Function and Probiotic	Effect	Model	Reference
S. boulardii	Prevent EHEC-induced apoptosis	T84	27
L. rhamnosus p40, p75	Inhibit cytokine-induced apoptosis	YAMC, HT29, mouse colon explant	160
L. rhamnosus p40, p75	Inhibit H_2O_2-induced ↓ TER and ↑ permeability	Caco-2, HT29, T84	135

↑, increased; ↓, decreased; EcN, *Escherichia coli* Nissle; hBD, human β-defensins; EPEC, enteropathogenic *E. coli;* EHEC, enterhemorrhagic *E. coli;* sIgA, secretogy IgA; TER, transepithelial resistance; ZO, zonula occludens; TJ, tight junction; DSS, dextran sodium sulfate.

does not guarantee its efficacy, it demonstrates the relative safety of this medically prepared probiotic in healthy individuals. Nevertheless, the immune status of the patient must remain a primary consideration, since additional enteric bacteria, even those that are normally nonpathogenic, can have potentially harmful side effects including causing an opportunistic infection in immunocompromised individuals (75, 137).

Although there are many studies that explore the benefits of probiotic use in preventing or ameliorating human enteric diseases, the subject area remains controversial, mainly because of small sample sizes in clinical trials and inconsistent dose and type of bacterial strains utilized. Meta-analyses can often help to clarify these problems and uncover medical significance. Probiotics have been shown by meta-analysis to protect against acute diarrhea in developed countries by at least 21% and, more specifically, to prevent antibiotic-associated diarrhea by 52% in healthy individuals. Furthermore, the beneficial effects did not differ significantly between strains or combinations (130). Similarly, the use of probiotics is effective in preventing pouchitis after restorative ileal pouch anal anastomosis and necrotizing enterocolitis in preterm infants weighing more than 1,000 g at birth (4, 31). Global symptoms of irritable bowel syndrome and abdominal pain were also decreased with this therapy; however, other individual symptoms were not consistently reduced across studies (92, 95). The maintenance of remission in IBD patients was not affected by probiotic treatment, and *Lactobacillus* species even caused adverse events (vomiting/nausea, epigastric pain, or constipation) in some studies of Crohn's disease patients (81, 123). Therefore, not all intestinal inflammation is benefited by probiotics.

Mechanisms of Probiotic Function

A common misconception is that probiotics must always colonize the intestinal tract to exert their effects. In fact, some probiotics (e.g., *Bifidobacterium longum* and *Bacteroides thetaiotamicron*) become part of the human intestinal microflora, whereas others (e.g., *Lactobacillus casei* and *B. animalis*) may not (111, 142). Noncolonizing probiotics must then indirectly exert their effects either in a transient manner as they pass through or, more likely, by remodeling or influencing the existing microbial community (112, 142).

For example, *S. boulardii* is present in the intestinal tract of human microbiota-associated mice during administration of the probiotic but only remained detectable for 2–5 days after cessation of treatment. This transient, nonmucosally associated colonization did not, however, detectably alter the amount of total bacteria or the proportion of the major groups of bacteria in these mice. Furthermore, after antibiotic treatment, mice treated with this probiotic yeast regained their normal microbiota quicker than control-treated animals. This indicates that *S. boulardii* can help restore healthy microbiota because it is not affected by antibiotic treatment (9). Similarly, *L. rhamnosus* RD20 was no longer detectable in the stool of six human volunteers after consumption was discontinued, indicating a transient colonization. This probiotic did, however, alter the proportion of lactobacilli and enterococci present in the fecal matter, but only during the treatment period (148).

These are only two examples of the many ways in which probiotics may alter the microflora balance, with or without colonization, to be beneficial. This review focuses on the mechanisms by which probiotics directly or indirectly (perhaps via alterations in microflora) enhance the host barrier function (Fig. 1).

Mucous layer. Goblet cells are found along the entire length of the intestinal tract, as well as other mucosal surfaces. These cells express rod-shaped mucins, which are abundantly core glycosylated (up to 80% wt/wt) and either localized to the cell membrane or secreted into the lumen to form the mucous layer (91, 121). Of the 18 mucin-type glycoproteins expressed by humans, MUC2 is the predominant glycoprotein found in the small and large bowel mucus. The NH_2- and COOH-termini are not glycosylated to the same extent, but are rich in cysteine residues that form intra- and inter-molecular disulfide bonds. These glycan groups confer proteolytic resistance and hydrophilicity to the mucins, whereas the disulfide linkages form a matrix of glycoproteins that is the backbone of the mucous layer (16, 65, 74).

