

Review

Probiotics and immunity

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Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, including the gastrointestinal tract. While this beneficial effect was originally thought to stem from improvements in the intestinal microbial balance, there is now substantial evidence that probiotics can also provide benefits by modulating immune functions. In animal models, probiotic supplementation is able to provide protection from spontaneous and chemically induced colitis by downregulating inflammatory cytokines or inducing regulatory mechanisms in a strain-specific manner. In animal models of allergen sensitization and murine models of asthma and allergic rhinitis, orally administered probiotics can strain-dependently decrease allergen-specific IgE production, in part by modulating systemic cytokine production. Certain probiotics have been shown to decrease airway hyperresponsiveness and inflammation by inducing regulatory mechanisms. Promising results have been obtained with probiotics in the treatment of human inflammatory diseases of the intestine and in the prevention and treatment of atopic eczema in neonates and infants. However, the findings are too variable to allow firm conclusions as to the effectiveness of specific probiotics in these conditions.

Key words: microflora, nutritional immunology, dietary supplement, innate immunity, vaccine

Introduction

The gastrointestinal tract constitutes an important interface between host and environment and, as such, has the

dual role of excluding pathogens while facilitating the absorption of nutrients. It is colonized by an estimated 10^{14} microbes, with the density of colonization increasing from the stomach to the distal colon. Only a minority (300–500) of the species present in the intestinal microflora can currently be cultured in vitro, whereas a vast majority of intestinal microorganisms cannot. Commensal bacteria participate in both tasks of the gastrointestinal tract: some help in the absorption of otherwise indigestible nutrients, especially complex carbohydrates, and some contribute to colonization resistance, that is, the ability to inhibit colonization or overgrowth of potentially pathogenic microorganisms, by producing antimicrobial substances, competing for adhesion sites and nutrients, and stimulating the immune system.

Recent research efforts have demonstrated that inflammation and immunity changes in general are critical to the development of nearly every complex condition, as well represented by the autoimmunity scenario, which now involves new, previously unsuspected clinical entities¹ and mechanisms.² Similarly, recent evidence has provided new insights into the immune-mediated mechanisms in metabolic diseases.^{3,4} Taken together, the cumulative data argue for the need to determine new tools to modulate immunity either by enhancing (as in the case of immunodeficiencies) or by suppressing (such as in the case of allergy) the immune response, and dietary components are ideal candidates in this regard.

Probiotics are frequently, though not necessarily, commensal bacteria. The most widely accepted definition of probiotics states that they are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.⁵ In order to qualify as probiotic, microorganisms should fulfill most, if not all, of the criteria listed in Table 1. The established probiotics that meet these criteria are generally lactic acid bacteria (LAB), most commonly *Lactobacillus* and *Bifidobacterium* species, but *Lactococcus*, *Streptococcus*,

Table 1. Criteria for the use of probiotics in humans**Identified at the genus, species, and strain level**

- The gold standard for species identification is DNA–DNA hybridization; 16S rRNA sequence determination is a suitable substitute, particularly if phenotypic tests are used for confirmation
- Strain typing should be performed by pulsed-field gel electrophoresis
- Strain should be deposited in an international culture collection

Safe for food and clinical use

- Nonpathogenic
- Not degrading the intestinal mucosa
- Not carrying transferable antibiotic resistance genes
- Not conjugating bile acids
- Susceptible to antibiotics

Able to survive intestinal transit

- Acid and bile tolerant

Able to adhere to mucosal surfaces**Able to colonize the human intestine or vagina (at least temporarily)****Producing antimicrobial substances****Able to antagonize pathogenic bacteria****Possessing clinically documented and validated health effects**

- At least one phase 2 study, preferably independent confirmation of results by another center

Stable during processing and storage

and *Enterococcus* species, as well as some nonpathogenic strains of *Escherichia coli*, and certain yeast strains also qualify. Numerous other LAB have shown probiotic potential in animal studies. In recent years, evidence has accumulated that probiotic strains can exhibit the same activities as commensal bacteria, including immunomodulation.

Gut-associated lymphoid tissue (GALT) is the largest lymphoid tissue of the human body. Its earliest and largest exposure to microbial antigens occurs during the initial intestinal colonization, which starts at birth. The type of colonizing organisms depends on the mode of birth and the mode of feeding, hygiene levels, and medication use. A fairly stable configuration of permanently colonizing bacteria is reached in children by approximately 4 years of age. The stimuli provided by colonization with commensal bacteria are essential for the development of a fully functional and balanced immune system, including not only homing of B and T cells to the lamina propria and expansion and maturation of IgA plasmocytes and IgA production but also the induction of tolerance toward innocuous food and microbial antigens.

GALT can be divided into areas where lymphocytes are scattered throughout the epithelium and the lamina propria and organized lymphoid tissues, including Peyer's patches and mesenteric lymph nodes (MLNs).⁶ Intestinal antigens are delivered to Peyer's patch dendritic cells (DCs) via specialized enterocytes called M cells. In addition, lamina propria DCs have been shown to directly sample antigens, including enteropathogenic and commensal bacteria, in the intestinal lumen via

transepithelial dendrites without jeopardizing the integrity of the epithelial barrier.^{7,8} This process can be induced by ligation of any of a variety of Toll-like receptors (TLRs) expressed on epithelial cells.⁹ Whereas priming of T cells in Peyer's patches or MLNs leads to effective local (mucosal) immune responses or tolerance depending on the encountered antigen, systemic immune responses or tolerance to ingested antigens are likely to be generated in the MLNs.⁶ The translocation efficiency of commensal (and presumably probiotic) bacteria depends on the bacterial strain and the luminal concentration,^{10,11} but the percentage of orally administered commensal bacteria that can be recovered from DCs in MLNs is very small.¹² Efficient killing by macrophages and sequestering in MLN DCs largely prevent commensal bacteria from gaining access to the systemic immune compartment.^{10,12} This indicates that intestinal DC populations are central to the immune-modulating effects of commensal and, by extension, probiotic bacteria.

DCs are critical players in both innate and adaptive immunity since they are the most potent antigen-presenting cells and have the ability to activate naïve T cells.¹³ In addition, they have a critical role in directing helper T-cell responses toward Th1 or Th2, or regulatory patterns.¹³ Th1 immune responses critically depend on the ability of DCs to produce interleukin (IL)-12 and are characterized by the production of interferon (IFN)- γ and IL-2, which induce cell-mediated immunity. Th2 immune responses involve IL-4, IL-5, IL-6, and IL-13 and induce humoral immunity. There is now substantial evidence that DCs can direct T cells to assume regula-

tory functions and thereby induce oral tolerance.^{6,14} One of the most extensively studied mechanisms for the induction of regulatory T cells by DCs is the release of IL-10 or transforming growth factor (TGF)- β , resulting in Tr1 and Th3 cells regulatory T cells that act through the secretion of IL-10 and TGF- β , respectively. Other mechanisms involve the production of immunosuppressive IFN- α or the induction of indoleamine 2,3-dioxygenase (IDO), which is an immunoregulatory enzyme with key functions in the interactions between DCs and regulatory T cells that ultimately result in immunosuppression. DC immaturity or partial maturation at the time of antigen presentation has also been implicated in tolerance induction,¹⁵ particularly as a result of their interaction with apoptotic cells.¹⁶ Since tolerance to innocuous food and commensal antigens is a prerequisite for gastrointestinal immune homeostasis, it is not surprising that intestinal DCs differ phenotypically, and functionally, from peripheral DCs in that they preferentially prime antigen-specific CD4+ T cells to produce Th2 cytokines or to differentiate into regulatory T cells and induce tolerance.^{6,14} Yet, it remains unclear whether this tolerogenic phenotype of intestinal DCs is due to their derivation from distinct lineages, their tissue microenvironment, or, in the case of commensal and other nonpathogenic bacteria, to differential stimuli provided by key bacterial components. For further data on mucosal immunity and its relationship to disease, we refer to the recent literature.¹⁷⁻²⁵

