

New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics—A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report

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The dietary approach to reducing the risk of atopic diseases in infancy is evolving from passive allergen avoidance to active stimulation of the immature immune system, the aim of which is to support the establishment of tolerance. The intestinal mucosa and the mucosa-associated immune system are the primary loci of allergen contact and induction of immune responsiveness. In this review we discuss cross-talk between the intestinal microbiota and the host as it pertains to healthy immunologic maturation. Understanding these complex phenomena provides the rationale for the use of probiotics in reducing the risk and nutritional management of atopic disease. (*J Allergy Clin Immunol* 2005;116:31-7.)

Key words: Allergy, atopic disease, host-microbe cross-talk, hygiene hypothesis, intestinal microbiota, probiotics

The basic foundation of nutrition lies in a healthy balanced diet to meet the needs for growth and development in children. The first goal here is directed toward the prevention of diet-related deficiencies, and the second target is reduction of the risk of nutrition-related chronic diseases. Dietary attempts to reduce the risk of allergic disease have hitherto focused on elimination diets, even though the results of different elimination procedures with dietary restrictions alone or in combination with some other environmental control measures have been inconclusive regarding the long-term prevention of atopic diseases.¹ Indeed, the association between antigen exposure and the development of atopic disease appears by no means direct or causal. In addition to elimination of potentially allergenic proteins, current research interest is directed toward intervention by novel immunomodulatory dietary compounds with specific effects in health promotion.

Abbreviations used

TLR: Toll-like receptor
Tollip: Toll-interacting protein

The development of this new allergy prevention strategy calls for a better understanding of the processes that regulate the maturation of immune defense mechanisms at an early age and thereby promote the nonatopic mode of immune responsiveness. Many of the immunoregulatory aberrations favoring sensitization instead of tolerance induction prevail in early infancy. The intestine's antigen exclusion, elimination, and immune regulation mechanisms are incomplete, predisposing to aberrant antigen uptake and inflammatory responses. During pregnancy, the cytokine profile is polarized away from cell-mediated immunity (T_H1 type) toward humoral immunity (T_H2 type) to protect the developing fetus. Thus the T_H2 responder phenotype is universal at an early age.² Allergic disease could thus be ascribed to a lack of immunologic counterregulatory processes and insufficient maturation signals endorsing the gut barrier function. The recent demonstration that the gut microbiota and, by the same token, probiotics have a strong effect on priming immunophysiologic regulation in the intestine's mucosal barrier has opened up new angles in the science of nutrition. In modulating specific target functions in the gut and the immune system, probiotics in the diet might exert clinical effects beyond the nutritional effect of food.

GUT MICROBIOTA: THE SOURCE OF PROBIOTICS

The microbiota of a newborn develops rapidly after birth and is initially markedly dependent on genetic factors, maternal microbiota, mode of delivery, and birth environment.³ The establishment of the gut microbiota is usually characterized by specific stages of development: early colonization by facultative anaerobes,

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such as enterobacteria, coliforms, and lactobacilli, succeeded by anaerobic genera, such as *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Eubacterium*.³ The greatest difference in the microbiota of breast-fed and formula-fed infants lies in the numbers and species composition of bifidobacteria. *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium longum* are species frequently found in fecal samples from breast-fed infants, whereas later in life, *Bifidobacterium adolescentis* becomes more common. After weaning, the microbiota becomes more diverse, resembling that of adults. The gastrointestinal tract of the human adult harbors more than 500 identified species of culturable and a large number of unculturable bacteria.

The relationship between intestinal bacteria and the host is referred to as host-microbe cross-talk, implying peaceful coexistence and mutual benefit. The importance of resident bacteria for the host's physiology has been clearly documented: major functions of the gut microbiota include metabolic activities, trophic effects on the intestinal epithelium, and protection of the host against the overgrowth of potential pathogens in the gastrointestinal tract. More recently, evidence from prospective studies in infancy demonstrate that specific aberrancies in the intestinal microbiota might predispose to disease later in life.

A probiotic is currently defined as a live microbial food supplement with a proved beneficial effect on human health.³ The aims of intervention are to avert deviant microbiota development, impaired gut barrier function, abnormal immune responsiveness, and immunoinflammatory disease. At an early age, probiotic supplementation aims to provide safe yet sufficient microbial stimulus for the immature immune system, contributing to the anti-inflammatory tone of the intestinal milieu.

Probiotics are selected from members of the normal healthy intestinal microbiota, most of them belonging to *Lactobacillus* or *Bifidobacterium* species, but new probiotic microbes from other species and genera have recently been introduced. Improved understanding of the host-microbe interaction at different ages will lead to the development of a new generation of probiotics, the action of which could be selected for defined disease-associated aberrancies. Identification of new *Bifidobacterium* and *Lactobacillus* species and strains from the gastrointestinal tract of healthy subjects might then allow us to devise better target- and age-specific probiotics, which might be recognized with risk reduction of human disease in the future. It is well established that different probiotic strains induce distinct responses, and thus specific strains might have specific targets in reducing the risk and treatment of human disease. Probiotic strains selected for their beneficial effects in providing maturational signals for the infant's immune system and propagating oral tolerance induction might be of no use in treating adults. Further research might also substantiate the clinical efficacy of genetically modified probiotic bacteria, as indicated by the initial studies on *Lactococcus lactis* engineered to produce IL-10 locally.⁴

Probiotic research exemplifies that the dietary approach to reduce allergic diseases is evolving from passive elimination diets to supplementation with active immunomodulatory compounds. Still, because of interaction between nutrients, no single supplement can be expected to resolve the challenge of allergic disease, and better understanding of the interaction between nutrients is required.

