


Mechanisms of Action of Probiotics

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ABSTRACT

Probiotics are living microorganisms that confer health benefits to the host when administered in adequate amounts; however, dead bacteria and their components can also exhibit probiotic properties. *Bifidobacterium* and strains of lactic acid bacteria are the most widely used bacteria that exhibit probiotic properties and are included in many functional foods and dietary supplements. Probiotics have been shown to prevent and ameliorate the course of digestive disorders such as acute, nosocomial, and antibiotic-associated diarrhea; allergic disorders such as atopic dermatitis (eczema) and allergic rhinitis in infants; and *Clostridium difficile*-associated diarrhea and some inflammatory bowel disorders in adults. In addition, probiotics may be of interest as adjuvants in the treatment of metabolic disorders, including obesity, metabolic syndrome, nonalcoholic fatty liver disease, and type 2 diabetes. However, the mechanisms of action of probiotics, which are diverse, heterogeneous, and strain specific, have received little attention. Thus, the aim of the present work was to review the main mechanisms of action of probiotics, including colonization and normalization of perturbed intestinal microbial communities in children and adults; competitive exclusion of pathogens and bacteriocin production; modulation of fecal enzymatic activities associated with the metabolism of biliary salts and inactivation of carcinogens and other xenobiotics; production of short-chain and branched-chain fatty acids, which, in turn, have wide effects not only in the intestine but also in peripheral tissues via interactions with short-chain fatty acid receptors, modulating mainly tissue insulin sensitivity; cell adhesion and mucin production; modulation of the immune system, which results mainly in the differentiation of T-regulatory cells and upregulation of anti-inflammatory cytokines and growth factors, i.e., interleukin-10 and transforming growth factor; and interaction with the brain-gut axis by regulation of endocrine and neurologic functions. Further research to elucidate the precise molecular mechanisms of action of probiotics is warranted. *Adv Nutr* 2019;10:S49–S66.

Keywords: bifidobacteria, lactic acid bacteria, lactobacilli, mechanism of action, probiotics, volatile fatty acids, brain-gut axis, immune system

Introduction

The term “probiotics” refers to microorganisms that confer health benefits to hosts when administered in adequate amounts (1–3). A true probiotic should preferably be of

human origin, safe, and free of vectors that are able to transfer resistance to antibiotics and of pathogenicity or toxicity factors. In addition, a probiotic should have great capacity to survive under intestinal conditions (acidic pH, enzymes, biliary salts, etc.). Moreover, a probiotic should exhibit antagonism against pathogens and stimulation of the immune system and, ultimately, must have demonstrable beneficial effects on the host. Finally, maintenance of the activity, viability, and growth efficacy of the probiotic upon technologic treatment should be demonstrated (4, 5).

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Abbreviations used: ASD, autism spectrum disorder; BCFA, branched-chain fatty acid; BSH, bile salt hydrolase; CD, Crohn disease; CMA, cow-milk allergy; DC, dendritic cell; EFSA, European Food Safety Authority; eHCF, extensively hydrolyzed casein formula; FoxP3, forkhead box P3; GPR, G protein-coupled receptor; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; LAB, lactic acid-producing bacteria; NAFLD, nonalcoholic fatty liver disease; NEC, necrotizing enterocolitis; NF- κ B, nuclear transcription factor κ B; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RCT, randomized clinical trial; Th, T-helper; TLR, Toll-like receptor; T-reg, regulatory T cell; T2D, type 2 diabetes.

Ilya Ilyich Mechnikov (6) performed the first investigations on lactic acid-producing bacteria (LAB) and their health effects in humans, and results from these first investigations suggested that LAB ingestion improved host health. LAB are a heterogeneous group of microorganisms that are often present in the human gut, being introduced via the ingestion of fermented foods, such as yogurt and other fermented milk products, various cheeses, and fermented cured meat by-products. Strains of *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Saccharomyces boulardii*, and *Escherichia coli* Nissle 1917 are the most widely used probiotic bacteria. However, other strains such as *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* are also used as probiotics (7–9).

In 2014, the International Scientific Association for Probiotics and Prebiotics stated that the development of metabolic by-products, dead microorganisms, or other microbe-based nonviable products has potential; however, these do not fall under the probiotic construct (3). Nevertheless, several studies have shown that dead bacteria and bacterial molecular components display probiotic properties (4, 5, 10). Currently, the term “postbiotic” refers to soluble components with biological activity that, could therefore be a safer alternative to the use of whole bacteria (11).

The effects of probiotics on host health have been reported in many articles, reviews, and systematic reviews (12, 13). These studies have documented the role of probiotics in the prevention of health problems, including digestive disorders such as diarrhea caused by infections (4), antibiotic-associated diarrhea (14), irritable bowel syndrome (IBS) (15), *Clostridium difficile*-associated diarrhea in adults and children (16), inflammatory bowel disease (IBD), only in ulcerative colitis (17), and allergic disorders such as atopic dermatitis (eczema) (18) and allergic rhinitis (19).

Even though many probiotic strains are well documented as safe or denoted “generally recognized as safe,” the European Food Safety Authority (EFSA) and the US FDA do not attribute the ability to prevent or treat diseases to probiotic administration. Probiotics are purchased as dietary supplements in many countries and follow current market policies.

The EFSA has not approved any product with health claims associated with probiotic administration. More than 300 approval requests have been submitted for 200 probiotic strains or combinations of strains, claiming >60 beneficial effects (20). The principal reasons for these approval requests being denied were as follows: insufficient characterization, undefined claims, nonbeneficial claims, lack of relevant human studies, lack of measurable outcomes that reflect direct benefit for humans, and finally, the quality of the presented studies (20). In addition, the FDA might regulate probiotic strains as a dietary supplement, food ingredient, or drug (21). Similarly to the EFSA, the FDA has not approved any probiotics to prevent or treat health problems (22). Both food agencies have emphasized the following notions: each health claim is unique for each probiotic strain; scientific requirements have to be considered in the context of each application; guidelines and past evaluations are valuable sources of information; it is important to understand the

rationale behind the principles being applied; no recipe for success can be provided; and finally, researchers and companies need to try, fail, learn, and try again (20, 22). Moreover, many studies of probiotics lack insight into the potential mechanism of action.

However, Health Canada approves a multistrain probiotic [*Streptococcus salivarius* subsp. *thermophilus* (SD5207), *Bifidobacterium breve* (SD5206), *Lactobacillus plantarum* (SD5209), *Lactobacillus paracasei* (SD5218), *Bifidobacterium animalis* subsp. *lactis* (SD5220, SD5219), *Lactobacillus acidophilus* (SD5212), and *Lactobacillus helveticus* (SD5210)] and a single strain of *B. animalis* spp. *lactis* LAFTI B94 as natural health products for relief of IBS symptoms, such as abdominal discomfort, gas, and bloating (23).

Currently, it is accepted that gut dysbiosis refers to changes in the quantitative and qualitative composition of microbiota, that these changes may lead to altered host microbial interaction that can contribute to a disease state often with inflammation, and that this is associated with the development of many noncommunicable human diseases, but the mechanisms via which homeostasis is maintained are not yet completely understood (4, 24). Recent investigations have proposed that, during homeostasis, epithelial hypoxia limits oxygen availability in the colon, leading to the maintenance of a balanced microbiota that functions as a microbial organ, producing metabolites that contribute to host nutrition, immune training, and niche security (24, 25).

Probiotics are a current strategy to treat dysbiosis, restoring microbial diversity and altering the perturbed intestinal microbiota with specific mechanisms of action that have not been completely elucidated (26, 27). For this reason, we performed a literature review of the varied mechanisms of action of probiotics to understand the role of various strains in host homeostasis. A comprehensive search of the relevant literature was performed with the use of electronic databases, including MEDLINE (PubMed), EMBASE, and the Cochrane Library. MEDLINE through PubMed was searched for scientific articles in English through the use of the terms “probiotics” combined with “mechanism of action,” “competitive exclusion,” “volatile fatty acids,” “mucin,” “immune system,” and “brain-gut axis.” The following mechanisms have been reviewed: 1) colonization and normalization of perturbed intestinal microbial communities in children and adults; 2) competitive exclusion of pathogens and bacteriocin production; 3) enzymatic activity and production of volatile fatty acids; 4) cell adhesion, cell antagonism, and mucin production; 5) modulation of the immune system; and 6) interaction with the brain-gut axis.