This gel layer provides protection by shielding the epithelium from potentially harmful antigens and molecules, while acting as a lubricant for intestinal motility. Mucins can also bind the epithelial cell surface carbohydrates and form the bottom layer, which is firmly attached to the mucosa, whereas the upper layer is loosely adherent (109). Mucus thickness can vary from 50 to 800 μm, but the 30-μm-thick area closest to the epithelium is essentially bacteria free in healthy individuals (146). The mucus is the first barrier that intestinal bacteria meet, and pathogens must penetrate it to reach the epithelial cells during infection. Microorganisms have developed diverse methods to degrade mucus, such as reduction of mucin disulfide bonds (*Helicobacter pylori*), protease activity (*Pseudomonas aeruginosa*, *Candida albicans*, and *Entamoeba histolytica*), and glycosidase activity (mixed oral and intestinal microbial communities) for invasion and/or uptake of mucus-derived nutrients (8, 15, 28, 77, 96, 156). Furthermore, the colonic mucous layer is thinner in areas of inflammation, allowing increased bacterial adherence and infiltration (146). Ulcerative colitis patients also exhibit reduced mucus thickness, particularly in areas of active inflammation, which is likely a consequence of the disease (65, 113, 144, 146).

Probiotics may promote mucus secretion as one mechanism to improve barrier function and exclusion of pathogens. In

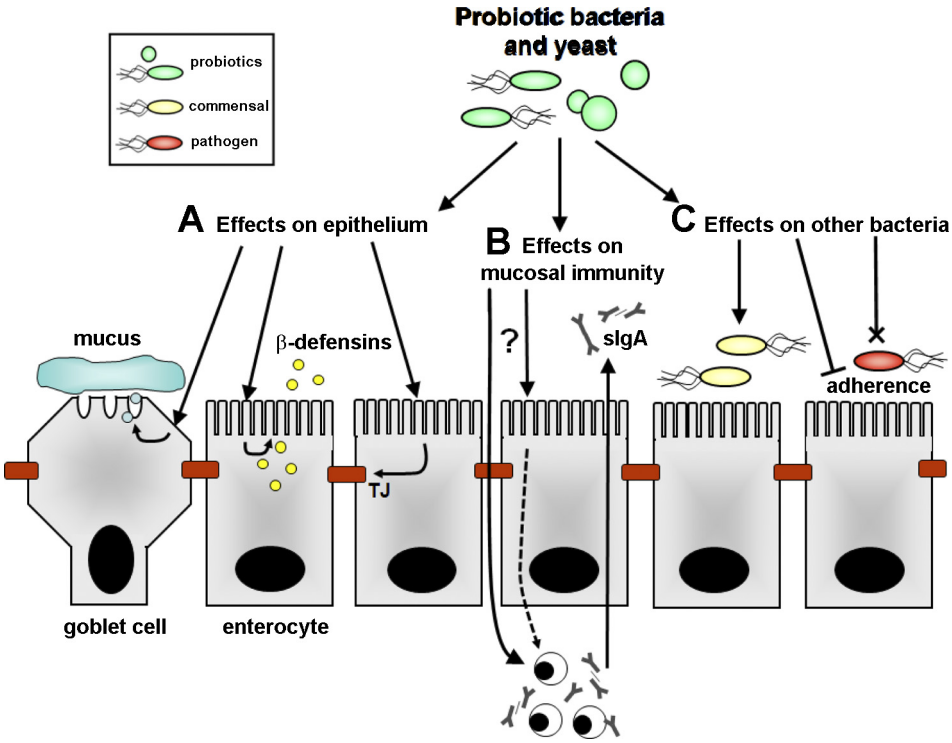


Fig. 1. Effects of probiotic bacteria and yeast on intestinal epithelial barrier function. Probiotics affect the epithelial barrier in numerous, diverse ways. This multifactorial approach to enhancing intestinal barrier function aids in developing and maintaining homeostasis. Depending on the strain of bacteria or yeast and the model used, probiotics target the epithelial barrier in the following 3 areas. *A*: direct effects on the epithelium. Probiotics can increase mucin expression and secretion by goblet cells, thereby limiting bacterial movement across the mucous layer. Augmentation of β-defensin expression and secretion into the mucus by epithelial cells can prevent the proliferation of commensals and pathogens, thus also contributing to barrier integrity. Finally, probiotics can enhance tight junction stability, which decreases epithelial permeability to pathogens and their products. *B*: effects on mucosal immunity. Probiotics can increase levels of IgA-producing cells in the lamina propria and promote secretory IgA (sIgA) secretion into the luminal mucous layer. These antibodies limit epithelial colonization by binding bacteria and their antigens, thus contributing to gut homeostasis. *C*: effects on other surrounding or infecting bacteria. Probiotics can alter the microbiota composition and/or gene expression, leading to indirect enhancement of the barrier through the commensal bacteria. Furthermore, some probiotics can directly kill or inhibit growth of pathogenic bacteria via expression of antimicrobial factors such as bacteriocins. Probiotics can also compete with pathogens or commensals for binding sites on mucins or epithelial cells, thereby preventing detrimental colonization and contributing to barrier function.

support of this concept, probiotics have been shown to increase mucin expression in vitro, contributing to barrier function and exclusion of pathogens. Several *Lactobacillus* species increased mucin expression in the human intestinal cell lines Caco-2 (MUC2) and HT29 (MUC2 and 3), thus blocking pathogenic *E. coli* invasion and adherence (78, 87). However, this protective effect was dependent on *Lactobacillus* adhesion to the cell monolayers, which likely does not occur in vivo. Conversely, another group showed that *L. acidophilus* A4 cell extract was sufficient to increase MUC2 expression in HT29 cells, independent of attachment (67). Additionally, VSL#3 (which contains some *Lactobacillus* species), but not EcN, increased expression of MUC2, 3, and 5AC in HT29 cells (105). In vivo studies are less consistent, in part because of the fact that very few studies have been performed. Mice given VSL#3 daily for 14 days did not exhibit altered mucin expression or mucous layer thickness (45). Conversely, rats given VSL#3 at a similar dose daily for 7 days had a 60-fold increase in MUC2 expression and a concomitant increase in mucin secretion (16). MUC1 and MUC3 expression were also elevated with probiotic stimulation, but to a lesser extent (16). Therefore, mucus production may be increased by probiotics in vivo, but further studies are needed to make a conclusive statement.