HOST-MICROBE INTERACTION IN THE GUT EPITHELIUM: A KEY TO INTESTINAL HOMEOSTASIS

The gastrointestinal epithelium is equipped with pattern recognition receptors, including Toll-like receptors (TLRs), which recognize specific conserved pathogen-associated molecular patterns, to sense the myriad of microorganisms found in the gastrointestinal lumen. Nonpathogenic microbes also possess these same structures, and therefore they might as well be called commensal-associated molecular patterns.^{5,6} Thus TLRs cannot distinguish between pathogens and commensals. Human primary colonic epithelial cells have been demonstrated to express TLRs 1 through 6, 8, and 9, which can recognize several different microbial structures, including lipopeptides, lipoteichoic acid, LPS, single- and double-stranded RNA, flagellin, and unmethylated cytosine-guanine-rich DNA.^{5,7} Most of the TLRs recognize more than one different pathogen-associated molecular pattern. Typically, an intracellular signaling domain known as the TIR domain initiates intracellular signaling pathways of TLRs that result in production of proinflammatory cytokines through activation of the transcription factors nuclear factor κ B, activator protein 1, and interferon regulatory factor 3.⁵ In addition to TLRs, intestinal epithelial cells also express a second group of pattern recognition receptors (ie, 2 intracellular nucleotide oligomerization domains, NOD1 and NOD2, the ligands of which are diaminopimelic acid of peptidoglycan in gram-negative bacteria and muramyl dipeptide, a breakdown product of the peptidoglycan component of all bacterial cell walls, respectively).⁵

Several molecular characteristics of the gut epithelium have been thought to prevent inappropriate immune responses toward indigenous gut microbiota. These include a relatively sparse expression of both certain TLRs and their essential coreceptors on the intestinal epithelium, as well as intracellular location of a few TLRs. This topic is, however, a matter of some controversy.⁷ A recent study suggests that a negative regulator of the TLR-signaling Toll-interacting protein (Tollip) might mediate some tolerogenic effects of commensal microbes.⁷ In that study Otte et al⁷ demonstrated that a continuous exposure of colonic epithelial cell lines to lipoteichoic acid and LPS resulted in a state of hyporesponsiveness (tolerance), whereas a short exposure elicited a proinflammatory response. In those hyporesponsive cells, Tollip expression was upregulated, although TLR expression was unaltered.

Moreover, upregulation of Tollip in transfected colonic epithelial cells reduced significantly the proinflammatory response of those cells, implying that Tollip expression regulates innate immune responses of the intestinal epithelium toward molecular structures found in huge amounts in commensal microbes.

Certain strains of commensal microbes and probiotics have been found to elicit anti-inflammatory responses in the intestinal epithelial cells *in vitro*, thus strengthening the intestinal homeostasis. Both some nonpathogenic salmonella strains and probiotic *Lactobacillus reuteri* were able to attenuate IL-8 secretion elicited by pathogenic salmonella or TNF- α in the polarized T84 model colonic epithelia.^{8,9} In either case this immunosuppressive effect was mediated by inhibition of the proinflammatory-antiapoptotic nuclear factor κ B pathway. In contrast, *Lactobacillus rhamnosus* GG has been shown to prevent cytokine-induced apoptosis in mouse and human colon cells *in vitro* through activation of antiapoptotic Akt and protein kinase B and inactivation of proapoptotic p38 mitogen-activated protein kinase signaling cascade.¹⁰ These examples imply that nonpathogenic microbes in the gut manipulate different intracellular signaling pathways of the intestinal epithelia to maintain equilibrium.

GUT BARRIER: THE TARGET OF PROBIOTIC EFFECTS

The intestinal barrier consists of physiologic and immunologic factors that restrict mucosal colonization by pathogens, prevent foreign antigens and pathogens from penetrating the mucosa, and regulate the antigen-specific immune responses.¹¹ The intestinal microbiota constitute an important aspect of the mucosal barrier through their metabolic activity and trophic effects on the intestinal mucosa, complex immunomodulatory properties, and resistance to colonization by microbial invaders. All these aspects of the gut barrier function are potential targets for probiotic intervention. Probiotics have been shown to colonize the mouth¹² and the intestine¹³ at least transiently and also alter the overall composition of the intestinal microbiota.¹⁴⁻¹⁶

Lactobacillus rhamnosus strain GG, ATCC 53103 (*Lactobacillus* GG), has been demonstrated to improve intestinal barrier function compromised by rotavirus infection¹⁷ or cow's milk antigens¹⁸ and the combination of *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 12246 stabilizes the impaired intestinal mucosal barrier in children with atopic dermatitis.¹⁹ Of note, the same combination has earlier been shown to reduce the extent of atopic dermatitis.²⁰