Colonization and Normalization of Perturbed Intestinal Microbial Communities in Children and Adults

Children

Early colonization of the infant gastrointestinal tract is likely to be a key determinant in the establishment of the gut

microbiome in later life (28). Assembly of the intestinal microbiota begins before childbirth and continues into childhood. Several factors influence initial intestinal colonization, such as the genetic constitution of the newborn, method of childbirth, use of antibiotics, type of feeding, and whether the mother is under stress or expresses an inflammatory condition (29). Bacteria isolated from the placenta, umbilical cord blood, and meconium (*Enterococcus faecium*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Escherichia coli*) are among those that might affect colonization (30, 31). However, the bacteria present in the vagina and in human milk seem to be more important for infant gut colonization (32, 33). These bacteria can spread from the digestive tract to extradigestive sites via dendritic cells (DCs), which can penetrate the epithelium and take the bacteria directly from the intestinal lumen. Once inside DCs or macrophages, the bacteria can be transported to other areas by immune cell circulation through the bloodstream (34). Adhesion of bacteria to host surfaces is a crucial aspect of host colonization because it prevents the mechanical clearing of pathogens. In addition to pili, which are polymeric hair-like organelles protruding from the surface of bacteria, and which represent a first class of structures involved in the binding of bacteria to host cells, a wide range of bacterial surface factors with adhesive properties have been described. These adhesins recognize various classes of host molecules including transmembrane proteins such as integrins or cadherins, or components of the extracellular matrix such as collagen, fibronectin, laminin, or elastin (35). Preclinical studies in children that used probiotics found positive results such as normalization of perturbed microbiota composition, intestinal maturation, decreased pathogenic load and infections, and improved immune response; however, only a few of these studies documented specific changes in the composition of the microbiota (13). In clinical studies in children, specific administered probiotic strains have shown promise in attenuation of the severity of different pathologies such as necrotizing enterocolitis (NEC), IBD, nosocomial and antibiotic-associated diarrhea, colic, and allergies (13, 36, 37).

Breastfeeding and formula feeding modify microbial succession in the gut in infants. Although commercially available formulas are supplemented with bacteria considered to be probiotics, little is known about the ability of these bacteria formulas to have a long-term impact on infant gut microbial composition and function (38). Specifically, changes in the composition of the gut microbiota have been observed to be directly correlated with increased concentrations of biomarkers of innate and acquired immunity after the use of a fermented milk product containing heat-killed cells of the probiotic strain *L. paracasei* CBAL74. Infants fed with this product showed higher amounts of *Bacteroides* and specific oligotypes of *Roseburia*, *Faecalibacterium*, and *Blautia*, which showed a positive correlation with secretory IgA (sIgA) and fecal defensin concentrations. In addition, an increase in the relative abundance of genes predicted to be involved in butyrate synthesis and higher fecal butyrate amounts associated with the consumption of this product have been described

(39). On the other hand, infants exposed to bifidobacteria-supplemented formula showed slight differences, such as decreased occurrence of *Bacteroides fragilis* and *Blautia* spp., compared with infants fed a placebo. To confirm colonization of the supplemented bifidobacteria, authors performed strain-specific analysis, detecting *Bifidobacterium bifidum*, *B. breve*, and *Bifidobacterium longum* in month 4. At 2 y of age, the strains were no longer detectable, suggesting that the supplemented bifidobacteria failed to stably colonize the infant gut due to competition within the ecosystem over time. The authors established these time points to study colonization and found that long-term colonization was not shown (38). This lack of probiotic colonization at 24 mo might be a benefit of their use, because the organism can be depleted from the gut through the effects of colonization resistance (40). Moreover, the most significant differences in the composition of the microbiota and in metabolite concentrations have been found between breastfed infants and those fed formula and between infants birthed vaginally and those birthed by cesarean delivery (38).

It has been suggested that pathogenesis in severe NEC must be multifactorial and may involve an overactive response of the immune system, causing an insult that might be ischemic, infectious, related to the introduction of enteric feeds, or a response to the translocation of normal enteric bacteria. Prophylactic treatment with probiotics in premature newborns has been shown to reduce the risk of severe NEC. Probiotic preparations containing *Lactobacillus* alone or in combination with *Bifidobacterium* have led to decreased mortality, days of hospitalization, and days after which exclusive enteral nutrition is achieved (41, 42). There are no reports in the literature about mechanisms associated with these positive effects on health; nevertheless, this treatment could help control the outgrowth of pathogenic bacteria due to the immature immune system of premature neonates (42).

With regard to infant colic, there is evidence that the use of *Lactobacillus reuteri* improves crying spells, but only after 2–3 wk of treatment, even with the natural evolution of this disorder (43–45). Other bacterial strains (bacilli and bifidobacteria) also appear to have some beneficial effects in alleviating the symptoms of infant colic and can lead to changes in the composition of the gut microbiota. *Lactobacillus rhamnosus* GG consumption resulted in increased abundance of different *Bifidobacterium* species compared with the effect seen upon consumption of a placebo. In general, *Bifidobacterium* was associated with differences between infants suffering from colic and healthy controls; infants with colic tended to be less frequently colonized with *B. breve* than healthy infants at a baseline level and at the end of the study, despite intervention (46).

With respect to probiotics for the prevention of pediatric diarrhea and antibiotic-associated diarrhea, it has been described that probiotics can restore microbial balance and thus inhibit the proliferation of pathogens such as *C. difficile*, acting as both preventive and treatment; however, most of the studies mainly provided clinical effects and tolerance and safety data but did not provide potential mechanisms of action (16, 45, 47, 48).

The intestinal microbiota has been analyzed to determine the long-term effects of *L. rhamnosus* GG intake on antibiotic use by preschool children and on antibiotic-associated gastrointestinal complaints. This intervention increased the abundance of *Prevotella*, *Lactococcus*, and *Ruminococcus* and decreased the abundance of *Escherichia*, appearing to prevent some of the changes in the microbiota associated with penicillin use but not those associated with macrolide use and preventing certain bacterial infections for ≤ 3 y after the trial (49). Recently, in children with acute watery diarrhea randomly assigned to receive *L. acidophilus* or placebo, no differences were observed in the daily fecal concentrations of rotavirus and norovirus or in *Lactobacillus* colonization in both groups (50).

Lactose intolerance usually leads to diarrhea; the effect of *L. acidophilus* strain LBKV-3 on fecal residual lactase activity in undernourished children <10 y of age was tested. Lactase activity increased over the course of the treatment (51). Some probiotics promote lactose digestion in lactose intolerance through increasing the overall hydrolytic capacity in the small intestine and increasing the colonic fermentation (52), and they can decrease lactose concentration in fermented products, and also increase active lactase enzyme entering the small intestine with the fermented products (53, 54).

The microbiome of lactose-intolerant individuals is represented by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Tenericutes*, *Elusimicrobia*, *Actinobacteria*, *Synergistetes*, *Cyanobacteria*, and *Lentisphaerae* (55). There is some evidence suggesting the clinical potential of probiotics against lactose intolerance, and *B. animalis* has been established among the most well-researched and effective strains (54, 56, 57).

Cow-milk allergy (CMA) is mostly a disease of infancy and early childhood. The majority of affected children have ≥ 1 symptoms involving ≥ 1 organ systems, mainly the gastrointestinal tract and/or skin (58). Many infants develop symptoms in ≥ 2 organ systems. Typical IgE-mediated symptoms include urticaria, angioedema, vomiting, diarrhea, and anaphylaxis. Dermatitis and rhinitis can be IgE and non-IgE mediated. Vomiting, constipation, hemosiderosis, malabsorption, villous atrophy, eosinophilic proctocolitis, enterocolitis, and eosinophilic esophagitis are non-IgE-mediated reactions (59). Samples from subjects with CMA showed that *Firmicutes* and *Clostridia* were enriched in the infant gut microbiome of subjects whose milk allergy resolved by age 8 y, whereas *Bacteroidetes* and *Enterobacter* were characteristic of subjects whose milk allergy did not resolve by age 8 y (60). Specifically, there seems to be a link between dysbiosis in the composition of the intestinal microbiota and the pathogenesis of CMA. The administration of probiotics such as *L. rhamnosus* GG in an extensively hydrolyzed formula led to increased tolerance in infants with CMA compared with those treated with hydrolyzed formula alone, which was due in part to changes in the structure of the bacterial community in the intestines of the infants (61).

The use of probiotics in adult diseases

After the administration or consumption of probiotic strains, the process of colonization begins. Few studies have assessed this step, and most have only evaluated major outcomes and drawn associations between those results and microbial administration.

In healthy adults, probiotic administration increases the production of SCFAs (see below), fecal moisture, frequency of defecation, and volume of stools (62). Recorded gastrointestinal symptoms, defecation frequency, and stool consistency were not influenced by *L. rhamnosus* PRSF-L477, indicating that this bacterium was well tolerated. The detection of *L. rhamnosus* in the feces of subjects in the probiotic-treated group was an important issue (63). Tolerance to *Lactobacillus salivarius* CECT5713 was assessed in healthy adults; the strain was tolerated, and no adverse effects were detected, but no attempt was made to evaluate intestinal colonization by this strain (64). However, intestinal persistence was observed in volunteers who received *L. rhamnosus* CNCM I-4036, as detected through the use of a specific primer for qRT-PCR (65). The effects of probiotics go beyond health status; subjects living with overweight and obesity are good candidates to receive probiotic strains, as individual treatments or as multistrain preparations. VSL#3 is a multistrain probiotic preparation that has been tested in subjects living with overweight. VSL#3 administration reduced the concentrations of lipids and inflammatory markers such as high-sensitivity C-reactive protein, enhanced insulin sensitivity, and produced changes in the composition of the gut microbiota (66). In patients living with obesity and hypertension, *L. plantarum* TENSIA decreased BMI and blood pressure (67).