In vitro effects of probiotics on DC phenotype and function

In their immature state, DCs are characterized by high capacity for antigen uptake.¹³ Once they capture an antigen in the presence of appropriate inflammatory

stimuli, they migrate to secondary lymphoid organs and simultaneously undergo a maturation process. This process involves the upregulation of major histocompatibility complex (MHC) molecules for antigen presentation and of costimulatory molecules, such as CD40, CD54, CD83, and B7.1 and B7.2 (CD80 and CD86) for effective T-cell stimulation. Phenotypic maturation is accompanied by stimulus-specific enhancement of the production of various cytokines that shape the subsequent T, B, and natural killer (NK) cell responses. In recent years, numerous studies have examined how known and potential probiotics influence DC maturation and cytokine secretion. Because intestinal DCs are difficult to isolate in sufficient numbers, these experiments have focused almost exclusively on human DCs derived from peripheral blood monocytes and on murine bone marrow-derived DCs (BMDCs). There are indications that DCs from the peripheral compartment respond differently to commensal bacteria than do MLN DCs.²⁶ However, there are also data suggesting that the responses of peripheral (human blood) and colonic lamina propria DCs to cell wall components of probiotic strains are qualitatively, though not quantitatively, similar.²⁷ However, blood myeloid DCs respond differently from monocyte-derived DCs (MDDCs) to probiotic bacteria of intestinal origin.²⁸ Therefore, it remains unclear to what extent the results obtained with MDDCs accurately reflect the responses of at least a subset of intestinal DCs. Nonetheless, as is discussed in other sections of this review, certain characteristics of human MDDCs and murine BMDCs correspond rather well to the results of in vivo studies.

Incubation of live or UV-killed probiotics and other LAB induces distinct and strain-specific maturation and cytokine patterns in both human MDDCs and murine BMDCs (see Tables 2-4). Overall, there are at least two types of LAB: strong and weak inducers of IL-12 and

Table 2. Influence of live probiotic and commensal bacteria on human MDDC maturation and cytokine production

Bacterium	Strain	Bacterial dose (bacteria/DCs)	CD80	CD83	CD86	CD40	MHC II	IL-10	IL-12	Ref
<i>Lactobacillus gasseri</i>	ATCC 19992	1000	↑	↑	↑	↑	↑	±	↑	34
<i>L. johnsonii</i>	ATCC 33200	1000	↑	↑	↑	↑	↑	±	↑	34
<i>L. plantarum</i>	NCIMB 8826	10	↑	↑	↑	↑	↑	↑	↑	201
<i>L. plantarum</i>	NCIMB 882	10	↑	↑	↑	↑	↑	↑	↑	41
<i>L. plantarum</i>	ATCC 8014	10	↑	↑	↑	↑	↑	↑	↑	145
<i>L. reuteri</i>	ATCC 23272	1000	↑	↑	↑	↑	↑	±	↑	34
<i>L. rhamnosus</i>	GG	5	↑	↑	↑	↑	↑	±	±	141
<i>L. rhamnosus</i>	Numico Research isolate	20	(low)	↑	↑	±	±	±	±	35
<i>L. salivarius</i>	W24	10	±	±	±	±	±	±	±	37
<i>Bifidobacterium bifidum</i>	W23	10	±	±	↑	±	↑	±	±	37
<i>B. infantis</i>	W52	10	±	±	↑	±	↑	±	±	37

MDDC, monocyte-derived dendritic cells; MHC, major histocompatibility complex; IL, interleukin; DCs, dendritic cells

Table 3. Influence of UV-killed probiotic and commensal bacteria on human MDDC maturation and cytokine production

Bacterium	Strain	Bacterial dose	CD80	CD83	CD86	CD40	MHC II	IL-10	IL-12	Ref
<i>L. acidophilus</i>	X37	30–50 µg/1.2 × 10 ⁶ DCs		↑	↑	↑	↑	↑	↑	30
<i>L. acidophilus</i>	X37	25 µg/10 ⁶ DCs	↑	↑	↑		↑	↑	↑	31
<i>L. acidophilus</i>	X37	10 ⁵	↑	↑		↑	↑	↑	↑	28
<i>L. paracasei</i>	DSM 12246	25 µg/10 ⁶ DCs	↑	↑		±	±	↑	↑ (Slight)	28,31
<i>L. reuteri</i>	DSM 12246	25 µg/10 ⁶ DCs	↑	↑	↑		↑	↑	↑ (Slight)	31
<i>L. reuteri</i>	DSM 12246	30–50 µg/1.2 × 10 ⁶ DCs		↑	↑	↑	↑	↑	±	30
<i>L. rhamnosus</i>	GG	30–50 µg/1.2 × 10 ⁶ DCs		↑	↑	↑	↑	↑ (Slight)	±	30
<i>B. bifidum</i>	S131	30–50 µg/1.2 × 10 ⁶ DCs		↑	↑	↑	↑	↑	±	30
<i>B. bifidum</i>	S13.1	25 µg/10 ⁶ DCs	↑	↑	↑		↑	↑	↑ (Slight)	31
<i>B. lactis</i>	Bb12	30–50 µg/1.2 × 10 ⁶ DCs	↑	↑	↑	↑	↑	↑	±	30
<i>B. longum</i>	Q45	30–50 µg/1.2 × 10 ⁶ DCs	↑	↑	↑	↑	↑	↑	±	30
<i>B. longum</i>	Q46	30–50 µg/1.2 × 10 ⁶ DCs	↑	↑	↑	↑	↑	↑	±	30
<i>B. longum</i>	Q46	25 µg/10 ⁶ DCs	↑	↑		±	±	↑	↑ (Slight)	28,31

Table 4. Effect of LAB on murine BMDCs

Bacteria	Strain	Bacterial dose	CD86	CD40	MHC II	IL-10	IL-12	Reference
<i>L. casei</i> ^a	CHCC3139	up to 10 µg/1.4 × 10 ⁶ DCs	↑		↑	↑	↑	202
<i>L. fermentum</i> ^a	Lb20	up to 10 µg/1.4 × 10 ⁶ DCs	↑		↑	↑	↑	202
<i>L. johnsonii</i> ^a	La1	up to 10 µg/1.4 × 10 ⁶ DCs	↑		↑	↑	↑	202
<i>L. plantarum</i> ^a	Lb1	up to 10 µg/1.4 × 10 ⁶ DCs	↑		↑	↑	↑	202
<i>L. plantarum</i> ^a	299v	up to 10 µg/1.4 × 10 ⁶ DCs	↑		↑	↑	Little effect	202
<i>L. reuteri</i>	DSM12246	up to 10 µg/1.4 × 10 ⁶ DCs	±		↑	↑	↑	202
<i>L. acidophilus</i>	NCFM	10:1	↑	↑	↑	↑	↑	107
<i>L. lactis</i>	MG1363	10:1	↑	↑	↑	↑	↑	107
<i>L. rhamnosus</i>	Lr32	10:1	Moderate effect	±	Little effect	Little effect	Little effect	107
<i>L. salivarius</i>	Ls33	10:1	Moderate effect	Moderate effect	Moderate effect	Little effect	Little effect	107

LAB, lactic acid bacteria; BMDCs, bone marrow-derived dendritic cells

^aNote that Christensen et al.²⁰² used γ -irradiated bacteria

tumor necrosis factor (TNF)- α production; however, almost all of them induce significant, though quite variable, levels of IL-10. Most *Bifidobacterium* strains stimulate the production of high levels of IL-10, but only modest levels of IL-12 and TNF- α , and this is also the pattern seen with Gram-negative bacteria of the intestinal microflora.^{28–30}

The results of coinubation experiments indicate that there are considerable interactions between bacterial strains that can result in the inhibition or enhancement of DC maturation and IL-12 and IL-10 induction, depending on the combination.^{30,31} Similar findings have been reported in LAB-stimulated peripheral blood mononuclear cells (PBMCs).^{32,33} This suggests that the intestinal microflora of the host may have a decisive influence on the response to treatment with a specific probiotic. Note, however, that this inhibitory effect of weak IL-12 inducers on the inducing capacity of strong

inducers is DC-subset dependent, with inhibition seen in MDDCs, but not in blood myeloid DCs.²⁸ Therefore, it remains to be established whether lamina propria DCs are sensitive to the inhibitory effect of the simultaneous presence of strong and weak IL-12 inducers.