Additionally, intestinal trefoil factor 3 (TFF3) is coexpressed with MUC2 by colonic goblet cells and is suggested to promote wound repair (45, 64, 85). However, healthy rats did not display increased colonic TFF3 expression after stimulation by VSL#3 probiotics (16). Furthermore, mice treated with 1% dextran sodium sulfate (DSS) to induce chronic colitis did not exhibit increased TFF3 expression or wound healing when subsequently treated with VSL#3 (45). This observation indicates that probiotics do not enhance barrier function by upregulation of TFF3, nor are they effective at healing established inflammation. Therefore, use of current probiotics is likely to be effective only in preventing inflammation as shown by studies in animal models (13, 42).

Host cell antimicrobial peptides. There are two main families of intestinal antimicrobial peptides: defensins and cathelicidins. The latter family consists of cationic, α-helical antimicrobial peptides constitutively expressed by gastrointestinal epithelial cells, which are involved in host defense against pathogens (65). The only microbial or inflammatory stimulus that appears to induce cathelicidin expression is butyrate, which is produced by the enteric microflora (131). Yet few if any studies have looked at the role of cathelicidin production in probiotic function. One study, however, used butyrate to treat established *Shigella* infection in rabbits and reported a signif-

icant reduction in dysentery that correlated with upregulation of cathelicidin (126). Further studies of nonpathogenic bacterial regulation of the cathelicidin family of antimicrobial peptides would be beneficial in defining the role and mechanisms by which probiotics may protect against infection.

Defensins are further classified into α-defensins expressed primarily by small bowel Paneth cells (HD-5 and -6) and β-defensins expressed by epithelial cells throughout the intestine (hBD-1 through -4) (155). The small (3–5 kDa), cationic peptides display antimicrobial activity against a wide variety of bacteria, fungi, and some viruses and are constitutively expressed to keep pathogens from reaching the epithelium. Lowered defensin production has been associated with the development of IBD and with increased susceptibility to bacterial infections (65, 94, 155).

In vitro studies have shown that hBD-2 expression and/or secretion was significantly upregulated in Caco-2 cells upon stimulation by EcN, the commensal *E. coli* strain DSM 17252 S2 G_2 (sold as Symbioflor 2), several *Lactobacilli* species, or VSL#3 (97, 132, 155). Expression of hBD-1, HD-5, and HD-6 was not altered by any of the probiotic bacteria (not tested with Symbioflor 2). Furthermore, healthy humans who received Symbioflor 2 twice daily for 3 wk were shown to have significantly increased fecal levels of hBD-2 protein, whereas placebo-treated individuals had no change. hBD-2 levels were still elevated 9 wk after cessation of probiotic treatment, although to a lesser degree (97).

By use of specific inhibitors, this enhanced secretion of hBD-2 was shown to require the MAP kinases ERK 1/2, p38, and JNK in vitro. NF-κB and activator protein-1 (AP-1) were also implicated, since deletion of their binding sites on the hBD-2 promoter completely inhibited probiotic-mediated defensin expression (132, 155). Additionally, flagellin was found to be the major stimulatory factor since purified EcN flagellin induced hBD-2 mRNA expression, but flagellin-negative mutants or EcN incubated with flagellin antiserum did not (133). Although activation of the proinflammatory NF-κB pathway by bacterial flagellin is characteristic of the Toll-like receptor 5 (TLR5) bacterial recognition signaling, the role of this pathway in defensin production is not yet known. Further studies of the host cell receptors involved are needed to fully understand this interesting probiotic effect on defensin production.

Probiotic antimicrobial factors. In addition to altering expression levels of host cell-derived antimicrobial peptides, probiotics can directly inhibit growth or killing of pathogens by production of antimicrobial molecules including short-chain fatty acids (SCFA) and bacteriocins or microcins. Given the intimate relationship between the commensal microbiota and host mucosal homeostasis, secreted commensal bacterial factors can be considered an integral part of the intestinal barrier, particularly when these factors are involved in suppressing or killing pathogenic organisms. Indeed, commensal microbes are essential to normal gut physiology, as shown by the severely altered intestinal function in germ-free mice (24). Thus these bacteria and their products must be considered when evaluating intestinal barrier function.