IBD is a term used to describe a group of systemic pathologies that affect the gastrointestinal tract. In these conditions, the function of the epithelial barrier is affected and is a main factor in the onset of the disease and in further complications (4). Alterations in the gut microbiota might be associated with the initiation and progression of IBD. Probiotic treatment trials in patients living with Crohn disease (CD) showed no remission effect; in contrast, probiotic consumption by patients with ulcerative colitis seems to be more effective in the remission of the pathology, especially upon treatment with VSL#3 and a combination of *Lactobacillus* and prebiotics (68).

Butyrate-producing bacterial strains (*Butyricoccus pullicaecorum* 25-3T, *Butyricoccus pullicaecorum* 1.20, *Faecalibacterium prausnitzii*, and a mix of *Butyricoccus pullicaecorum* 25-3T, *Faecalibacterium prausnitzii*, *Roseburia hominis*, *Eubacterium hallii*, and *Anaerostipes caccae*) were tested in patients with CD to evaluate mucus stimulation. All the assayed strains exhibited increased butyrate production and improved the integrity of the epithelial barrier (69).

With regard to *C. difficile*-associated diarrhea, moderate-quality evidence suggests that probiotic administration results in efficient alleviation of this condition (70).

Competitive Exclusion of Pathogens and Bacteriocin Production

Competitive exclusion refers to the situation in which 1 species of bacteria competes for receptor sites in the intestinal tract more vigorously than other species (71). The specific pathways and key regulatory mechanisms underlying these effects of probiotics are largely unknown. Reduction in luminal pH, competition for nutritional sources, and production of bacteriocin or bacteriocin-like substances are among the main proposed mechanisms for competitive exclusion of pathogens (72).

Most studies have focused on the reduction of human pathogens such as *Salmonella typhi* and *E. coli* (73). Hence, some probiotic metabolites appear to play a role in the modulation of diverse signaling and metabolic pathways in cells. Indeed, components of the probiotic metabolome (organic acids, bacteriocins, hydrogen peroxide, amines, etc.) have been reported to interact with multiple targets in some metabolic pathways that regulate cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis, and metastasis (74).

Some lactobacilli and bifidobacteria can produce antimicrobial peptides known as bacteriocins, which prevent the proliferation of selected pathogens. The term “colonization resistance” refers to the use of probiotics to prevent or treat enteric pathogens (71). Bacteriocins are small cationic molecules composed of ~30–60 amino acids. These molecules act at bacterial cytoplasmic membranes and target energized membrane vesicles to disrupt the proton-motive force (75). Bacteriocins are classified into 4 main types based on their primary structures, molecular weights, post-translational modifications, and genetic characteristics (76). In particular, some of these compounds produced by *L. plantarum* and *L. acidophilus* have been shown to inhibit the growth of *Helicobacter*, *C. difficile*, rotaviruses, and multidrug-resistant *Shigella* spp. and *E. coli* in some gastrointestinal conditions (77) and have activity against a number of uropathogens (76).

Enzymatic Activities and Production of Volatile Fatty Acids

Enzymatic activities

The enzymatic activities of probiotics in the gut lumen can play a role in the biological effects of these probiotics. Lactobacilli and bifidobacteria exhibit >20 different enzymatic activities, with β -galactosidase activity being the most typical.

Intestinal bacterial β -glucuronidase hydrolyzes glucuronidated metabolites to their toxic forms in intestines, resulting in intestinal damage. In addition, low β -glucuronidase activity in fecal material has been linked to an increase in the amounts of substances such as carcinogens in the colonic lumen (78). *B. longum*, when added to the diet, contributes to changes in the intestinal microbiota, lowering the activity of β -glucuronidase, which is associated with the inhibition of aberrant crypt formation and is an early preneoplastic marker of malignant potential

in the process of colon carcinogenesis (79). Moreover, in a systematic review of randomized clinical trials (RCTs) testing probiotics, prebiotics, or both (synbiotics) for the treatment of nonalcoholic fatty liver disease (NAFLD) in adult patients, a reduction in liver aminotransferase activity was documented (80).

In an RCT involving 30 healthy adults to evaluate the effects of a fermented product containing 2 probiotic strains (*Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711), compared with standard yogurt, on host intestinal function, 19 enzymatic activities were detected in the feces of volunteers. The pattern of enzymatic activity exhibited by the control and the probiotic-treated groups was very stable throughout the study. However, the naphthol-AS-BI-phosphohydrolase activity, a typical feature of lactobacilli, was augmented in the feces of the probiotic-treated group. In addition, the leucine arylamidase activity, which is characteristic of probiotic strains, also increased, whereas the β -glucuronidase activity exhibited a decreasing trend (62).

Probiotics interact with bile acids in the gut lumen, modifying bile acid metabolism and in turn influencing cholesterol absorption. Bile salt hydrolase (BSH) is an enzyme produced by bacterial species of several genera associated with the gastrointestinal tract and by most of the known probiotics; this enzyme may participate in the first reaction of the deconjugation of biliary salts (81). Considering these beneficial effects of BSH-containing bacteria, BSH activity has been included in FAO/WHO guidelines for the evaluation of probiotics for food use (1). Enzymatic deconjugation of bile acids by BSH from probiotics has been considered to be one of the main mechanisms of the hypocholesterolemic effect attributed to probiotics (81, 82).

Volatile fatty acids

In an RCT with adult volunteers, the treatment group that received *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 exhibited increased production of fecal butyrate compared with a group who received yogurt. Similarly, production of propionic and acetic acid was higher in the probiotic-treated group after 2 wk of treatment. At the end of the washout period, the production of butyrate in the probiotic-treated group was still higher than that in the control group (62, 83).

Another RCT study, conducted to determine the impact after 4 wk of daily consumption of a capsule containing $\geq 24 \times 10^9$ viable *L. paracasei* DG on the intestinal microbial ecology of healthy volunteers, reported that participants with a butyrate concentration of >100 mmol/kg of wet feces had a butyrate reduction of $49\% \pm 21\%$ (mean \pm SD) and a concomitant decrease in the total abundance of 6 genera of Clostridiales, namely, *Faecalibacterium*, *Blautia*, *Anaerostipes*, *Pseudobutyrvibrio*, *Clostridium*, and *Butyrvibrio*, after the probiotic intervention. However, in subjects with initial butyrate concentrations of <25 mmol/kg of wet feces, the probiotic contributed to a very high increase in butyrate concentrations concomitantly with a ~55% decrease in *Ruminococcus* abundance and a 150% increase in an abundantly represented unclassified Bacteroidales genus.

Therefore, the authors concluded that the impact of the intake of *L. paracasei* DG on the microbiota and on SCFAs seems to depend on the initial characteristics of the intestinal microbial ecosystem, and specifically, fecal butyrate content might represent an important biomarker for identifying subjects who may benefit from probiotic treatment (84).

Another RCT study recruited 33 healthy subjects, including young (mean age of 26 y), middle-aged (mean age of 51 y), and elderly (mean age of 76 y) volunteers, who were given a single daily oral dose of *L. plantarum* Lp-8. The concentrations of both acetate and propionate, but not butyrate, increased significantly and peaked at week 5 in all 3 age groups. After Lp-8 consumption was terminated, the concentrations of both acetate and propionate gradually decreased but remained higher than the baseline concentrations (85). Hence, the production of fecal butyrate by different probiotics appears to strictly depend on the specific bacteria used.

Worthley et al. (86) carried out a 4-wk crossover RCT of resistant starch and *Bifidobacterium lactis*, either alone or as a combined synbiotic preparation, in 20 human volunteers. This synbiotic supplementation at the doses used induced unique changes in the fecal microbiota but did not significantly alter any other fecal, serum, or epithelial variables: for example, fecal SCFA concentrations were unchanged from the baseline. In contrast, a 4-wk crossover RCT carried out in 43 older volunteers that used a synbiotic comprising the probiotic *B. longum* and an inulin-based prebiotic reported increased production of acetate, succinate, butyrate, and isobutyrate compared with the placebo-treated group at the end of the treatment. Thus, short-term synbiotic use could be effective in improving the metabolic activity of the colonic bacterial microbiota in older people (83). Osmotic diarrhea and antibiotic-associated diarrhea are significant problems in patients receiving total enteral nutrition, particularly elderly patients, because enteral feeding may change the intestinal microbiota and SCFA composition (87).

The results of an RCT showed that short-term treatment (~6 d) with the probiotic yeast *S. boulardii* may decrease the incidence of diarrhea in patients receiving total enteral nutrition. Fecal butyrate concentrations were lower in patients than in controls, and treatment with *S. boulardii* increased the total fecal SCFA concentrations in the patients. At the end of the treatment with *S. boulardii*, the patients had higher fecal and total SCFA concentrations, which remained high 9 d after treatment was discontinued. Thus, the increase in fecal SCFA concentrations, particularly butyrate, may contribute to explaining the preventive effects of *S. boulardii* on total enteral nutrition-induced diarrhea (88).