DCs that undergo partial or full maturation after incubation with a particular LAB strain also differ in their capacity to stimulate allogeneic/autologous T-cell proliferation and cytokine production^{27,34–36} and to enhance NK cell cytokine production and cytotoxicity.^{28,31} In addition, when DC maturation is induced by lipopolysaccharide (LPS) with or without added TNF- α and IL-1 β , the mere presence of certain LAB strains during this maturation process affects the ability of DCs to polarize the cytokine production of CD4+ T cells.³⁷ Using a similar protocol, it can be shown that *Lactobacillus rhamnosus* GG (LGG) is able to induce T-cell hyporesponsiveness³⁸ and that *Lactobacillus reuteri*

ASM20016 or *Lactobacillus casei* NIZO B255, but not *Lactobacillus plantarum* NIZO B253, are capable of priming DCs for the induction of regulatory T cells.³⁹ Similar findings have been reported for *Lactobacillus paracasei* NCC2461 in mixed lymphocyte reactions using murine splenic CD4⁺ T cells and allogeneic splenocytes as accessory cells.⁴⁰

Interactions between bacteria, intestinal epithelial cells, and DCs

There are complex and dynamic interactions between the intestinal epithelium and bacteria on the luminal side, and between the epithelium and the underlying immune system on the basolateral side. Intestinal epithelial cells are able to distinguish pathogenic from non-pathogenic bacteria on the basis of their invasiveness and the presence of flagella,⁴¹ although the exact mechanisms that allow them to do so are not fully elucidated and likely involve additional mucosal factors.¹⁷ Intestinal epithelial cells that have encountered certain bacteria participate in the immune response by producing chemokines and cytokines and upregulating adhesion molecules, thereby attracting and activating immune cells.

This aspect has been largely neglected in investigations of the influence of probiotics on DC function. There are, however, studies showing that probiotics and other nonpathogenic LAB or components thereof strain-specifically affect the production of the chemokine IL-8 and of proinflammatory cytokines by intestinal epithelial cells.^{42–48} This ability is further modulated by the presence of leukocytes on the basolateral part of the culture plates.⁴⁹

In turn, experiments where bacteria are added to the apical side and DCs to the basolateral side of an intestinal epithelial cell monolayer show that the presence of intestinal epithelium affects the ability of probiotic and other bacteria to induce DC maturation, and particularly the ratio of IL-10 to IL-12 that these DCs release.^{41,42} In addition, the small fraction of DCs that directly sample bacteria by extending dendrites across the intestinal epithelial layer are activated to a different extent than those that only come into contact with soluble mediators produced by epithelial cells that have encountered these bacteria.⁴¹ Interestingly, DCs treated with supernatant fractions of epithelial cell–bacteria cocultures or of epithelial cells alone were found to preferentially drive Th2 or regulatory T-cell responses, that is, to induce a “mucosal” DC phenotype, even though peripheral MDSCs were used in these experiments.^{41,42} The production of TGF- β and thymic stromal lymphopoietin by intestinal epithelial cells has been implicated in the induction of this tolerogenic DC phenotype.^{42,50}

Probiotics in clinical practice

Probiotics in inflammatory diseases of the bowel

Crohn’s disease (CD) and ulcerative colitis (UC) are two distinct clinical forms of inflammatory bowel disease (IBD), characterized by chronic relapsing intestinal inflammation that is thought to result, similar to other autoimmune diseases, from complex interactions between genetic,^{51–54} environmental,^{55–60} and immunological factors.^{19,61–64} Loss of tolerance to the patient’s own commensal flora has been implicated in the development of both diseases. Decreased levels of *Bifidobacterium* and *Lactobacillus* strains have been described in fecal samples, whereas raised counts of *Enterococcus* and *Bacteroides* species are found in inflamed mucosa of patients with IBD. High levels of inflammatory cytokines (TNF- α and IL-1 β) and IL-8 are produced by inflamed colonic mucosa of both UC and CD patients,^{65–69} while the role of autoantibodies remains to be determined.⁷⁰ In addition, lamina propria T cells from IBD patients secrete elevated levels of IL-6, and IL-6/STAT-3 signaling is upregulated.^{71,72} However, there are differences between the two clinical forms of IBD, with lamina propria T cells from CD patients showing Th1 polarization, whereas those of UC patients express Th2 cytokines.⁷³ In addition, there is enhanced production of IL-10 in colonic tissue of UC patients. Interestingly, the vast majority of studies report that natural FoxP3⁺ CD4⁺CD25⁺ regulatory T cells (Tregs) are markedly expanded in colonic lamina propria and MLNs of both UC and CD patients, particularly during active disease, and are functionally normal *in vitro*.^{74–78} While data in celiac disease are awaited,^{25,79} imbalanced microbiota and increased production of proinflammatory cytokines have also been implicated in irritable bowel syndrome (IBS) and in pouchitis. The latter is an inflammatory condition of the ileal reservoir in patients with ileal pouch anal anastomosis, a surgical procedure used in the management of UC. We note recent literature that reviews the issues of inflammation, redox, and autoimmunity.^{80–88}

The effectiveness of probiotics in clinical trials with UC and CD patients

Because of the potential of certain probiotic strains to influence the intestinal microbial balance, improve mucosal barrier function, and modulate immune responses, there have been numerous randomized controlled clinical trials of probiotic supplementation in IBD patients. Their results indicate that certain probiotics, including *E. coli* Nissle 1917, LGG, and the probiotic mixture VSL#3 are as effective as standard therapy (most frequently mesalazine) in inducing or maintaining remission in UC or maintaining remission

in CD.^{73,89–91} However, when added to standard therapy, these and other probiotics do not provide additional benefits compared with standard therapy alone, and most of the probiotics tested to date are not more effective than placebo in inducing or maintaining remission in CD or UC. Only the results of some small trials suggest that various combinations of *Bifidobacterium* strains are superior to placebo in preventing relapse in UC.

Supplementation of UC patients with a combination of *Bifidobacterium breve* strain Yakult, *Bifidobacterium bifidum* strain Yakult, and an unspecified strain of *Lactobacillus acidophilus* was associated with significantly lower clinical activity index, endoscopic activity index, and histological scores compared with mesalazine alone.⁹⁰ In a small study of patients with UC, symbiotic treatment with *Bifidobacterium longum* plus fructooligosaccharides and inulin, in addition to their existing medical therapy, tended to lower the sigmoidoscopy score ($P = 0.06$ compared with placebo).⁹² Supplementation with a variety of probiotic strains was also reported to be associated with beneficial effects on IBS.⁷³ In addition, VSL#3 was reported to prevent the onset of pouchitis.

The mechanisms by which probiotics might ameliorate IBD, IBS, and pouchitis are rarely addressed in these clinical trials. The symbiotic treatment that tended to lower the sigmoidoscopy score was associated with significantly reduced mRNA expression of TNF- α and IL-1 α in biopsy specimens.⁹² In an open-label study of VSL#3 in patients that had recovered from acute pouchitis after antibiotic treatment, supplementation with this probiotic mixture also decreased the pouch levels of proinflammatory cytokines (TNF- α , IL-1 α and IFN- γ), while it increased the production of IL-10.⁹³ Similarly, in patients with IBS, supplementation with *Bifidobacterium infantis* 35624 or *L. rhamnosus* GR-1 plus *L. reuteri* RC-14 decreased IL-12 production and normalized the IL-10:IL-12 ratio.^{94,95} In addition, administration of VSL#3 resulted in the expansion of colonic Foxp3+ Tregs in conjunction with significantly reduced pouchitis disease activity scores,⁹⁶ and supplementation with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was associated with an increased frequency of peripheral Tregs in IBS patients.⁹⁵

In healthy volunteers, it was shown that consumption of LGG led to downregulation of the production of proinflammatory cytokines and enhanced the ex vivo synthesis of IL-4 and IL-10 of their CD4+ T cells in response to stimulation with intestinal bacterial preparations.⁹⁷ Since inflammatory reactions to the host's own microflora are implicated in IBD, the ability of LGG to skew microflora-induced cytokine production toward an anti-inflammatory profile could be of great benefit. Note, however, that intestinal CD4+ T cells from CD

patients and healthy volunteers show differential IFN- γ and IL-10 responses to stimulation with autologous intestinal bacteria or with sonicates of probiotics.⁹⁸ Therefore, it needs to be established whether LGG supplementation can have the same anti-inflammatory effects in IBD patients as those seen in these healthy volunteers.