It has been shown that both *Lactobacillus* and *Bifidobacterium* strains can directly kill *Salmonella typhimurium* in vitro (34, 72). Probiotic bacteria can lower the luminal pH through secretion of acetic and lactic acids, which inhibits the growth of some pathogens, including enterohemorrhagic *E. coli* (EHEC) (104). SCFA production also decreased EHEC Shiga toxin expression, providing an additional barrier to infection (17). Furthermore, these SCFA can disrupt the outer membranes of gram-negative pathogens such as EHEC, *P. aeruginosa*, and *S. typhimurium*, causing inhibition of pathogen growth. This permeabilization potentiates the activity of other antimicrobial molecules by allowing them to more easily penetrate the cell wall (3).

However, the protective effects of *Lactobacilli* probiotics are predominantly via nonlactic acid mechanisms (34). Bacteriocins and microcins are similar peptides with bactericidal (kills bacteria) or bacteriostatic (inhibits growth) activity and are produced in a strain-specific manner by a wide variety of both probiotic and commensal bacteria (74). Typically, the term "bacteriocin" refers to peptides produced by gram-positive bacteria, whereas gram-negative bacteria are said to produce microcins, thus named because they are <10 kDa in size. Bacteriocins can either permeabilize the inner membrane of gram-negative bacteria, leading to disruption, or interfere with cell wall synthesis and cause the formation of pores by binding to the peptidoglycan precursor lipid II. Microcins, on the other hand, can target the inner membrane, enzymes that are involved in DNA or RNA structure and synthesis, or protein synthesis enzymes (30). All of these mechanisms induce rapid bacterial death and thus contribute to maintaining a pathogen-free intestinal barrier.

L. salivarius produces a two-peptide bacteriocin, ABP-118, which inhibits the growth of *Bacillus*, *Listeria*, *Enterococcus*, and *Staphylococcus* species. However, growth of most *Lactobacillus* species was not inhibited by these peptides, thus imparting a selective advantage for intestinal colonization and limiting the growth of pathogenic bacteria (40). *L. lactis* also produces a two-peptide bacteriocin, lacticin 3147. This antimicrobial peptide forms selective ion pores and is a broad-spectrum inhibitor of gram-positive bacteria, including clinical *Clostridium difficile* isolates (90, 117, 127).

Other molecules are produced by probiotic bacteria in addition to bacteriocins but have not been as well characterized. For example, *L. delbrueckii* produce at least four bacteriostatic agents in vitro. Two of these are H_2O_2, which kills bacteria by oxidation, and lactic acid as described above. Another molecule was characterized as bacteriocin-like because it inhibited the growth of two similar *Lactobacillus* strains, was sensitive to heat and protease activity, and was larger than 50 kDa. The fourth, however, is heat stable and inhibits the growth of *Streptococcus thermophilus* (153).

The antimicrobial potential of two strains of *Bifidobacterium* isolated from human feces has also been characterized. The inhibitory activity of both strains was not due to a protein given that it was not affected by proteases, but instead by a lipophilic molecule(s) of unknown identity. This molecule found in culture supernatant was able to decrease the viability of *E. coli*, *Klebsiella pneumoniae*, *Yersinia pseudotuberculosis*, *Staphylococcus aureus*, and *S. typhimurium*. Furthermore, this antimicrobial component prevents invasion of Caco-2 cells by *S. typhimurium* and can kill intracellular *S. typhimurium* in a treatment model. Similarly, monoassociation of gnotobiotic mice with either of these *Bifidobacteria* significantly reduced *S. typhimurium*-induced death (72).

Interestingly, a strain of *L. acidophilus* also secretes a protease-insensitive, nonlactic acid, antimicrobial component active against many of the same pathogens as the above mentioned *Bifidobacterium* strains. This component did not inhibit growth of other normal gut flora, such as *Lactobacilli* and *Bifidobacteria*. Moreover, monoassociation of gnotobiotic mice with this bacterium reduced *S. typhimurium* illness (11). Perhaps both *Bifidobacterium* and *L. acidophilus* employ a similar component to inhibit *Salmonella* infection; however, this cannot be known by these studies alone. It will be interesting to see future results from these models, further characterizing the molecule(s) involved.

Epithelial adherence. Probiotic bacteria can also contribute to intestinal barrier function against invading pathogens in a strain-specific manner by competing for binding sites to epithelial cells and the overlying mucous layer. *L. rhamnosus* and *L. acidophilus* can adhere to intestinal epithelial cells in vitro (HEp-2 and T84 cell lines), and pretreatment of these probiotic strains reduced the binding of enteropathogenic *E. coli* (EPEC) and EHEC. However, *L. gasseri*, *L. casei*, and *L. plantarum* do not block EHEC adherence (139). Furthermore, *L. rhamnosus* pretreatment inhibited the EHEC-induced increase in permeability, a measure of monolayer integrity (62). Although this preventative effect was inhibited by heat killing the bacteria, surface layer proteins (which form crystalline lattice on the outer membrane of many bacterial species and can mediate cell adhesion) from other *Lactobacilli* can bind epithelial cell lines in the absence of live bacteria. In fact, surface layer proteins purified from *L. helveticus* similarly inhibited EHEC adherence and the subsequent rise in permeability, without altering growth of the pathogen. This demonstrates the strain specificity of probiotic function (63).