L. plantarum 299v has been shown to enhance the concentrations of fecal SCFAs in patients with recurrent *C. difficile*-associated diarrhea, contributing to reducing the adverse effects of antibiotics. In fact, after consumption of metronidazole, a significant decrease in total SCFA concentrations was observed in the placebo-treated group but not in the probiotic-treated group. Moreover, the concentration of fecal butyrate was higher in the *Lactobacillus*-treated group

than in the placebo-treated group. At the end of the study and after cessation of placebo or probiotic treatment, the total SCFA concentrations were restored to the pre-antibiotic-treatment levels in the placebo-treated group (89).

Nagata et al. (90) conducted an RCT in 77 elderly people (mean age of 84 y) at a long-stay health service facility. The study evaluated the effect of the intake of probiotic-fermented milk containing *Lactobacillus casei* strain Shirota on norovirus gastroenteritis, which occurs in the winter season, during the intake period. While the duration of norovirus-gastroenteritis-related processes decreased, *Bifidobacterium* and *Lactobacillus* were found to be the dominant genera; the abundance of Enterobacteriaceae decreased in the fecal samples of the probiotic-treated group; and a significant increase in fecal acetic acid concentration was observed. In a more recent RCT carried out by Nagata et al. (91) with elderly residents who randomly received either *L. casei* Shirota or a placebo beverage once daily for 6 mo, the counts of *C. difficile* were significantly lower and the fecal acetic acid concentration and total acidity were significantly higher in the *L. casei* Shirota-treated group, and these results were associated with significantly lower incidence of fever and improved bowel movements.

Changes in fecal SCFA or branched-chain fatty acid (BCFA) concentrations can partially explain the effect of probiotics and the role of probiotics in the nutritional status of, and risk of diarrhea in, children. *L. paracasei* Lpc-37 or *B. lactis* HN019 consumption by 2- to 5-y-old children was found to reduce the risk of diarrhea and was associated with higher concentrations of selected SCFAs and BCFAs in subjects who had experienced diarrhea. The concentrations of SCFAs, namely, acetate, propionate, and butyrate, were found to correlate with each other. Likewise, the concentrations of the BCFAs isobutyrate, 2-methylbutyrate, and isovalerate also correlated with each other. After this intervention, *L. paracasei* Lpc-37 abundance correlated positively with total *Bifidobacterium* counts and isovalerate concentrations. *B. lactis* HN019 counts were found to correlate positively with total bacterial counts and negatively with propionate concentrations (92).

Riezzo et al. (93), in an RCT, evaluated the effects of probiotic-enriched artichokes, compared with ordinary artichokes, on SCFA patterns in constipated subjects. Each patient consumed 180 g of ordinary artichokes/d or artichokes/d enriched with *L. paracasei* IMPC 2.1 for 15 d. Propionic acid concentrations were higher than baseline in the probiotic-treated group, and this result was associated with a lower constipation score (93).

The addition of prebiotics or probiotics to infant formula to improve the intestinal microbiota of formula-fed infants is a matter of great interest for consumers and stakeholders and for the food industry.

An RCT evaluated the effects of an infant formula containing *L. salivarius* CECT5713 compared with a control standard formula over 6 mo. Consumption of the probiotic formula led to an increase in the fecal lactobacilli content and the fecal concentration of butyric acid over 6 mo (94).

Abnormal colonization of low-birth-weight infants usually occurs because of a number of reasons, including cesarean delivery, prolonged hospital stay, and immature intestines, and can have adverse effects on health. An RCT evaluated the oral application of a probiotic, usually called *B. lactis* Bb12 (true name *B. animalis*), on selected indicators of health status in preterm infants. The fecal pH in the probiotic-treated group was significantly lower than that in the placebo-treated group, and the fecal concentrations of acetate and lactate were 42% and 38% higher in the probiotic-treated group than in the placebo-treated group, respectively (95). The lower fecal pH in feces of infants, as seen in breastfed infants, is associated with a lower incidence of diarrhea. Another RCT compared the effect of 2 prebiotic/probiotic products on the weight gain, stool microbiota, and stool SCFA content of premature infants. Even the bifidobacteria content was higher in the infants who were fed the probiotic formulas, and significant differences in fecal SCFA content were detected between the groups (96).

Based on the roles of probiotics in the modulation of the immune system (see below), various studies have investigated the potential effects of probiotics in the prevention of childhood eczema and allergies. Probiotic supplementation (*B. bifidum* W23, *B. animalis* subsp. *lactis* W52, and *Lactococcus lactis* W58) resulted in fewer children developing eczema at the age of 3 mo compared with the controls. In addition, the probiotic-treated group exhibited higher concentrations of lactate and SCFAs (acetate, butyrate, propionate, and isobutyrate) and lower concentrations of lactose and succinate than the group who had received the placebo. Moreover, lower concentrations of SCFAs, succinate, phenylalanine, and alanine were detected in fecal samples of the children who later developed eczema, whereas the amounts of glucose, galactose, lactate, and lactose were higher than those in the children who did not develop eczema (78). These results emphasize the roles that SCFAs and other probiotic metabolites may play in the regulation of the immune system.

Extensively hydrolyzed casein formula (eHCF) represents an elective treatment for infants diagnosed with CMA, and supplementation with probiotics, particularly *L. rhamnosus* GG (eHCF + LGG), seems to accelerate antigenic tolerance (61). Regardless of changes in the gut microbial communities, after treatment with eHCF, most tolerant infants showed a significant increase in fecal butyrate concentrations, which was associated with enrichment of *Blautia* and *Roseburia* species (61). Thus, eHCF + LGG treatment seems to promote tolerance in infants with CMA by influencing the bacterial community structure and the capacity to produce SCFAs, mainly butyrate.

Both human and experimental obesity is associated with changes in the intestinal microbiota, as characterized by relatively lower abundances of *Firmicutes* and higher abundances of *Bacteroidetes*. In addition, some obesity-associated comorbidities, namely type 2 diabetes (T2D) and NAFLD, also exhibit perturbation of the intestinal microbiota. In these conditions, both probiotics and synbiotics may

provide beneficial health effects because these treatments can influence the intestinal microbial ecology and immunity. A recent article by our group reviewed the effects of probiotics and synbiotics on obesity, insulin resistance syndrome, T2D, and NAFLD in a human RCT (97). Selected probiotics and synbiotics exhibited beneficial effects in patients with obesity, mainly affecting the BMI and fat mass. Some probiotics had beneficial effects on insulin resistance syndrome, decreasing the concentrations of some biomarkers of cardiovascular disease. Moreover, selected probiotics improved the carbohydrate metabolism, fasting blood glucose concentrations, insulin sensitivity, and antioxidant status and reduced the metabolic stress in subjects with T2D. Some probiotics and synbiotics also improved the liver and metabolic markers in patients with NAFLD (97).

An RCT has evaluated the effects of *L. salivarius* Ls-33 on the fecal microbiota of obese adolescents over 12 wk. The ratio of bacteria of the *Bacteroides-Prevotella-Porphyromonas* group to *Firmicutes*, including *Clostridium* cluster XIV, *Blautia coccoides*, *Eubacteria rectale*, and *R. intestinalis*, was significantly increased after Ls-33 administration. However, the abundances of the *Lactobacillus* group and *Bifidobacterium* were not significantly altered by the intervention, and similarly, the SCFA concentrations remained unaffected (98).

VSL#3 is a high-concentration probiotic preparation of 8 live freeze-dried bacterial species that are normal components of the human gastrointestinal microbiota, including 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), 3 strains of bifidobacteria (*B. longum*, *B. breve*, and *B. infantis*), and *S. salivarius* subsp. *thermophilus*. VSL#3 treatment induced changes in the NAFLD urinary metabolic phenotype; these changes occurred primarily at the level of host amino-acid metabolism (i.e., valine, tyrosine, 3-amino-isobutyrate, or β -aminoisobutyric acid), nucleic acid degradation (pseudouridine), creatinine metabolism (methylguanidine), and also at the level of gut microbial amino-acid metabolism (i.e., 2-hydroxyisobutyrate from valine degradation). Furthermore, the concentrations of some of these metabolites correlated with clinical primary and secondary trial endpoints after VSL#3 treatment, particularly alanine aminotransferase and active glucagon-like peptide 1 (99). Thus, in addition to the beneficial effects of some probiotics in lowering the concentrations of liver lipids (100), the induced changes in fecal metabolite concentrations may play an important role in the pathogenesis of NAFLD, and some of these metabolites may be considered as noninvasive effective biomarkers to evaluate the response to treatment (99).

Consistent with in vitro and in vivo data, SCFAs have been reported to have numerous physiologic, biochemical, and molecular effects in many tissues, including intestine, liver, adipose, muscle, and brain tissues (101). It has been proposed that acetate produced by bifidobacteria can improve the intestinal defense mediated by epithelial cells and can protect the host against lethal infection. Thus, genes encoding an ATP-binding-cassette-type carbohydrate

transporter present in certain bifidobacteria contribute to protecting mice against death induced by *E. coli* O157:H7, and this effect can be attributed, at least in part, to increased production of acetate and to translocation of the *E. coli* O157:H7 (102).