Data from in vitro studies with inflamed ileal tissue from CD patients suggest that *L. casei* DN-114001 can not only downregulate TNF- α and IL-6 production but also increase the percentage of lymphocytes undergoing apoptosis, which may contribute to its ability to diminish the number of activated T cells in the lamina propria.^{67–69} Downregulation of IL-6, which can exhibit antiapoptotic activity, may play a role in this process.⁶⁹ Indeed, in a mouse model of chronic colitis, treatment with neutralizing IL-6 receptor antibodies prevented macroscopic signs of inflammation in the colon while markedly increasing the number of lamina propria CD4+ T cells undergoing apoptosis.⁹⁹

Probiotics in animal models of colitis

There are numerous animal models of colitis. Mice deficient in IL-10 or IL-2 (IL-10^{-/-} and IL-2^{-/-} mice) and HLA-B27 transgenic rats spontaneously develop colitis if they are not kept in germ-free conditions, which highlights the importance of the intestinal microflora in these models. Other models of chronic colitis include the transfer of CD4⁺CD45RB^{high} or CD4⁺CD62L⁺ T cells into severe combined immunodeficiency (SCID) mice. In addition, chronic colitis can be induced by the intrarectal administration of trinitrobenzene-sulfonic acid (TNBS). In all of these models, colitis is T-cell mediated and involves mononuclear infiltration of the colonic mucosa along with enhanced production of proinflammatory cytokines. Administration of dextran sodium sulfate (DSS) in the drinking water induces a model of acute colitis that requires neither B nor T cells, but is largely macrophage mediated. Although a mononuclear infiltrate is also seen in the colon of DSS-treated mice, the damage is thought to be due mostly to the toxic effects of DDS rather than the inflammation.

Most available studies focused on the role of various probiotic in the prevention of colitis. None of the strains examined to date was completely effective. However, downregulating the production of proinflammatory cytokines and other inflammatory mediators seems to constitute important mechanisms for the partial amelioration of colitis seen with numerous LAB strains in various models.^{48,100–102} Of note, TNF- α blocking agents are also quite successful in the treatment of patients with CD.

In addition, the ability to dampen Th1 responses (IL-12 and IFN- γ production) has been implicated in the preventative effect of *Lactobacillus salivarius* subspe-

cies (ssp.) *salivarius* 433118 and *B. infantis* 35624,¹⁰³ the therapeutic effect of DNA isolated from VSL#3,⁴⁸ and the partial protection provided by preventative and therapeutic administration of *L. plantarum* 299v in IL-10^{-/-} mice.¹⁰⁴ Interestingly, in SCID mice reconstituted with activated CD4⁺ T cells or CD4⁺CD62L⁺ T cells, preventative treatment with a combination of *L. reuteri* DSM 12246 and *L. rhamnosus* 19070-2 or with *E. coli* Nissle was associated with significantly decreased production of Th2 cytokines by polyclonally stimulated MLNs.^{105,106} However, the release of IFN- γ was also lower in both studies, although this reached significance only with *E. coli* Nissle. In both cases, IL-10 levels were not significantly affected.

A recent study investigated whether the transfer of LAB-treated DCs could protect mice from the development of TNBS-induced colitis.¹⁰⁷ *Lactobacillus salivarius* Ls33 and *L. rhamnosus* Lr32 were found to be weak inducers of DC maturation and cytokine production. Administration of BMDCs that had been incubated with one of these two LAB strains partially protected mice from TNBS-induced colitis, and this protection was greater than that achieved with intraperitoneal (i.p.) administration of the same LAB strain. This amelioration of colitis was shown to depend on the presence of CD4⁺CD25⁺ regulatory T cells, but to be independent of IL-10. Instead, it was associated with highly increased expression of IDO and IFN- γ . Production of this cytokine by DCs is known to induce IDO expression. This suggests that the partial maturation of DCs in the presence of *L. salivarius* Ls33 and *L. rhamnosus* Lr32 resulted in the induction of a tolerogenic DC phenotype.

Whereas the preceding studies do not provide evidence for a significant role of IL-10, other data suggest an important function for this anti-inflammatory and regulatory cytokine in the protection from recurrence of colitis in HLA-B27 transgenic mice associated with consumption of LGG.¹⁰² In addition, the ability of various LAB strains to induce a high ratio of IL-10/IL-12 production in human PBMCs correlated with their capacity to provide significant protection from TNBS-induced colitis.^{108–110} Increasing the production of IL-10 by lamina propria T cells was also found to be an important mechanism in the ability of VSL#3 to reduce the severity of recurrent colitis induced by readministration of TNBS.¹¹¹ This induction of IL-10 was essential for the expansion of a population of regulatory T cells that was identified as a critical mediator of the amelioration of colitis and possessed characteristics of “natural” Tregs. Note that a role for IL-10 in the expansion of natural Tregs has, to our knowledge, not been previously described, although there are indications that it might be necessary for their functional activation, in particular their ability to release TGF- β .¹¹² Given the increased

frequency and intact in vitro function of Foxp3⁺ Tregs in colonic lymphoid tissue of IBD patients, the question arises as to whether the induction of such “natural” Tregs is likely to benefit such patients. There are, however, indications that the expansion of Tregs may have protective effects in patients with IBS or pouchitis.^{95,96}

Cell wall components of the probiotic mixture VSL#3 can stimulate the maturation of murine BMDCs³⁶ and strongly induce IL-10 production from human blood and colonic lamina propria DCs.²⁷ In addition, they are capable of decreasing basal IL-12 secretion in human blood DCs and of downregulating the LPS-induced increase in the percentage of IL-12-producing blood DCs, while leaving the percentage of IL-10⁺ DCs unchanged.²⁷ When naïve allogeneic CD4⁺ T cells were activated in the presence of DCs stimulated with VSL#3 cell wall components, the proportion of IFN- γ -producing cells was significantly reduced compared to either LPS-treated or unstimulated DCs, while the proportion of IL-4- or IL-10-producing cells was not significantly altered.²⁷ These results indicate that cell wall components of VSL#3 do not prime DCs for the generation of Th1 T-cell responses, but they do not predict the IL-10-dependent induction of regulatory T cells as seen in TNBS-induced colitis.¹¹¹ It is possible, however, that cell wall components are not responsible for the observed effects.

VSL#3 also significantly inhibited the development of DSS-induced colitis and had remarkable therapeutic effects in established colitis.¹¹³ These effects were shown to be due to probiotic DNA, which when given orally could inhibit disease activity and improve histologic scores to a similar extent as whole live bacteria. In addition, a single subcutaneous (s.c.) administration of probiotic DNA protected against TNBS-induced colitis and spontaneous colitis in IL-10^{-/-} mice. Of note, intragastric or s.c. administration of *E. coli* (DH5a) DNA also provided significant protection from DSS-induced colitis. This confirms that immunostimulatory DNA (ISS-DNA) sequences are a common property of certain bacterial and viral genomes. ISS-DNA sequences and their synthetic oligodeoxynucleotide (ODN) analogs contain unmethylated CpG dinucleotides within consensus sequences, termed CpG motifs, and can exert a variety of effects on macrophages, NK cells, B cells, and DCs. Studies with other ISS-ODNs in the DSS-induced model of colitis suggest that protection is mainly mediated by the production of immunoregulatory type 1 IFN (IFN- α/β) by plasmacytoid DCs.¹¹⁴ In this model, type 1 IFN acts mainly, but not exclusively, by suppressing the inflammatory response of activated macrophages. Since IFN- α has also been implicated in the regulation of T-cell responses, it will be of interest to determine whether such regulatory mechanisms are operative in VSL#3-

associated amelioration of T cell-mediated models of colitis. In addition, organ culture experiments of colonic biopsy specimens from patients with active UC suggest that reduced production of proinflammatory cytokines is another potential mechanism by which VSL#3 DNA can protect against intestinal inflammation.⁶⁶