Additionally, *Lactobacillus* strains can directly compete with other pathogens, such as *Salmonella* species, for binding sites on human mucins or Caco-2 cell surfaces. They can also displace bound pathogens, although more slowly and to a lesser extent (71). EcN secretes a nonbacteriocin component that may act on either the pathogen or the host cell to inhibit adherence of several pathogens (5), and *B. longum* secretes an unknown substance that binds EHEC Vero toxin to inhibit its adherence (66). These studies demonstrate the diverse pathways employed by probiotics to inhibit pathogen adherence to enhance epithelial barrier function.

Comparable inhibitory effects with probiotic bacteria have been seen with some in vivo studies. In a chronic stress model, pretreatment of rats with *L. rhamnosus* and *L. helveticus* reduced commensal bacterial adherence and translocation (164). Pretreatment with *L. rhamnosus*, but not *L. fermentum*, in a rat hemorrhagic shock model also reduced bacterial translocation and actin cytoskeleton rearrangement (76).

S. boulardii, on the other hand, has no known effect on pathogen attachment in vitro but appears to inhibit adherence in vivo in a noncompetitive manner. Cotreatment of T84 cells with *S. boulardii* and either EPEC, EHEC, or *Shigella flexneri* does not affect cellular adhesion of the pathogens (25, 26, 99). However, the probiotic does decrease levels of intracellular EPEC, likely by preventing ERK1/2 activation (25), but not intracellular *S. flexneri* (99). In vivo, this probiotic prevented *Citrobacter rodentium* adherence to intestinal epithelial cells by inhibiting secretion of key virulence factors involved in attachment (EspB and Tir), while exhibiting no bactericidal activity. Probiotic treatment of infected mice attenuated disease, including prevention of epithelial damage, infiltration of granulocytes, and loss in body weight. A heat-labile factor secreted by *S. boulardii* was found to be responsible for the decreased bacterial adherence (157). Thus inhibition of pathogenic adherence is one of the mechanisms for probiotic functionality in preventing intestinal infection.

Secretory IgA. Approximately 80% of all plasma cells are found in the intestinal mucosa, where more IgA is produced than any other isotype (in humans, 40–60 mg per kg per day). Peyer's patches are the main generation site for IgA^+ B cells. IgM^+ B cells are recruited into Peyer's patches, where they are activated and proliferate through interactions with local T cells and/or dendritic cells. IL-6, IL-10, and TGF-β in the local environment promote class switching of these B cells to IgA production, then terminal differentiation into IgA^+ plasma cells. IgA dimers can then be shuttled into the lumen by epithelial cells via the polyimmunoglobulin receptor, where they are referred to as secretory IgA (sIgA) (reviewed in Refs. 19, 79).

Although both IgG and IgA are found in intestinal mucosal secretions, IgA is the more abundant isotype in healthy individuals and is historically considered to be the primary element of the mucosal immune response to microbial antigens (59, 128). However, IgG has recently been recognized as an important part of the mucosal innate immune response (125). It is upregulated upon antigenic stimulation in many mucosal sites (nasal, tracheal, urogenital, and intestinal), is transported into the lumen via the neonatal Fc receptor, which is specific for IgG, and has been found to be protective against viral and bacterial infections (22, 38, 89, 106, 118, 125, 162). Researchers have begun to exploit this fact by developing vaccines that target mucosal IgG production along with serum IgG and mucosal IgA, particularly for respiratory and urogenital infections (52, 59, 61, 100). However, the majority of information available addresses the protective effects of mucosal IgA, which will therefore be the focus of this review.

In the luminal mucous layer, sIgA protects the intestinal epithelium against colonization and/or invasion by binding antigens on pathogens or commensals. This surrounds the microorganism with a hydrophilic shell that is repelled by the epithelial glycocalyx, thus providing immune exclusion of bacteria (79, 98). The antigen-complexed IgA can also bind the receptor FcαRI (CD89) constitutively expressed on immune cells such as neutrophils, interstitial dendritic cells, monocytes, and some macrophages (19, 166). Receptor binding can initiate antimicrobial activity (including antibody-dependent cell-mediated cytotoxicity, phagocytosis, and generation of bactericidal superoxides), anti-inflammatory signaling, or proinflammatory signaling depending on the cell type and nature of IgA ligand involved (98, 125). Immune exclusion not only protects the epithelium from invading pathogens, it is also important in maintaining gut homeostasis by preventing overgrowth of the enteric microflora (108, 145, 150). Furthermore, sIgA can protect against intracellular pathogens by binding and neutralizing viral or bacterial components during transcytosis of the epithelium. The immune complexes are then secreted apically and invasion is inhibited (37).