SCFAs are an important source of energy for enterocytes and are key signaling molecules for the maintenance of gut health. In addition, SCFAs can enter the systemic circulation and interact with cell receptors in peripheral tissues. In fact, SCFAs have an important role in the regulation of energy homeostasis and metabolism. Increasing evidence, mainly derived from animal and in vitro studies, has suggested a role for SCFAs in the prevention and treatment of obesity and obesity-related disorders in glucose metabolism and insulin resistance (101). SCFAs can interact with the SCFA receptors G protein-coupled receptor (GPR) 41 and GPR43, leading to an increase in the intestinal secretion of polypeptide YY and glucagon-like peptide 1, respectively, which, in turn, can enhance satiety (101, 103). Moreover, SCFAs might reach the adipose tissue and contribute to decreasing fat accumulation by interacting with GPR43, which would result in decreased lipolysis and inflammation and increased adipogenesis and leptin release. Propionate can increase free fatty acid uptake, possibly by affecting the lipoprotein lipase inhibitor angiopoietin-like 4. Acetate and propionate might also attenuate intracellular lipolysis via decreased hormone-sensitive lipase phosphorylation by interacting with the SCFA receptor GPR43. Similarly, acetate, propionate, and butyrate might increase peroxisome proliferator-activated receptor (PPAR)- γ -mediated adipogenesis, which is possibly regulated by a GPR43-related mechanism. In addition, it has been proposed that acetate, propionate, and butyrate, especially the latter 2, could reduce the secretion of proinflammatory cytokines and chemokines, likely by reducing local macrophage infiltration (101). Furthermore, SCFAs seem to activate AMP kinase in muscles, increasing insulin sensitivity and fatty acid oxidation and decreasing lipid accumulation (101). **Figure 1** summarizes the potential biological effects of SCFAs in humans.

Other miscellaneous biological activities of SCFAs might be attributed to probiotics as a result of epigenetic alterations, which may explain the wide range of anticarcinogenic effects attributed to probiotics (104). However, further study is needed in this area, particularly in humans.

Cell Adhesion and Mucin Production

When a microbial strain is indicated to be a probiotic, there are some specific prerequisites that need to be addressed. One of them is adhesion to the intestinal mucosa for colonization and further interaction between the administered probiotic strains and the host (71). This specific interaction is required for the modulation of the antagonism against pathogens and for actions in the immune system (4, 105).

Intestinal epithelial cells secrete mucin to avoid the adhesion of pathogenic bacteria (72). Several *Lactobacillus* proteins have been shown to promote this adhesion (106),

exhibiting surface adhesins that facilitate attachment to the mucous layer (107).

Over the last 30 y, the Caco-2 cell line has been extensively used to determine the adhesion capacity of probiotics in vitro (108). These cells form a homogeneous monolayer that resembles that of mature enterocytes in the small intestines of humans (109) and form crypts, which are typical structures of the epithelial monolayer (7, 110).

L. rhamnosus ATCC 7469 was tested in the presence of an F4-expressing *E. coli* strain (serotype O149: K91, K88ac) in intestinal porcine epithelial J2 cells. The expression of Toll-like receptor (TLR)-4 and nucleotide-binding oligomerization-domain-containing protein (NOD) 2 (NOD2) was augmented by the presence of *E. coli*, and these increases were attenuated by *L. rhamnosus* treatment (111). Pretreatment with *L. rhamnosus* enhanced Akt phosphorylation and increased zonula occludens-1 and occludin protein expression. The probiotic maintained the epithelial barrier and promoted intestinal epithelial cell activation in response to bacterial infection (111).

In another study, the effects of 3 *L. plantarum* strains were evaluated on in vivo small intestinal barrier function and gut mucosal gene transcription in human subjects. *L. plantarum* TIFN101 modulated gene transcription pathways; notably, this probiotic upregulated the matrix metalloproteinase 2, tissue inhibitors of metalloproteinase 1 and 3, and *muc2* genes and downregulated genes involved in the tricarboxylic acid cycle II pathway (112).

Modulation of the Immune System

The gut microbiota modulates the immune system via the production of molecules with immunomodulatory and anti-inflammatory functions that are capable of stimulating immune cells. These immunomodulatory effects are due to the interaction of probiotic bacteria with epithelial cells and DCs and with monocytes/macrophages and lymphocytes (113). One of the major mechanisms of action of probiotics is the regulation of host immune response. Thus, the immune system is divided into the innate and adaptive systems. The adaptive immune response depends on B and T lymphocytes, which bind to specific antigens. In contrast, the innate system responds to common structures, called pathogen-associated molecular patterns (PAMPs), shared by a majority of pathogens. The primary response to pathogens is produced by pattern recognition receptors (PRRs), which bind to PAMPs. Consequently, PRRs comprise TLRs, which are transmembrane proteins that are expressed on various immune and nonimmune cells, such as B-cells, natural killer cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells. Furthermore, PRRs comprise nucleotide-binding oligomerization domains, adhesion molecules, and lectins (114). In addition to TLRs, PRRs include NOD-like intracellular receptors (NODLRs), which guard the cytoplasmic space (115). Other PRRs have also been described, such as C-type lectin receptors, formylated peptide receptors, retinoic acid inducible-like helicases, and intracellular IL-1-converting enzyme protease-activating factor (116). In

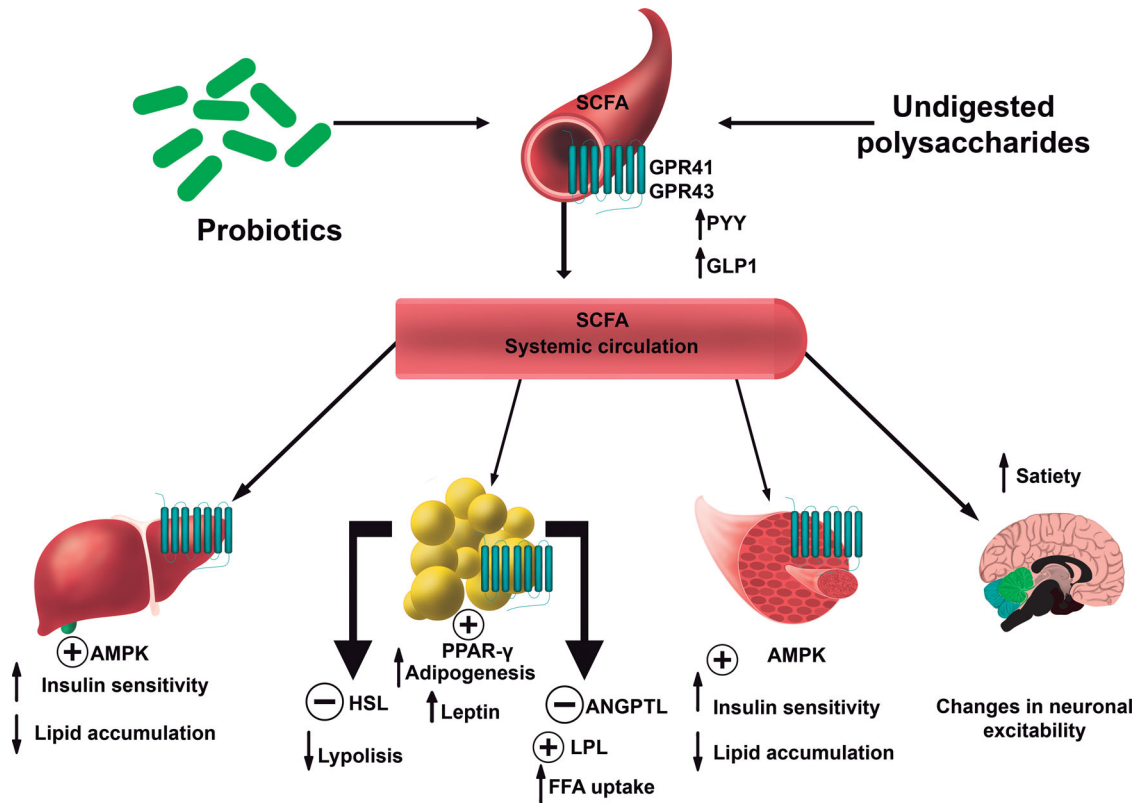


FIGURE 1 Potential biological effects of SCFAs in humans. AMPK, AMP kinase; ANGPTL, angiopoietin-like; GLP1, glucagon-like peptide 1; GPR, G protein-coupled receptor; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPAR- γ , peroxisome proliferator-activated receptor- γ ; PYY, polypeptide YY.

general, the T cell subset, which is involved in regulating the immune balance, is finely tuned by the host and the microbes with which the host interacts, and disequilibrium between the effector T-helper (Th) cells and regulatory T cells (T-regs) leads to impaired immune response (117). Probiotics help to preserve intestinal homeostasis by modulating the immune response and inducing the development of T-regs (118).