Probiotics in allergic diseases

Recent decades have witnessed a marked increase in the incidence of atopic diseases in industrialized countries. According to the “hygiene hypothesis,” a relative lack of microbial stimuli in infancy and early childhood due to increased hygiene results in an imbalance between Th1- and Th2-type immune responses that favors the development of IgE-mediated allergies. However, over the same period of time, there has also been an increase in the incidence of diseases that are characterized by a predominantly Th1-polarized T-cell response. This suggests that immunoregulatory mechanisms capable of controlling both Th1 and Th2 responses do not fully mature in the relative absence of microbial stimuli. While much research has focused on early exposure to infectious agents, it is becoming increasingly recognized that commensal bacteria, which provide the first and major source of microbial exposure, play a central role not only in Th1/Th2 polarization but also in inducing the appropriate regulatory mechanisms. It is also under investigation what role (if any) probiotics have on T regulatory cells.^{115,116} There are indications that the composition of commensal bacteria differs

between children who develop atopic diseases and those who do not, and between children from countries with a high or low incidence of atopic disease.¹¹⁷ However, to date, the differences identified are not consistent between studies. In addition, a recent large multinational prospective study failed to detect an association between the time of colonization with any particular culturable bacterial group and the development of atopic eczema or sensitization to food allergens during the first 18 months of life.¹¹⁸

Primary allergy prevention trials with probiotics

The hope of altering intestinal microbial balance and thereby modulating the polarization of helper T-cell responses have prompted numerous studies examining the potential role of probiotic strains in the primary prevention or treatment of atopic diseases. To date, the results of five randomized placebo-controlled clinical trials of probiotics in the primary prevention of allergic disease have been published (see Table 5). All of these trials enrolled infants at high risk of developing allergy, usually defined as ≥ 1 family member having an allergic disease. In all studies except one,¹¹⁹ the mothers received the supplement for the last weeks of pregnancy. After that, the study design becomes more variable, with the supplement being given to the lactating mother for varying periods, or to the infant directly until 6 months postnatally. Only one study supplemented until 12 months postnatally.¹²⁰ Assessment of allergic manifestations occurred at various time points during the first and/or second year of life. In 2001, Kalliomäki et al.¹²¹

Table 5. Risk of eczema/atopic dermatitis in randomized placebo-controlled primary prevention trials

Study location	Number of subjects randomized/completed	Bacterial strains	Daily bacterial dose (cfu)	Eczema	IgE-associated eczema	Other significant results	Reference
Turku, Finland	159/132	LGG	1×10^{10}	RR 0.51 (CI, 0.32–0.84)			121
Freiburg, Germany	105/94	LGG	1×10^{10}	OR, 0.96 (CI, 0.38–2.33)			125
Perth, Australia	231/178	<i>L. acidophilus</i> LAVRI-A1	3×10^9	No significant difference	Significantly higher rate	Significantly higher rate of sensitization;	119
Sweden	232/188	<i>L. reuteri</i> ATCC 55730	1×10^8	No significant difference	OR, 0.36 , <i>P</i> = 0.047		120
Helsinki, Finland	1223/925	LGG, <i>L. rhamnosus</i> LC705, <i>B. breve</i> Bb99, <i>P. shermanii</i> JS plus galactooligosaccharides	2.44×10^{10}	OR, 0.74 (CI, 0.55–0.98)	OR, 0.66 (CI, 0.46–0.95)	Tended to reduce IgE-associated diseases: OR, 0.71 (0.5–1.00); <i>P</i> = 0.052	124

Values in bold type designate significant risk reduction

P. shermanii, *Propionibacterium freudenreichii* ssp. *shermanii*; RR, relative risk; OR, odds ratio; CI, confidence interval; LGG, *Lactobacillus rhamnosus* GG

reported that LGG supplementation of pregnant women and later their infants resulted in a 50% reduction in the risk of eczema by the age of 2 years. This protective effect was maintained at the ages of 4 and 7 years.^{122,123} However, others were unable to confirm a preventative effect of LGG supplementation,^{124,125} and the results of primary prevention trials using other probiotics are variable and not as impressive as those obtained by Kalliomäki et al.¹²¹ (as summarized in Table 5).

In the trials that examined the risk of other manifestations of allergic disease, probiotic supplementation did not show significant effects on allergic rhinitis, food hypersensitivity, or asthma.^{119–121,124} In three of the trials, the rates of sensitization also did not differ significantly between the probiotic and the placebo group.^{121,124,125} Supplementation with *L. reuteri* ATCC 55730 lowered the incidence of skin prick reactivity in infants with mothers with allergies.¹²⁰ In contrast, ingestion of *L. acidophilus* LAVRI for the first 6 months of life raised the rate of sensitization to a variety of food and inhalant allergens tested.¹¹⁹ In addition, this and the recent LGG trial showed a significantly greater frequency of wheezing or wheezing bronchitis in the probiotic compared with the placebo groups.^{119,125} Wheezing in infancy may be a risk factor for developing asthma later. Of note, Kalliomäki et al.¹²³ reported that the risk of both allergic rhinitis and asthma showed a tendency to be increased in the probiotic (LGG) compared with the placebo group at the 7-year follow-up. This makes it advisable to exert great caution in the use of probiotics in the prevention of allergic disease. Several other primary prevention trials are underway,¹²⁶ and it will be of great interest whether any of them reveal similarly concerning findings.

In the original primary prevention trial with LGG,¹²¹ analysis of data from a subset of women who breast-fed their infants for at least 3 months suggested that LGG supplementation of mothers during late pregnancy and lactation reduced the incidence of atopic eczema in their infants by increasing immunosuppressive TGF- β 2 levels in breast milk.¹²⁷ In another study, however, low, rather than high, TGF- β 2 levels in colostrum and milk tended to be associated with reduced sensitization and decreased incidence of IgE-associated eczema.¹²⁸

The induction of low-grade inflammation has been proposed as a mechanism of the prevention of eczema by supplementation with LGG, *L. rhamnosus* LC705, *B. breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* in combination with a prebiotic.^{124,129} This was based on an analysis of plasma levels of cytokines and inflammatory markers in a randomly selected subgroup of participants in a large primary prevention trial, which indicated that increased plasma C-reactive protein (CRP) concentrations in infants at 6 months of age were associated with decreased risk of eczema and allergic

disease at 2 years of age after adjustment for probiotic use.¹²⁹ This was also observed in a separate analysis of the placebo group. Plasma IL-10 concentrations were higher in the probiotic group and, paradoxically, correlated positively with CRP. Induction of low-grade inflammation has also been proposed as a mechanism for therapeutic effects of probiotic supplementation in atopic dermatitis.¹³⁰ Interestingly, in that trial the very same combination of probiotics was used, but without added prebiotic, and was not found to have an inflammatory effect but to also increase serum IL-10 levels. In contrast, treatment with LGG alone was associated with increased serum CRP and IL-6 concentrations in the subgroup of children with IgE-associated atopic eczema/dermatitis syndrome.