Probiotics have been shown to augment total and pathogen-specific sIgA levels upon infection, while typically not inducing production of probiotic-specific sIgA. Mice given *L. casei*

displayed significantly increased numbers of IgA^+- and IL-6-producing cells (which can stimulate B cell class switching to IgA) in the small bowel lamina propria. Specific antibodies against *L. casei* were not produced, indicating the nonresponsiveness of the gut immune system to this beneficial bacteria (44). However, another study found that mice monoassociated with *L. casei* did not have increased levels of sIgA (84). Pretreatment of mice with *Bifidobacterium* species significantly reduced illness after challenge with rotavirus (*B. bifidum* and *B. infantis* used) or EHEC (*B. lactis* used). Gut mucosal pathogen-specific IgA antibody titers were increased in animals given probiotic pretreatment in both experiments (115, 140), and total levels of sIgA were increased in mice monoassociated with *B. animalis* or *E. coli* EMO in a different study (84). In infant rabbits pretreated with *L. casei*, morbidity of subsequent EHEC infection was reduced due to increased mucosal levels of anti-EHEC and anti-Shiga toxin IgA antibodies compared with controls (103). Interestingly, peptides released by *L. helveticus* during milk fermentation can also increase the sIgA response. Rats treated with this bioactive peptide before challenge with EHEC displayed increased numbers of intestinal lamina propria IgA^+ B cells and levels of sIgA (70). Purification and identification of the peptide, as well as future investigations into the presence of peptide- or EHEC-specific IgA responses in this model will be informative.

However, not all probiotics are equal in terms of their effects on sIgA production. A combination of *L. rhamnosus* and *B. lactis* did not increase sIgA levels in rats. But rats given prebiotics (oligofructose-enriched inulin) or synbiotics (*L. rhamnous*, *B. lactis*, and inulin) did exhibit increased sIgA production (124), indicating the diversity of stimuli that can lead to enhanced immune exclusion in the gut. Furthermore, *S. boulardii* differs somewhat from probiotic bacteria in its effect on sIgA. This yeast similarly increases total levels of sIgA in monoassociated and conventionally raised mice, but levels of anti-*S. boulardii* sIgA in the rat model were also increased, showing an immune response to the probiotic itself (84, 114, 122).

Probiotics have many other immunomodulatory effects in the human intestine, including promoting tolerogenic dendritic cell and regulatory T cell phenotypes, inhibiting inflammatory cytokine production, and enhancing natural killer cell activity (101). However, these effects have been the subject of other reviews (35, 101) and will not be covered here.

Epithelial cell tight junctions. After navigating the mucous layer containing antimicrobial peptides and sIgA, and competing for binding sites with the commensal microflora, many pathogens next penetrate the epithelium and cause disease. Enterocytes express pathogen-associated molecular pattern receptors, including TLRs and nucleotide-binding oligomerization domain (NOD)-containing proteins (reviewed in Refs. 10, 41). These receptors sense the presence of conserved bacterial motifs and can initiate a proinflammatory cascade for defense. To differentiate between commensal and pathogenic bacteria, these molecules are located intracellularly (e.g., NODs and TLR9), basolaterally (e.g., TLR5), or in limited numbers on unstimulated enterocytes (e.g., TLR2 and TLR4) (10, 12, 41, 48, 57). Therefore, only after a breach of the barrier will pathogens or commensal bacteria that passively diffuse across activate these pathways in healthy adults.

Epithelial cell-cell adhesion, however, is an essential component of enterocyte barrier function. Several components make up the intercellular junctional complexes: tight junctions (TJ), adherens junctions (AJ), gap junctions, and desmosomes, the best characterized of which are TJ (reviewed in Refs. 50, 83). Two main types of transmembrane proteins are found in TJ, occludin and claudins, which link adjacent enterocytes through interactions of their extracellular loops (55). Occludin localization to TJ is regulated by phosphorylation at multiple sites by the nonreceptor tyrosine kinase c-Yes or PKC (7, 20, 129). The extracellular loops of the claudin family directly regulate passive paracellular flux of ions and small molecules according to size and charge, preferentially allowing small cations to move across the barrier (51, 60, 147). TJ also include the intercellular zonula occludens (ZO) scaffolding proteins that link the transmembrane junctional proteins to the actomyosin cytoskeleton and several cytoplasmic regulatory proteins. Furthermore, ZO proteins provide a link between TJ and AJ through interactions with the AJ cytoskeleton connector α-catenin (55). The junctional adhesion molecule family is composed of single-pass transmembrane proteins with two extracellular immunoglobulin-like domains involved in the formation of TJ, regulation of permeability, and leukocyte-endothelial cell interactions (50, 55, 136). These proteins interact with ZO-1 via PDZ-binding domains, which provide anchorage to the actin cytoskeleton (50).