Modulation of sIgA and cytokine production

sIgA is secreted by intestinal B cells and is expressed on the basolateral surface of the intestinal epithelium as an antibody transporter. sIgA facilitates the translocation of IgA dimers to the luminal surfaces of epithelial cells. Several studies have reported that probiotics show potent stimulation of the production of sIgA, thereby enhancing barrier function (119). Regardless, probiotics interact with intestinal and specific immune cells, which results in the production of selected cytokines. Thus, *L. salivarius* CECT5713 consumption augmented the percentages of NK cells and monocytes as well as the plasmatic concentrations of immunoglobulins M, A, and G and IL-10 in healthy adult volunteers (64). In addition, *L. casei* Shirota increased the expression of the CD69 activation marker on circulating T cells and NK cells and induced an increase in mucosal salivary IFN- γ , IgA1, and IgA2 concentrations in healthy adults (120). In addition, administration of *B. breve* CNCM

I-4035 resulted in a significant increase in fecal sIgA content; the plasmatic concentrations of IL-4 and IL-10 also increased, whereas the concentrations of IL-12 decreased, in the sera of volunteers treated with this strain. Similar results have been obtained with 2 other probiotic strains, *L. rhamnosus* and *L. casei* (65).

Recently, a probiotic strain (*E. faecium* AL41) was proposed to be effective against *Campylobacter jejuni* infection in chickens. *E. faecium* modulates the expression of transforming growth factor (TGF)- β 4 but downregulates the relative expression of IL-17 and activates IgA-producing cells in the caeca of chicks infected with *C. jejuni* (121).

In mice, treatment with *L. rhamnosus* RC007 for 10 d increased the phagocytic activity of peritoneal macrophages and the number of IgA cells in the lamina propria of the small intestine. Consequently, higher concentrations of monocyte chemoattractant protein 1 (MCP-1), IL-10, and TNF- α were observed, and the ratio of anti- to proinflammatory cytokines (IL-10/TNF- α) in the intestinal fluid increased after *L. rhamnosus* RC007 treatment (122). Another *Lactobacillus* strain, *L. plantarum* 06CC2, is capable of increasing the concentration of IL-12 in co-culture with J774.1 cells, and oral administration induced Th1 cytokine production, activating the Th1 immune response associated with intestinal immunity in normal mice (123). Aktas et al. (124) investigated 7 different *L. casei* strains for their ability to alter the murine gut microbiota. They observed that *L. casei*

species are capable of modulating the host gut microbiota and the host immune system because there is a relation between the ability of a strain to alter the composition of the gut microbiota, PRR regulation, and antimicrobial peptide regulation (124).

In addition, bifidobacteria strains also modulate the immune system. Accordingly, *B. longum* subsp. *infantis* 35624 is a probiotic with immunoregulatory effects, and it has been described that the consumption of *B. longum* subsp. *infantis* 35624 resulted in the induction of T-regs and attenuation of nuclear transcription factor κ B (NF- κ B) activation, preventing the excessive inflammation induced by *Salmonella* infection in mice. Induction of T-regs by the strain has also been shown in humans, and reduction of systemic proinflammatory biomarkers has been seen in patients with psoriasis, IBS, chronic fatigue syndrome, or ulcerative colitis (125, 126). *B. breve* C50 releases soluble factors that alleviate the secretion of proinflammatory cytokines by immune cells. Thus, a study carried out by Heuvelin et al. (127) elucidated that *B. breve* C50 and the soluble factors secreted by this bacterium contribute positively to intestinal homeostasis by attenuating chemokine production, such as CXCL8 secretion by epithelial cells driven by Jun proto-oncogene, AP-1 transcription factor subunit and I κ B- α and decreased phosphorylation of p38-MAPK and I κ B- α molecules (127).

A probiotic mixture, VSL#3, induces NF- κ B nuclear translocation in epithelial cells, which is followed by the release of TNF- α , and this effect correlates with reduced epithelial permeability and susceptibility to CD-like ileitis in SAMP1/YitFc mice that spontaneously develop the disease. It has been recently shown that TNF- α can stimulate epithelial cell proliferation, and this stimulation occurs only when TNF- α induces epithelial cell apoptosis in combination with IFN- γ . Hence, it is possible that probiotics can participate in epithelial barrier regeneration by upregulating TNF- α (118, 128). A number of selected species of lactobacilli and bifidobacteria have been demonstrated to be prominent probiotics with anti-inflammatory properties, suppressing proinflammatory responses by increasing the concentrations of IL-10 and Th1-type cytokines. Kwon et al. (129) identified a mixture of probiotics that upregulates CD4⁺forkhead box P3 (FoxP3)⁺ T-regs. Thus, administration of the probiotic mixture induced both T cell and B cell hyporesponsiveness and downregulated Th1, Th2, and Th17 cytokines without inducing apoptosis. The probiotic mixture also induced the production of CD4⁺FoxP3⁺ T-regs from the CD4⁺CD25⁻ population and increased the suppressor activity of naturally occurring CD4⁺CD25⁺ T-regs. Conversion of T-cells to FoxP3⁺ T-regs is directly mediated by regulatory DCs that express high levels of IL-10, TGF- β , cyclooxygenase (COX)-2, and indoleamine 2,3-dioxygenase. Administration of the probiotic mixture had therapeutic effects in experimental treatments of IBD, atopic dermatitis, and rheumatoid arthritis. Overall, administration of probiotics that enhance the generation of regulatory DCs and T-regs represents an applicable treatment for inflammatory immune disorders (129).

Interaction of probiotics with TLRs and cell cascade signaling

TLRs are a family of evolutionarily conserved PRRs that recognize a wide range of microbial components. In mammals, the TLR family includes 11 proteins (TLR1–TLR11), and the activation of TLRs occurs after the binding of the ligand to extracellular leucine-rich repeats. In humans, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are associated with the outer membrane and primarily respond to bacterial surface-associated PAMPs. TLR3, TLR7, TLR8, and TLR9 are found on the surfaces of endosomes, where they respond primarily to nucleic-acid-based PAMPs from viruses and bacteria. The TLR signaling pathway, with the exception of TLR3, involves the recruitment of myeloid differentiation primary response 88, which activates the MAPK and NF- κ B signaling pathways. TLR3 utilizes the adaptor protein TIR-domain-containing adapter-inducing IFN- β , leading to the expression of type 1 IFNs. TLR-mediated signaling has been shown to control DC maturation. TLR9 signaling is essential for the mediation of the anti-inflammatory effect of probiotics (71, 114). **Figure 2** summarizes the interaction of probiotics with TLRs.

Probiotics are capable of suppressing intestinal inflammation via the downregulation of TLR expression, secretion of metabolites that may inhibit TNF- α from entering blood mononuclear cells, and inhibition of NF- κ B signaling in enterocytes (130). In this sense, signaling by cell wall components of lactobacilli can potentially occur via the binding of TLR2 and TLR6, stimulating cytokine production. In addition, TLR2 recognizes peptidoglycan, which is the main component of gram-positive bacteria, including those of the *Lactobacillus* genus. *L. casei* 431 interacts with epithelial cells via TLR2, and the interaction between *L. casei* and gut-associated immune cells induces an increase in the number of CD-206 and TLR2 receptors (131). Indeed, several strains, such as *L. plantarum* CCFM634, *L. plantarum* CCFM734, *L. fermentum* CCFM381, *L. acidophilus* CCFM137, and *S. thermophilus* CCFM218, stimulate TLR2/TLR6, and these interactions between PRRs such as TLRs are strain specific. Thus, TLR2/TLR6 signaling is essential in immune regulatory processes (132). In addition, Shida et al. (133) showed that *L. casei* induces a high amount of IL-12 production in both wild-type and TLR2-deficient macrophages and that peptidoglycan induces low amounts of IL-12 production in wild-type macrophages and even lower amounts in TLR2-deficient macrophages (133). Moreover, *L. rhamnosus* GG and *L. plantarum* BFE 1685 enhance TLR2 activity in human intestinal cells, and *L. casei* CRL 431 has similar effects in mice infected with *Salmonella enterica* serovar *typhimurium* (134, 135). Furthermore, *L. plantarum* was shown to activate TLR2 signaling, and subsequently, protein kinase C- α and - δ activation has also been implicated in tight-junction modulation and epithelial permeability (136).