Clinical trials of probiotics in the treatment of allergic diseases

Studies in adults have largely not revealed any significant effect of probiotic supplementation in allergic rhinitis or asthma,¹²⁶ with the exception of several Japanese trials that suggest improved symptom/medication scores in patients with allergic rhinitis. However, the above studies are difficult to critically evaluate because of a lack of detailed information on the methods and procedures. Several clinical trials addressed the effectiveness of probiotic supplementation compared with placebo in the treatment of eczema or atopic dermatitis in infants or children. Ingestion of LGG for periods ranging from 4 to 12 weeks has been associated with significant decreases or greater changes in Scoring Atopic Dermatitis (SCORAD) scores compared with placebo in some small studies, but not in several more recent and larger trials. Others were unable to detect significant differences in the group overall, but found the LGG-associated improvements to be significant in subgroups of children with IgE-associated disease.¹³¹ In the same study, a combination of four probiotics (LGG, *L. rhamnosus* LC705, *B. breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii*) did not have a significant effect.¹³¹ Interestingly, the same four probiotics given in combination with a prebiotic reduced the incidence of eczema and atopic eczema and tended to reduce IgE-associated diseases overall in a primary prevention trial.¹²⁴ This may suggest that probiotics have different effects in developing compared with in established disease. Alternatively, the addition of prebiotic galactooligosaccharides may have been the decisive difference, although clear evidence of its bifidogenic effect is still lacking. The combination of *L. rhamnosus* plus *Bifidobacterium lactis* (the strains were not specified) also significantly improved SCORAD scores only in the subset of food-sensitized children.¹³² In young children (aged 6–18 months) with moderate or severe atopic dermatitis, supplementation with *Lactobacillus fermentum*

tum VRI 003 PCC or placebo for 8 weeks was associated with a significant reduction in the SCORAD index in the probiotic group, but not in the placebo group, although the difference between the groups after 16 weeks was not statistically significant.¹³³

Cytokine production by PBMCs in response to polyclonal stimuli was not affected in participants of trials where probiotic supplementation did not show a significant effect.^{134,135} In contrast, improvements in SCORAD scores were associated with increased IFN- γ production in studies where probiotic supplementation was beneficial at least in certain subgroups of patients.^{136,137} In addition, the induction of low-grade inflammation, as indicated by increased serum CRP and IL-6 levels, has been proposed as a mechanism.¹³⁰ Note, however, that in the above study, serum cytokine concentrations were below the detection limit in the majority of samples, including in almost 80% of the IL-6 samples. In the other studies, sufficient blood samples were available only from subsets of study participants and no information was provided on how representative these children were of the whole study population.^{136,137} In addition, in one of these investigations significant differences in baseline IFN- γ production between the probiotic and placebo groups were not taken into account, raising the question of whether the observed effects were really attributable to probiotic supplementation, particularly given that allergen-specific IFN- γ responses were not significantly altered.¹³⁷

Probiotics in animal models of sensitization and allergic manifestations

Because of these methodological shortcomings, the available data from clinical trials provides little insight into the mechanisms accounting for the strain-specific benefits of probiotic supplementation. Animal studies are beginning to unravel some of the immunological changes that accompany ingestion of established and potential probiotics and that may be of benefit in allergic diseases. Most of these studies have focused on the sensitization process, which generally is not affected by probiotic supplementation in primary prevention trials. The most frequently used animal model of sensitization takes advantage of the fact that Balb/c mice are genetically predisposed toward the elaboration of Th2-polarized T-cell responses. These mice are injected i.p. with antigen, most commonly ovalbumin (OVA), in conjunction with aluminum hydroxide or a similar adjuvant. The effects of oral supplementation with various probiotic strains are then assessed by measuring serum levels of antigen-specific and total IgE production and the elaboration of Th1 and Th2 cytokines in splenocytes restimulated with OVA *ex vivo*.

The available data indicate that numerous LAB strains are capable of inhibiting total and OVA-specific

IgE production when administered orally before or concomitant with sensitization (see Table 6). Notably, many strains are effective even when heat-killed (see Table 7). The associated cytokine patterns in OVA-stimulated splenocytes from LAB-fed mice are highly strain dependent and indicate that different mechanisms are involved. As summarized in Tables 6 and 7, reduced serum levels of OVA-specific IgE can be associated with enhanced production of Th1 cytokines, decreased synthesis of Th2 cytokines, or both. However, in several studies IFN- γ production has been reported to be unaffected or even reduced, and in some instances both Th1 and Th2 cytokines are downregulated. In contrast, oral administration of *L. rhamnosus* HN001 before and during OVA sensitization increased both Th1 (IL-12, IL-18, and IFN- γ) and Th2 (IL-4 and IL-5) cytokines in splenocytes restimulated with OVA *ex vivo*.¹³⁸ Unfortunately, OVA-specific or total IgE levels were not determined in this study.

Two studies examined the effect of oral probiotic administration in murine models of asthma, in which i.p. sensitization with OVA was followed by intranasal OVA challenge.^{139,140} Note that one of these studies used a design resembling that of human primary prevention trials in that the probiotic supplementation was started at birth and continued throughout sensitization and challenge.¹⁴⁰ In these studies, oral administration of *L. reuteri* ATCC 23272,¹³⁹ LGG, or *Bifidobacterium animalis* ssp. *lactis* Bb-12¹⁴⁰ significantly decreased airway hyperresponsiveness and the influx of eosinophils and macrophages in bronchoalveolar lavage fluid obtained 24 h after intranasal challenge. This was not associated with a Th1 polarization of the cytokine response in either of the studies. Instead, upregulation of systemic, though not pulmonary, activity of IDO was observed after oral administration of *L. reuteri* ATCC 23272.¹³⁹ IDO is an important immune control enzyme and mediator of peripheral tolerance, and its stimulation suggests the induction of regulatory mechanisms. Such a mechanism was shown more directly to be involved in the dampening of airway hyperreactivity after oral administration of LGG or *B. animalis* ssp. *lactis* Bb-12.¹⁴⁰ Both probiotics significantly increased the number of natural Tregs (Foxp3+ CD4+ T cells) in the lungs of asthmatic mice, with Bb-12 showing greater effectiveness. Note that both Bb-12 and LGG induce only weak or partial maturation and little IL-12 production in human MDDCs.^{30,141} Whereas Bb-12 strongly stimulates MDDC synthesis of IL-10, LGG only moderately enhances it. Similar results have been reported from *in vitro* stimulation of human PBMCs with these LAB strains.^{33,142-144} These *in vitro* results are in keeping with the ability of these probiotic strains to induce regulatory mechanisms, rather than Th1 cytokine polarization, *in vivo*.

Table 6. Effect of oral supplementation with live LAB on total and OVA-specific IgE levels and cytokine production by OVA-stimulated splenocytes from OVA-sensitized Balb/c mice

Bacterium	Strain	Feeding regimen	OVA-specific IgE	Total IgE	Th1 cytokines	Th2 cytokines	Reference
<i>L. casei</i>	911	0.2% of the diet; 2 weeks before initial oral sensitization for 7 weeks	↓	↓			148
<i>B. bifidum</i>	BGN4	0.2% of the diet; 2 weeks before initial oral sensitization for 7 weeks	↓	↓	↑ IFN- γ	↓ IL-6	148,203
<i>B. bifidum</i>	BGN4	0.2% of the diet; 2 weeks after initial oral sensitization for 2 weeks	↓		↑ IFN- γ	↓ IL-6	203
<i>L. acidophilus</i>	B	0.9–1.1 $\times 10^9$ cfu by gavage; 1 week before initial sensitization for 8 weeks	↓		± IL-2 ± IFN- γ	↓ IL-4	204
<i>L. bulgaricus</i>	Lb	1 week before initial sensitization for 8 weeks	↓		± IL-2 ↑ IFN- γ	↓ IL-4	204
<i>L. bulgaricus</i>	448	1 week before initial sensitization for 8 weeks	↓		± IL-2 ± IFN- γ^a	↓ IL-4	204
<i>B. longum</i>	B6	1 week before initial sensitization for 8 weeks	↓		± IL-2 ↑ IFN- γ	↓ IL-4	204
<i>Streptococcus thermophilus</i>	MC	1 week before initial sensitization for 8 weeks	↓		± IL-2 ↑ IFN- γ	↓ IL-4	204
<i>B. bifidum</i>	G9-1	10 ¹⁰ cfu by gavage; 1 week before and 1 week after initial sensitization	↓	↓	↑ IFN- γ (↓ IFN- γ nonsignificantly)	(↓ IL-4 and IL-5 nonsignificantly)	205

OVA, ovalbumin; IFN, interferon

^aNote that it increased spontaneous IFN- γ production.^bCytokine production in response to OVA restimulation was assessed in mesenteric lymph nodes

Table 7. Effect of oral supplementation with heat-killed LAB on total and OVA-specific IgE levels and cytokine production by OVA-stimulated splenocytes from OVA-sensitized Balb/c mice