Regulation of TJ structure, and therefore epithelial barrier permeability, is achieved via myosin light chain II (MLC) phosphorylation and contraction of the perijunctional actomyosin ring (88, 138, 151). MLC kinase (MLCK), MAP kinases ERK1/2 and p38, and Rho kinase (ROCK; activated by Rho GTPases) can all phosphorylate MLC causing increased permeability (50, 54). Interestingly, pathogens can alter barrier function by altering MLC phosphorylation. For example, both EPEC and *H. pylori* can activate MLCK, causing actomyosin ring contraction, disruption of TJ proteins, and increased permeability (36, 69, 110, 149, 163). The regulation of Rho GTPases is tightly monitored in the cell, and either activation or inhibition of its ROCK phosphorylating capabilities can result in aberrant barrier function. *C. difficile* toxins inactivate small Rho GTPases by glucosylation, which leads to cytoskeletal and junctional rearrangement (46, 102). On the other hand, pathogenic *E. coli* strains, *S. typhimurium*, and the mouse pathogen *Citrobacter rodentium* activate Rho GTPases, which leads to perijunctional actin contraction (14, 39, 47, 86). Both of these mechanisms cause increased permeability as demonstrated by decreased transepithelial resistance (TER). Furthermore, actin polymerization by PKC is enhanced by the ZO toxin of *Vibrio cholerae*, again leading to disrupted TJ and increased permeability (33). The number of different mechanisms that pathogens have evolved to cross the epithelial barrier demonstrates how crucial this function is to homeostasis since a breach of this barrier can result in acute inflammation.

Chronic inflammation is often associated with altered TJ barrier function that allows for the passage of microbial antigens into underlying immune tissues. A dysregulated immune response to normal enteric microflora and/or microbial dysbiosis is thought to contribute to the pathogenesis of IBD in genetically susceptible individuals (e.g., *NOD2/CARD15* polymorphism) (21, 68, 159). However, it is still widely debated

whether disrupted TJs are a cause or a consequence of this disease. Proinflammatory cytokines seen in IBD patients (e.g., TNF-α, IFN-γ, IL-1β, and IL-13) can increase epithelial permeability, which could exacerbate inflammation (2, 6, 154, 161). On the other hand, preexisting barrier dysfunction could initiate this inflammation by allowing diffusion of bacterial antigens into the immune cell-rich lamina propria. In ulcerative colitis patients, barrier disruption appears to be secondary to inflammation, although the sequence of events is difficult to assess (82, 93, 134). In Crohn's disease patients, TJ structure is restored during remission (165), and disruption of this structure appears to precede clinical relapse (58, 141, 158). Moreover, both types of IBD patients with active inflammation display increased expression of the pore-forming claudin 2, whereas sealing claudins 5 and 8 were decreased in active Crohn's disease (56, 165).

Many studies have shown that pretreatment with probiotic bacteria can inhibit the decrease in resistance and TJ alteration caused by stress, infection, or proinflammatory cytokines (1, 25, 26, 32, 73, 80, 99, 120, 139). Of interest is the finding that probiotics can directly alter epithelial barrier function by influencing the structure of TJ. A study by Resta-Lenert and Barrett (119) found that *S. thermophilus* and *L. acidophilus* independently increased TER and decreased permeability of HT-29 and Caco-2 cells. These bacteria also induced activation of occludin and ZO-1, as shown by increased levels of phosphorylated proteins without a significant change in the total levels. Bacterial cultured medium and killed bacteria (by antibiotics or heat) failed to elicit any of the same responses, suggesting that live *S. thermophilus* and *L. acidophilus* are required for enhancement of barrier function.

Similarly, conditioned medium from several bacteria strains found in VSL#3 were found to independently increase TER of T84 cells after 4 h of incubation (32). *B. infantis*-conditioned medium exerted the biggest effect, which was maximal after 6 h, and also decreased cell monolayer permeability as shown by mannitol flux. Claudin-2 protein expression was decreased, whereas ZO-1 and occludin total protein expression was increased upon exposure. Expression of claudins-1, 3, and 4 was not altered at this time point. *B. infantis*-conditioned medium also rapidly increased levels of phospho-ERK1 and 2 and decreased phospho-p38, thus indicating that TJ tightening is achieved via a MAPK pathway. This was further verified by use of an ERK1/2 inhibitor that blocked the increase in TER. Pretreatment with the probiotic-conditioned medium also prevented a decrease in TER (corresponding to claudin and occludin redistribution), caused by TNF-α or IFN-γ exposure, and was blocked by ERK inhibition (32).

In contrast, treatment of HT-29 and Caco-2 cells with *S. thermophilus* and *L. acidophilus* activated p38, ERK, phosphatidylinositol 3-kinase (PI3K), and JNK pathways (119, 120). Pretreatment of monolayers with this combination of probiotics similarly prevented the deleterious effects of cytokine (IFN-γ or TNF-α) exposure. This included decreased ion secretion (as measured by short-circuit current), decreased TER, increased permeability, and activation of inflammatory pathways (STAT1 and 3, SOCS3, and IκB-α) commonly seen as a result of these cytokines. The reversal of cytokine-induced decrease in TER and ion secretion was shown to be dependent on ERK, p38, and PI3K activation by the probiotic bacteria (120).

Later studies by Zyrek et al. (167) showed that EcN also enhances the TER of T84 cells in vitro, which was associated with increased expression and TJ localization of ZO-2. PKC-ζ is the only PKC isoform located at TJ complexes and is recruited to the membrane during EPEC infections. Here, it can phosphorylate ZO-1, causing removal of this TJ scaffolding protein to the cytosol (149). Inhibition by use of a PKC-ζ pseudosubstrate protects against TJ disruption by EPEC (167). Oddly, exposure of cells to EcN causes an increase in PKC-ζ expression; however, EcN does not cause membrane translocation that would disrupt TJ structures. Furthermore, the decreased TER caused by EPEC infection can be reversed by removal of pathogenic *E. coli* and incubation with probiotic EcN (167). Despite this, other than noting an increase in ZO-2 expression, this study did not look into the mechanisms involved in barrier enhancement by probiotics.