With regard to lactobacilli, *L. casei* DG and its postbiotic modulate the inflammatory/immune response in postinfection IBS in an ex vivo organ culture model. Thus, IL-1 α , IL-6, and IL-8 mRNA levels and TLR4 protein expression were significantly higher, whereas IL-10 mRNA levels were

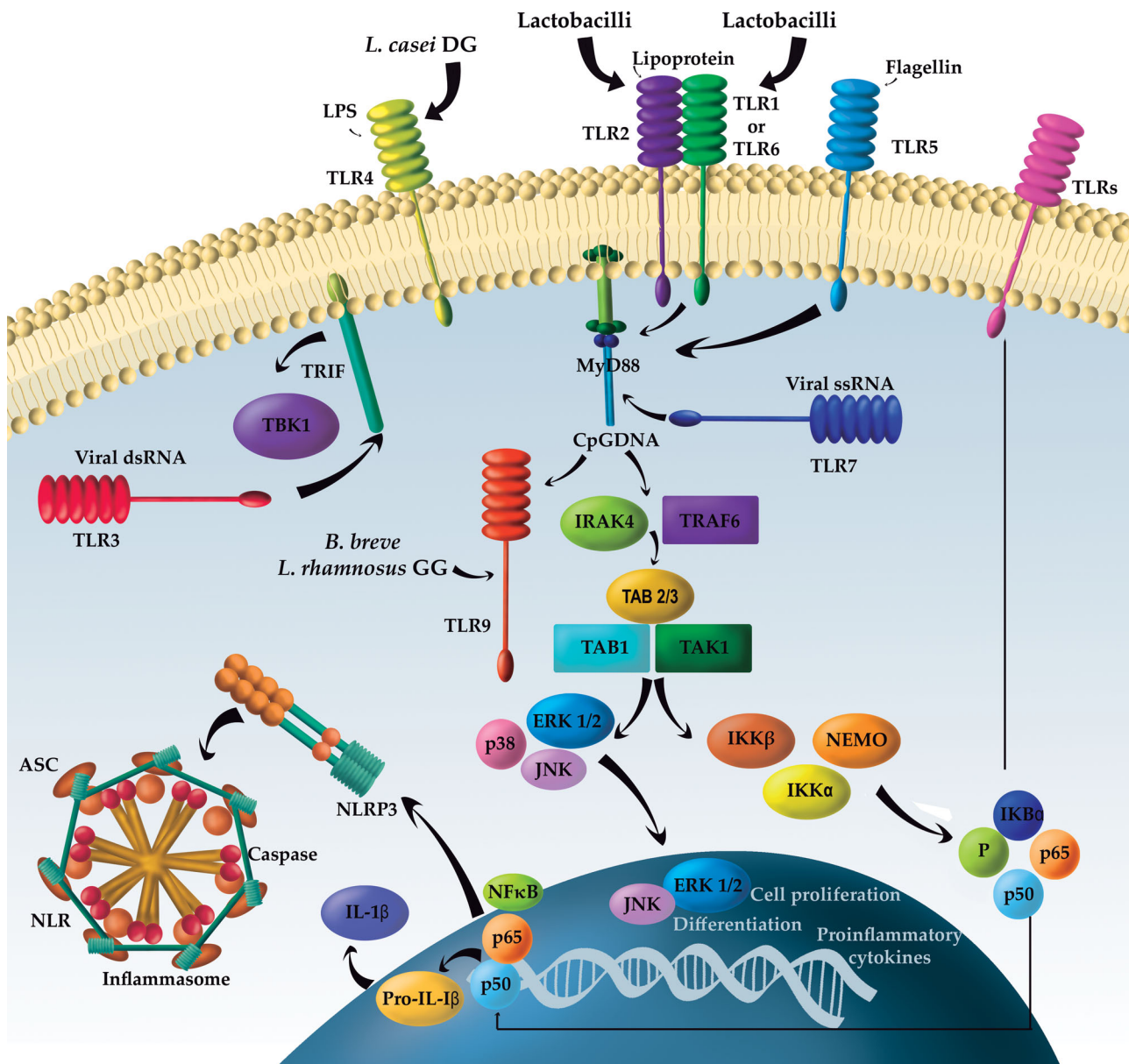


FIGURE 2 Main effects of probiotics on the immune system. ASC, apoptosis-associated Speck-like protein containing a CARD; *B. breve*, *Bifidobacterium breve*; CpGDNA, Cytosine-phosphate-guanosine DNA; dsRNA, Double strand DNA, ERK, extracellular regulated kinase; IKK, I κ B kinase; IRAK4, IL-1 receptor-associated kinase 4; JNK, Jun N-terminal kinase; *L. casei*, *Lactobacillus casei*; *L. rhamnosus*, *Lactobacillus rhamnosus*; MyD88, myeloid differentiation primary response 88; NEMO, NF- κ B essential modulator; NF- κ B, nuclear transcription factor; NLR, nucleotide-binding oligomerization domain-like receptors, in short NOD-like receptors; NLRP3, NLR family pyrin domain containing 3; P, Phosphate; ssRNA, TAB1/2/3, TAK binding proteins; TAK1, ubiquitin-dependent kinase of putative mitogen-activated protein kinase (MKK) and IKK; TBK1, serine/threonine-protein kinase 1; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon- β ; Viral ssRNA: Viral single strand DNA.

lower, in postinfection IBS than in healthy controls in both the ileum and colon. *L. casei* DG and postbiotic significantly reduced the mRNA levels of the proinflammatory cytokines IL-1 α , IL-6, and IL-8 and TLR4, whereas these bacteria increased the mRNA levels of IL-10, in both the ileum and colon after LPS stimulation. Therefore, there was an attenuation of inflammatory mucosal response in an ex vivo organ culture model of postinfection IBS (137).

Bifidobacteria also stimulate TLR2, and specifically, *B. breve* C50 induces maturation and IL-10 production and

prolongs DC survival (138). Similarly, Zeuthen et al. (139) showed that TLR2^{-/-} DCs produce more IL-2 and less IL-10 in response to bifidobacteria, and the authors concluded that the immunoinhibitory effect of bifidobacteria is dependent on TLR2 (71, 139). *B. bifidum* OLB 6378 stimulates TLR2 expression in the ileal epithelium and enhances COX-2 expression, increasing the production of prostaglandin E2 in rats with NEC. However, the specific mechanism for this phenomenon has not been elucidated (140). In another study, in which dysbiosis was induced in the rat intestine, treatment

with a probiotic mixture of 4 strains, namely *B. breve* DM8310, *L. acidophilus* DM8302, *L. casei* DM8121, and *S. thermophilus* DM8309, ameliorated the injury to the mucosal barrier, reduced the concentrations of proinflammatory factors and cytokines, and reduced neutrophil infiltration. These results are closely associated with the re-establishment of intestinal microbial homeostasis and alteration of the TLR2 and TLR4 signaling pathways (141).

TLR9 is another relevant TLR that is activated by probiotics, and in vivo, TLR9 exhibits anti-inflammatory effects at the epithelial surface. Hence, TLR9 activation induces intracellular signaling pathways via the apical and basolateral surfaces, and TNF- α -induced NF- κ B is expressed. Thus, the abilities of different probiotic species to stimulate TLR9 are likely to be different. TLR9 triggers I κ B α degradation and NF- κ B pathway activation, whereas apical TLR9 induces cytoplasmic accumulation of ubiquitinated I κ B and inhibition of NF- κ B activation (71, 142).

Different strains such as *B. breve*, *L. rhamnosus*, and *L. casei* induce different amounts of cytokine production in human and mouse primary immune cells. Thus, *B. breve* induces cytokine production in a TLR9-dependent manner, and the lower inflammatory profile is due to the inhibitory effects of TLR2 (143). In addition, purified genomic DNA from *L. plantarum* inhibits LPS-induced TNF production and reduces *TLR2*, *TLR4*, and *TLR9* gene expression in THP-1 cells (144).

A recent study demonstrated that transplantation of the human gut microbiota into pigs via different dosing regimens of *L. rhamnosus* GG affected intestinal bacterial communities and modulated the responses of the immune signaling pathway to an oral attenuated human rotavirus vaccine. The authors reported that pigs treated with 9 doses, but not those treated with 14 doses, of *L. rhamnosus* GG exhibited enhanced IL-6, IL-10, TNF- α , and TLR9 mRNA levels and p38 MAPK and extracellular regulated kinase expression in ileal mononuclear cells. Therefore, 9 doses of *L. rhamnosus* GG were more effective in activating the TLR9 signaling pathway than 14 doses in human-gut-microbiota-containing pigs vaccinated with attenuated human rotavirus vaccine (119).

Our research group has previously reported that *L. paracasei* CNCM I-4034 and the culture supernatant of *L. paracasei* CNCM I-4034 modulate *Salmonella*-induced inflammation in a novel trans-well co-culture of human intestinal-like dendritic and Caco-2 cells. *L. paracasei* CNCM I-4034 significantly increased the IL-1 β , IL-6, IL-8, TGF- β 2; regulated upon activation, normal T cell expressed and secreted (RANTES); and IP-10 levels and decreased the IL-12p40, IL-10, TGF- β 1, and macrophage inflammatory protein (MIP)-1 α levels in DCs through a physical barrier of Caco-2 cells. In contrast, incubation of the co-culture with cell-free culture supernatants increased IL-1 β , IL-6, TGF- β 2, and IP-10 production only when *S. typhi* was present. This induction was correlated with an overall decrease in the expression of all TLR genes except *TLR9*, which was strongly upregulated (145).

With regard to intestinal diseases, *L. rhamnosus* HN001 has been reported to have beneficial activity for the treatment

of inflammatory diseases such as NEC. Hence, the microbial DNA of *L. rhamnosus* HN001 can activate TLR9, attenuating NEC in vitro, and no evidence of toxicity has been described (146).