Bacterium	Strain	Bacterial dose	Total IgE	OVA-specific IgE	Cytokine changes		Reference
<i>L. acidophilus</i>	L-92	5.0 mg/animal per day; 2 weeks after initial immunization for 8 weeks	↓	↓	↓ IFN- γ	↓ IL-4 ↓ IL-10	206
<i>L. brevis</i>	SBC8803	0.5% of diet; 1 week before initial sensitization for 4 weeks	↓	↓	↓ IFN- γ ↓ IL-12	↓ IL-4 (↓ IL-5 and IL-6 nonsignificantly)	207
<i>L. casei</i>	Shirota	0.1 or 0.05% of the diet; at initial sensitization for 3 weeks	↓	↓	↑ IFN- γ ↑ IL-2	↓ IL-4 ↓ IL-5 ↓ IL-6 ↓ IL-10	208
<i>L. gasseri</i>	OLL2809	0.1% of the diet; at initial sensitization for 3 weeks	↓		± IFN- γ ↑ IL-12	↓ IL-4	178
<i>L. paracasei</i>	KW3110	0.03% of the diet starting at initial sensitization for 14 weeks	↓	↓	± IFN- γ ↑ IL-12	(↓ IL-4 nonsignificantly)	209
<i>L. pentosus</i>	S-PT84 (FERM ABP-1002)	0.075% of the diet; 1 week before initial sensitization for 5 weeks	↓	↓	± IFN- γ ± IL-12	↓ IL-4 ↑ IL-10	210

In one of the few studies to investigate the therapeutic rather than preventative effects of oral LAB supplementation, *L. plantarum* ATCC 8014 was identified as a strain that strongly induces IL-10 and far less IL-12 in human MDDCs, although the resulting DCs primed naïve allogeneic CD4+ T cells for the production of high levels of IFN- γ along with IL-10.¹⁴⁵ When *L. plantarum* was used as an adjuvant in sublingual immunotherapy in established murine asthma, airway hyperresponsiveness was significantly reduced compared with phosphate-buffered saline, whereas OVA alone or *L. plantarum* alone only induced nonsignificant reductions. This was not due to effects on OVA-specific IgE titers, the percentage of OVA-specific IL-5-producing splenocytes, or the activity of natural Tregs. Instead, *L. plantarum* markedly increased the proportion of OVA-specific proliferating T cells in cervical/maxillary lymph nodes. What role, if any, this enhanced proliferation plays in its beneficial effect in this therapeutic asthma model remains to be established.

Few other studies addressed the therapeutic rather than preventative effects of oral LAB administration. In a model of established allergic rhinitis, in which i.p. OVA sensitization and s.c. boosting were followed by intranasal OVA challenge, 3 days of supplementation with *L. acidophilus* L92 and *L. fermentum* CP34 were sufficient to significantly reduce allergen-specific IgE.¹⁴⁶ Milk fermented with these or several other *Lactobacillus* strains was also effective. In a similar experimental model of allergic rhinitis, but with a longer duration of sensitization and also of probiotic administration, *L. acidophilus* L-55 reduced OVA-specific IgE as measured by passive cutaneous anaphylaxis.¹⁴⁷ It also less-

ened sneezing and nasal rubbing, the effect becoming significant 2 weeks after the beginning of probiotic treatment and lasting throughout the treatment period. In a murine model of food allergy where mice were orally sensitized by intragastric administration of OVA together with cholera toxin, therapeutic administration of *B. bifidum* BGN4 inhibited OVA-specific IgE production, while increasing IFN- γ and decreasing IL-6 secretion by OVA-stimulated splenocytes and diminishing the number of degranulated mast cells in ear and tongue tissue.¹⁴⁸ These effects were somewhat smaller than those seen after preventative treatment with the same strain, but this may have been due to the much shorter treatment period, which was 2 weeks compared with 7 weeks in the prevention study.

Probiotics in liver diseases and cancer

Although beyond the aims of the present manuscript, a brief mention of the potential impact of probiotics on chronic liver diseases and cancer is in order.

The effectiveness of lactulose as the only available treatment for encephalopathy in liver cirrhosis has provided a rationale for the use of probiotics in chronic liver diseases. Indeed, this is mostly based on two major mechanisms, that is, the ability to augment the intestinal barrier function and to prevent the translocation of bacteria.¹⁴⁹ Both mechanisms represent the rationale for the use of probiotics to prevent major complications of liver cirrhosis such as the development of a hyperdynamic circulatory state or spontaneous bacterial peritonitis, with the former being the background for the establishment of portal hypertension.¹⁵⁰ While solid data

from human studies are awaited to confirm animal evidence,¹⁵¹ the use of probiotics is considered safe in patients with chronic liver diseases, with the possible exception of end-stage functionally impaired liver cirrhosis. Based on the immunomodulatory impact of probiotics, the possibility that these may manifest significant effects on the rapidly growing clinical problem of non-alcoholic fatty liver disease should not be ruled out.¹⁵²

It is well established that the etiopathogenesis of cancer involves several immune-mediated pathways,¹⁵³ particularly in the mucosal epithelia.^{17,154} A significant efficacy of probiotics on cancer development is suggested by several lines of scientific evidence and hypotheses, including the increase in immune cell activation and the suppression of bacteria converting procarcinogens.¹⁵⁵ Most data were obtained from cases of colon and breast carcinomas. In the former, prevention was first achieved in animal models and was ascribed to the significant anti-inflammatory effect of probiotics.¹⁵⁶ Of note, this is in agreement with data obtained in clinical entities characterized by chronic inflammation and higher carcinoma prevalence such as UC, as previously illustrated. In the case of breast cancer, on the other hand, suggested beneficial effects of probiotics appear to be mediated by an increase of the antitumor surveillance immune activity.¹⁵⁷ In both cases, however, more solid data are encouraged and awaited in prospective randomized studies of sufficient comparison power. Careful evaluation is also recommended in specific intensive care cases, as strongly suggested by data in patients with severe acute pancreatitis.¹⁵⁸

Probiotic supplementation in healthy volunteers

A number of studies have addressed the effects of supplementation with various probiotic strains in healthy volunteers. Increases in NK activity have been observed after supplementation with *L. casei* Shirota,^{159–161} *L. casei* DN114001,¹⁶² *L. rhamnosus* HN001,^{163,164} or *B. lactis* HN019.^{164–166} Increases in polymorphonuclear and/or macrophage phagocytic activity have also been reported after supplementation with *L. casei* DN114001,¹⁶² *B. lactis* HN019,^{165,166} *L. rhamnosus* HN001,¹⁶³ a combination of *L. acidophilus* 74-2 and *B. animalis* ssp. *lactis* 420,¹⁶⁷ or a combination of those two strains with *L. paracasei* Lpc-37.¹⁶⁸ Interestingly, a similar mixture, but consisting of different strains (*B. animalis* ssp. *lactis* Bb-12 and *Lactobacillus paracasei* ssp. *paracasei* CRL-431) did not enhance phagocytosis at any of the four doses tested.¹⁶⁹ The effect size is frequently small and of questionable clinical relevance, although subjects with low innate immune responses may benefit significantly.^{160,161,166}

Of note, even though several small studies indicated that *L. casei* Shirota supplementation could increase

NK cell and phagocytic activity, one of the probiotic supplementation studies with the highest methodological quality did not.¹⁷⁰ In this randomized placebo-controlled trial, all volunteers were kept on a controlled diet throughout the 2-week stabilization, 4-week treatment, and 2-week follow-up, periods.¹⁷⁰ None of the immune parameters under investigation was significantly affected by supplementation with *L. casei* strain Shirota. Besides NK cell activity, phagocytic, and respiratory burst activity, these included lymphocyte subset distribution, serum levels of IgM, IgG, IgA, and IgE, PBMC production of IL-1 β , IL-2, and IFN- γ in response to LPS stimulation, and delayed-type hypersensitivity responses to eight recall antigens.

A few other studies have addressed the ex vivo cytokine production of PBMCs from volunteers who had consumed probiotics, and they generally found few, if any, effects.^{169,171} In contrast, supplementation of volunteers with *L. fermentum* ECET5716 for 2 weeks before and 2 weeks after vaccination with an inactivated trivalent influenza vaccine increased serum IL-12 levels before vaccination, and enhanced vaccine-induced elevations in serum TNF- α .¹⁷² It also induced significantly higher levels of specific IgA, but not IgG or IgM, compared with the placebo group. Minor adjuvant effects have also been reported for LGG or *L. paracasei* ssp. *paracasei* CRL431,¹⁷³ a combination of probiotics (LGG, *L. rhamnosus* LC705, *B. breve* Bbi99, and *P. freudenreichii* ssp. *shermanii*) plus galactooligosaccharides,¹⁷⁴ and *L. paracasei* ssp. *paracasei* F19.¹⁷⁵ Overall, these results suggest that several probiotic strains may have the ability to enhance nonspecific immune responses, but the effects on adaptive cellular and humoral immune responses seem to be for the most part minor.