In vivo studies by Ukena et al. (152) demonstrate that colonization of gnotobiotic mice with EcN causes an increase in ZO-1, but not ZO-2, expression. In conventionally colonized mice challenged with DSS to induce colitis, probiotic pretreatment lessened disease and induced ZO-1 expression. The authors did not observe alterations in TER due to DSS or EcN; however, pretreatment with EcN significantly reduced DSS-mediated dye uptake into the colonic mucosa, an indicator of permeability. Conversely, another group briefly investigated this subject and found that *L. rhamnosus* and *L. helveticus* did not alter ileal or colonic permeability in rats (164).

The impact of the study by Ukena et al. (152) would be strengthened by inclusion of additional controls. No conventionally raised mice were treated with EcN without subsequent DSS challenge to establish appropriate baseline measurements of ZO-1 expression, TER, and dye uptake. In addition, studying colonization effects of probiotics in only germ-free mice is a concern because these mice display numerous morphological and immunological differences that could affect results. Specific pathogen-free mice or antibiotic-treated and monoassociated with a specific bacteria would be an important complement to germ-free models and perhaps more clinically relevant.

Increased permeability of the epithelial barrier can also be caused by apoptosis via caspase-3 activation (23). Although there are studies implicating activation of TLRs by commensal bacteria in the inhibition of apoptosis and upregulation of intestinal epithelial cell proliferation, this may not be the case in healthy adults since TLRs are thought to be sequestered away from the microflora (18, 43, 116). Nevertheless, after inflammatory insult or antibiotic treatment, TLR signaling by commensal or probiotic bacteria may play a role in restoring homeostasis. Probiotics have also been found to modulate apoptosis initiation by harmful stimuli. *S. boulardii* pretreatment prevented EHEC-induced apoptosis in T84 cells. This probiotic prevented pathogenic cleavage of procaspases-3, -8, and -9 and DNA fragmentation, likely through inhibition of TNF-α production and secretion (27).

Another study found that two proteins (p40 and p75) secreted from *L. rhamnosus* inhibited cytokine-induced apoptosis in epithelial cell lines by activating the EGF receptor and its downstream target Akt, as well as inhibiting p38 MAPK activation, in vitro and ex vivo (160). Akt promotes cell survival by inactivating proapoptotic proteins, including caspases 3 and 9 (53). Expression of p40 and p75 is strain specific because *L. casei*, but not *L. acidophilus*, also produces these proteins (160). Addi-

tionally, apical or basolateral pretreatment with either p40 or p75 protected several cell lines from H_2O_2-induced disruption of barrier function, as measured by TER and paracellular permeability. This effect was via inhibition of H_2O_2-induced cytosolic relocalization of the TJ proteins occludin and ZO-1 and the AJ proteins E-cadherin and β-catenin. These effects were all dependent on activation of PKCε, PKCβI, and the MAP kinases ERK1/2 (135). Therefore, bacterial proteins isolated from *L. rhamnosus* cultures effectively block the induction of apoptosis, helping to enhance epithelial barrier function. However, this group did not look at the effect of p40 or p75 on TJ structure in the absence of noxious stimuli, which would be informative.

Although there are many studies indirectly looking at barrier function in the presence of probiotics, most focus on the preventative effects to subsequent bacterial or inflammatory challenge. Therefore, future studies should examine the direct effects of various probiotic bacteria on TJ structure and function. This could include examining localization of other junctional proteins such as the claudins (especially the pore-forming claudin 2) and the effects on intercellular signaling pathways that regulate TJ structure. MLCK activity and the phosphorylation state of MLC after exposure of either cell lines or mucosal tissue will give direct evidence of probiotic influence on barrier function. Furthermore, future investigations into the bacterial factors involved (such as *Lactobacillus* p40 and p75) would be useful in developing probiotic-derived products to use as therapy in immunocompromised individuals who cannot use live probiotics.

Summary

Probiotics have long been used as an alternative to traditional medicine with the goal of maintaining enteric homeostasis and preventing disease. However, the actual efficacy of this treatment in still debated. Clinical trials have shown that probiotic treatment can reduce the risk of some diseases, especially antibiotic-associated diarrhea, but conclusive evidence is impeded owing to the wide range of doses and strains of bacteria used. So while probiotics represent a promising alternative or adjunct therapy, further studies will be needed before they can be widely used. The mechanism of action is also an area of interest. Many studies, as discussed above, have shown that probiotics increase barrier function in terms of increased mucus, antimicrobial peptides, and sIgA production, competitive adherence for pathogens, and increased TJ integrity of epithelial cells. Hopefully, understanding the effect that probiotics have on basic physiology will influence how they are used clinically in the future. By recognizing the strain specificity of probiotic function, perhaps development of a complementary mix of probiotics to protect against several insults will be within our grasp.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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