With regard to the role of probiotics in reducing allergies, the underlying mechanisms might include shifting the lymphocyte Th1/Th2 balance toward a Th1 response and consequent decreased secretion of Th2 cytokines, such as IL-4, IL-5, and IL-13, as well as decreased IgE concentrations and increased production of C-reactive protein and IgA (56).

Interaction with the brain-gut axis

In social groups, individuals who interacted physically through social grooming harbored more similar communities of gut bacteria to each other (147). This degree of social interaction explained why there was variation in the gut microbiota even after controlling for diet, host genetics, and shared environment. Social transmission of the microbiota may be beneficial for propagating the microbes themselves, and some evidence suggests that a socially transmitted microbiota could confer beneficial effects to the host communities as well (148).

The intestinal microbiota, the brain-gut signaling system, and the interaction of the microbiota with genetic receptors have been shown to be associated with the health of children and with the development of short- and long-term behavior (149). The role of the gut microbiota in health and disease in the first years of life has become very relevant because of evidence that the gut microbiota can influence many aspects of human behavior (150). Preterm infants differ from term infants in that preterm infants are particularly vulnerable to the effects of stress and pain. Stress activates the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, which increases intestinal permeability and allows bacteria and bacterial antigens to cross the epithelial barrier, activate the mucosal immune response, and alter the composition of the microbiome (151). In addition, oxidative stress in the intestine modulates the process of microbiome establishment in preterm infants (152).

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder that impairs a child's ability to communicate and interact with others. Children with neurodevelopmental disorders, including ASD, are regularly affected by gastrointestinal problems and dysbiosis of the gut microbiota (153). For example, Hsiao et al. (154) demonstrated that *B. fragilis* may play a role in the improvement in ASD-associated behaviors (154). Recently published data have linked the incidence of ASD with maternal obesity and diabetes (155, 156). A high-fat maternal diet was administered to mice with the objective of inducing impaired social behavior in the offspring, and subsequently the animals were administered *L. reuteri*. Administration of *L. reuteri* failed to mitigate anxiety but was able to restore oxytocin concentrations, the mesolimbic dopamine reward system, and social behaviors in the offspring that were fed a high-fat maternal diet (157).

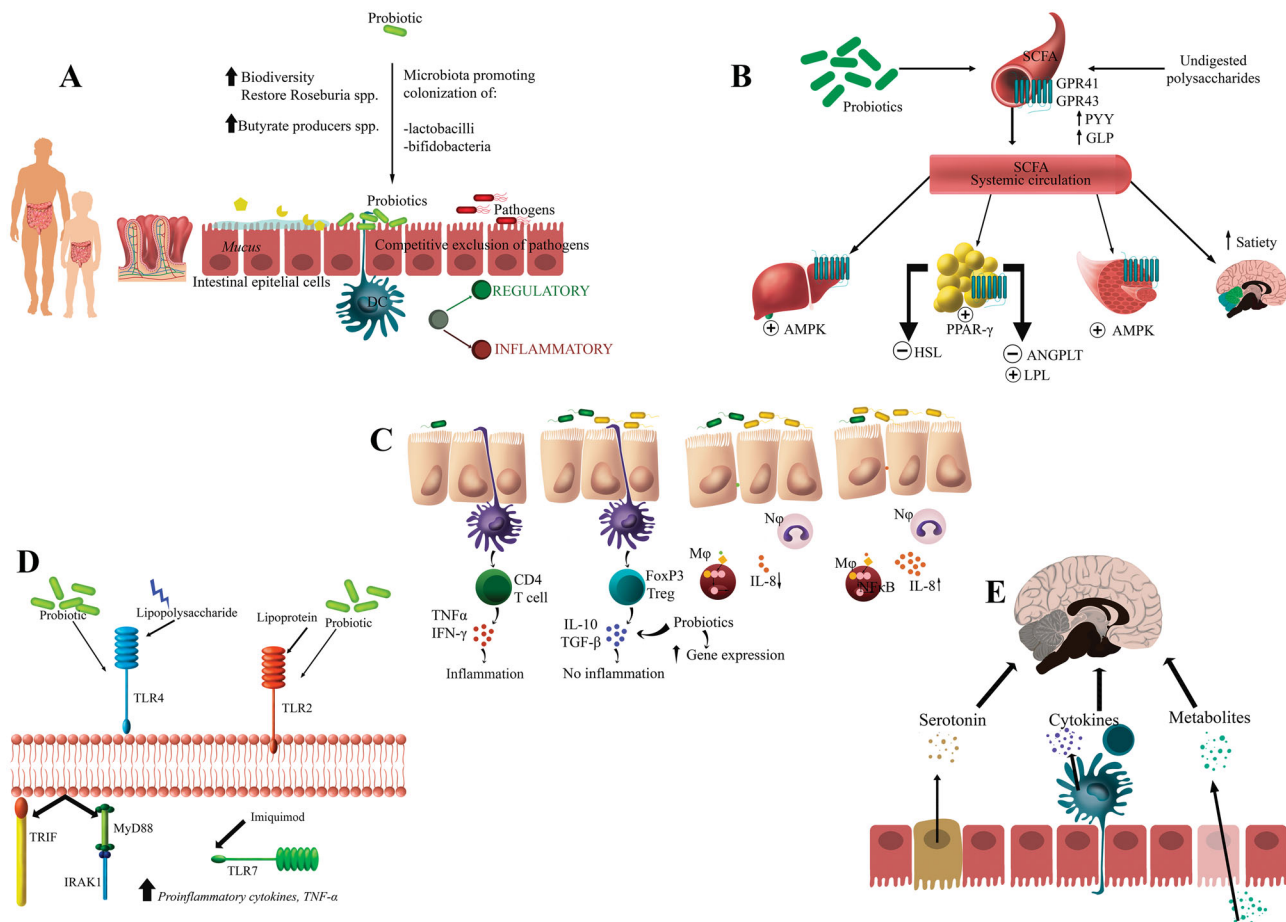


FIGURE 3 Probiotic mechanisms of action. (A) Colonization and normalization of perturbed intestinal microbial communities in children and adults and competitive exclusion of pathogens and bacteriocin production; (B) enzymatic activity and production of volatile fatty acids; (C) cell adhesion, cell antagonism, and mucin production; (D) modulation of the immune system; and (E) interaction with the brain-gut axis. AMPK, AMP kinase; ANGPLT, angiopoietin-like; DC, dendritic cell; FoxP3, forkhead box P3; GLP, glucagon-like peptide; GPR, G protein-coupled receptor; HSL, hormone-sensitive lipase; IFN, interferon; IRAK1, IL-1 receptor-associated kinase 1; LPL, lipoprotein lipase; MyD88, myeloid differentiation primary response 88; NF- κ B, nuclear transcription factor κ B; PPAR- γ , peroxisome proliferator-activated receptor- γ ; PYY, polypeptide YY; TGF, transforming growth factor; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon- β .

Dinan et al. (158) demonstrated that stress caused by physical or psychological factors might be directly associated with the imbalance of the microbiota-brain-gut axis. Messaoudi et al. (159) showed that the consumption of *L. helveticus* R0052 and *B. longum* R0175 reduced symptoms of depression in healthy human volunteers (160). Recently, changes in brain structure were found to be associated with diet-dependent changes in gut microbiome populations through the use of a machine learning classifier to quantitatively assess the strength of microbiome-brain region associations (161).

In general, the mechanisms underlying the effects of the gut intestinal microbiota on the central nervous system are multifactorial (neural, endocrine, and immunologic), but these effects are believed to principally occur via the generation of bacterial metabolites (161). SCFAs alter neuronal excitability, and gut bacteria manufacture a wide

spectrum of neuroactive compounds, including dopamine, γ -aminobutyric acid, histamine, acetylcholine, and tryptophan, which is a precursor in the biosynthesis of serotonin.

Although additional research is needed to test the causality and directionality of the association between the microbiota and social behavior, these initial studies have asked whether microbiota-mediated changes in social behavior affect social transmission of the microbiota and whether these interactions have consequences for both host and microbial fitness.

Finally, **Figure 3** summarizes the mechanisms of action considered in the present review.

Conclusions

Probiotics are safe microorganisms that when administered to human subjects in adequate doses and at appropriate periods confer some beneficial effects to the host. The

mechanisms of action of probiotics involve colonization and normalization of perturbed intestinal microbial communities in both children and adults; competitive exclusion of pathogens and bacteriocin production; modulation of enzymatic activities related to metabolization of a number of carcinogens and other toxic substances; and production of volatile fatty acids, namely, SCFAs and BCFAs, which play a role in the maintenance of energy homeostasis and regulation of functionality in peripheral tissues. In addition, probiotics increase intestinal cell adhesion and mucin production and modulate the activity of gut-associated lymphoid tissue and the immune system. Similarly, probiotic metabolites are able to interact with the brain-gut axis and play a role in behavior. All the aforementioned mechanisms of action should encourage investigators, companies, stakeholders, and consumers to learn about the effects of probiotics as a whole and evaluate those strains that show promising results. These steps toward establishing “good science” may result in the approval of health claims in the near future.

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