Identification of active probiotics components and receptor usage

The sometimes identical, sometimes differential effects of viable and nonviable bacteria suggest that metabolic or secreted factors as well as structural or other cellular components mediate certain immunomodulatory activities of probiotics^{47,139,176} (Tables 3, 4, 6, and 7). But the viability requirement depends not only on the immunomodulatory function under investigation but also on the bacterial strain.^{44,177} This suggests, and other data confirm,¹⁷⁸ that even members of the same species differ substantially in their composition or their production of secreted mediators. Various experiments indicate that the ability to induce the secretion of various cytokines is mediated to a large extent by cell wall components.^{177,179,180} In particular, peptidoglycans have been implicated in IL-12 induction in splenocytes,¹⁷⁸ while lipoteichoic acids are involved in the stimulation of

Table 8. Involvement of TLR-2, -4, -9, and the adaptor molecule MyD88 in probiotic activities

Bacterium	Strain	Activity	TLR2	TLR4	TLR9	MyD88	Reference
<i>L. casei</i>	NIZO B255	Luciferase reporter in HEK-23 cells	–	–	–		39a
<i>L. plantarum</i>	NIZO B253	Luciferase reporter in HEK-23 cells	–	–	–		
<i>L. reuteri</i>	ASM 20016	Luciferase reporter in HEK-23 cells	–	–	–		
<i>L. acidophilus</i>	L-2	IL-12 production in Peyer's patch and BMDCs from mice deficient in the respective TLR	–	–	–	+	190
<i>L. casei</i>	Shirota	IL-12 production in macrophages from mice deficient in the respective TLR	–	–	–	+	190
<i>L. johnsonii</i>	La1		–	–	–	+	
<i>L. paracasei</i>	KW3110		–	–	–	+	
<i>L. rhamnosus</i>	GG		–	–	–	+	
<i>L. casei</i>	Shirota		–	–	–	+	180
<i>L. casei</i>	ATCC 334		–	–	–	+	
<i>L. fermentum</i>	ATCC 14931		–	–	–	+	
<i>L. rhamnosus</i>	ATCC 7469		–	–	–	+	
<i>L. plantarum</i>	Unspecified	TNF- α production of human macrophages in the presence of anti-TLR2 or anti-TLR4 antibodies	++	±			189
<i>L. plantarum</i>	NCIMB 8826	IL-10 production by BMDCs of mice deficient in the respective TLR or MyD88	+	–		+	211
		IL-12 production by BMDCs of mice deficient in the respective TLR or MyD88	±	–		±	211
<i>B. animalis</i> ssp. <i>lactis</i>	Bb-12	IL-6 production in mouse embryonic fibroblasts from TLR2-deficient mice	+				212
<i>B. animalis</i> ssp. <i>lactis</i>	Bb-12	IL-1b production in mouse embryonic fibroblasts from TLR2-deficient mice	–				212

TLR, Toll-like receptor; TNF, tumor necrosis factor

^aThey also did not activate TLR1, -6, or -7

TNF- α production in macrophages and splenocytes.¹⁸¹ In addition, DNA has been shown to mediate a variety of immunomodulatory effects of certain probiotic strains both in vitro and in vivo.^{48,113,177} Note that ISS-DNA sequences have been identified in a variety of probiotic strains, including LGG,¹⁸² *Lactobacillus delbrueckii* ssp. *bulgaricus* NIAIB6,¹⁸³ *Lactobacillus gasseri* OLL2716,¹⁸⁴ and *B. longum* BB536.¹⁸⁵ The ISS-ODN from *B. longum* BB536 and LGG exhibited immunomodulatory activity in OVA-immunized mice after oral and i.p. administration, respectively.^{186,187}

Recognition of bacteria is mediated by a variety of pattern recognition receptors, the most important of which are TLRs and nucleotide-binding oligomerization domain (NOD) proteins. TLR usage by LAB strains is species- as well as activity-specific (see Table 8), probably partly attributable to the considerable variation in the cell wall composition even of members of the same species.¹⁷⁸ The TLRs identified as mediating certain activities of probiotics include TLR2, TLR9, and NOD2¹⁸⁸ (see Table 8). Their ligands are peptidoglycans and lipoteichoic acid from Gram-positive bacteria in the case of TLR2, and CpG DNA in the case of TLR9, while NOD2 recognizes muramyl dipeptide, a constituent of peptidoglycans from both Gram-positive and Gram-

negative bacteria. This further supports an important role for cell wall components and DNA in the immunomodulatory activities of probiotics. TLR4 recognizes LPS of Gram-negative bacteria, and its implication in macrophage TNF- α production in response to *L. plantarum*¹⁸⁹ is therefore unexpected. Note that some in vitro and in vivo effects of probiotics were found not to require TLR2, TLR4, or TLR9.^{107,180,190} However, deficiency of MyD88, an adapter molecule for TLR signaling, abolished the investigated activities. It remains to be established whether other TLRs or a MyD88-dependent, but TLR-independent, pathway is involved.

Final comments and thoughts

While there is substantial evidence from in vitro and animal studies that known and potential probiotics have strain-specific immunomodulatory capacities, the results from human intervention trials are far less convincing. This may be partly a question of the dose, which is often similar in animal studies and human trials despite the enormous difference in weight and intestinal size. Dose–response studies are urgently required, especially

since there are indications that certain in vitro effects are seen only at low bacterial doses³⁹ and that high doses may produce opposite effects to those obtained at low doses.⁴⁷ The results of a human supplementation trial also suggest that lower doses can be more effective than higher doses.¹⁶⁶ It might be advisable to determine whether a probiotic has any effect and whether it retains this effect when used in combination with another probiotic before proceeding with a dose–response study that ultimately shows that a probiotic combination has no effect at four different doses.¹⁶⁹

Another potential explanation for the differential effectiveness of probiotics in animals and humans might be that the composition of the intestinal microflora is likely to vary to a much greater extent between individual humans than between individual mice that are kept in the same environment and fed the same diet. Since in vitro studies suggest considerable interactions between different commensal and probiotic strains,^{30,31} human responses to probiotic treatment might differ depending on the composition of the individual's endogenous flora. Genetic differences in the expression of pattern recognition receptors and other factors contributing to the response to bacterial signals are also likely to contribute to the variable responses to probiotic treatment.

Despite the mostly minor immunomodulatory effects of probiotics in human supplementation studies, there are some concerning findings, such as the increased rate of sensitization or greater frequency of wheezing in participants of primary intervention trials who received *L. acidophilus* LAVRI-A1 or LGG,^{119,143} or the more severe endoscopic recurrence seen in CD patients treated with LGG.⁸⁹ Note also that there is some concern that strongly Th1-promoting strains may aggravate Th1-mediated autoimmune diseases. There are several studies showing the exacerbation of experimental autoimmune encephalomyelitis (EAE), a Th1-mediated model of multiple sclerosis, with certain *Lactobacillus* strains, including the well-established probiotic *L. casei* Shirota.^{191–193} However, other probiotic strains are capable of ameliorating EAE,¹⁹⁴ and other experimental autoimmune diseases that are known to be Th1 mediated, such as various models of rheumatoid arthritis^{195–197} or type 1 diabetes.¹⁹⁸ Together, these findings highlight the need for care in choosing appropriate probiotic strains for particular applications. In this process, it needs to be taken into account that the response to probiotic supplementation depends on the immunological status of the host, as demonstrated by in vitro^{98,199} and in vivo experiments.^{168,200} What ultimately counts in the search for functional foods to modulate the immune response is that well-tolerated and effective nutrients are needed, and probiotics are currently among the best candidates to play this critical role in future epidemics of metabolic and inflammatory conditions.

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