Lactobacilli maintain gut barrier function by exerting a protective effect against brush border lesions caused by diarrhoeagenic *Escherichia coli*.²¹ Probiotics have also been demonstrated to improve colonization resistance by inhibiting adherence and invasion by potential pathogens.^{22,23} Lactobacilli adhere to intestinal epithelial cells and induce mucin secretion *in vitro*, which inhibits

adherence of enteropathogenic *E coli*.^{24,25} In parallel, VSL#3, a probiotic compound containing 4 strains of lactobacilli, 3 strains of bifidobacteria, and *Streptococcus thermophilus*, has also been demonstrated to upregulate production of mucins in intestinal epithelial cells *in vitro* through the mitogen-activated protein kinase signaling pathway.²⁶ *E coli* Nissle 1917 and a few lactobacilli, but none of the 40 enteropathogenic *E coli* strains studied, strongly induced the gene expression of the antimicrobial peptide human defensin- β_2 in intestinal epithelial cells in a time- and dose-dependent manner.²⁷ A secreted proteinaceous soluble factor of VSL#3 increased gut epithelial barrier impermeability and resistance to salmonella invasion. The same probiotic was capable of reducing salmonella-induced alterations in the cellular cytoskeleton of the intestinal epithelium by modulating the distribution of the intercellular tight-junction protein zonula occludens 1.^{26,28} Still, administration of *Lactobacillus plantarum* failed to reduce the rate of bacterial translocation, gastric colonization, or infections in a randomized placebo-controlled study of patients undergoing abdominal surgery.²⁹ The dilemma of whether colonization is mandatory for probiotic effects needs to be unraveled. However, oral consumption of a mixture of probiotics has been shown to result in a reduction in nasal colonization by the potentially pathogenic bacteria *Staphylococcus aureus*, *Streptococcus pneumoniae*, and β -hemolytic streptococci in human subjects without evidence of colonization by the probiotic strains.³⁰ Moreover, these apparently discrepant data demonstrate that probiotic effects might not only be strain specific but also that the effects might be different in health and on inflamed mucosa.

Abundant IgA antibody production at mucosal surfaces contributes to the intestinal barrier function by binding to and excluding antigens. Maturation of dendritic cells carrying commensals and subsequent secretion of cytokines and chemokines then influence the polarization of T_H cells and thereby the adaptive immune responses, ensuring a local IgA response.³¹ This type of immune response has been suggested to prevent commensals from breaching the gut mucosal barrier, whereas pathogenic bacteria preferably destroy it.³¹⁻³³ A recent series of experimental studies in mice deficient in MyD88, an adaptor molecule essential for the TLR-mediated induction of inflammatory cytokines, demonstrated that TLR signaling pathways control the homeostasis of the epithelium and appear critical for protection of the host against gut injury in controlling cytoprotective factors and epithelial cell proliferation.³⁴

ANTI-INFLAMMATORY EFFECTS OF COMMENSALS AND PROBIOTICS IN THE GUT-ASSOCIATED LYMPHATIC TISSUE

Intestinal barrier function and immune regulation are closely linked and have common characteristics. The gut-associated immune system represents the largest mass of lymphoid tissue in the human body. Consequently, it comprises an important element of the total immunologic

capacity of the host. Anti-inflammatory and regulatory mucosal immune responses are essential for withholding detrimental inflammatory reactions to dietary antigens and nonpathogenic microbes, a phenomenon referred to as mucosal tolerance. There are several active mechanisms through which mucosal tolerance is established and maintained, including clonal deletion, anergy, and antigen-specific suppression by T cells. Distinct regulatory mechanisms modulating both T_H1 and T_H2 responses have recently been discovered as novel T-cell subclasses with suppressive and regulatory functions have emerged. Gut-derived T_H3 and T_R1 cells exert their effects through the production of cytokines, mainly TGF- β and IL-10, respectively. The thymus-originated $CD4^+CD25^+$ regulatory T cells act through contact-dependent mechanisms probably involving membrane-bound TGF- β .³⁵

TGF- β -producing regulatory T cells have been implicated in the establishment and maintenance of oral tolerance,³⁶ and indeed, infants with food allergy have been reported to display a defect in TGF- β -producing cells in the intestine.³⁷ TGF- β has direct suppressive effects on both T_H1 and T_H2 responses.³⁸ In addition, TGF- β is the initial trigger for the production of IgA antibodies^{39,40}; IL-10, on the other hand, has been implicated in tolerance toward intestinal microbiota.

The indigenous microbiota contribute to the anti-inflammatory tone of the mucosal immune system. The requirement of innate immune responses to enteric bacteria in the maintenance of tolerance has been demonstrated in an experimental animal model.⁴¹ The importance of host-microbe interaction is most vital in the neonatal period when the establishment of a normal microbiota provides the host with the most substantial antigen challenge, with a strong stimulatory effect for the maturation of the gut-associated lymphoid tissue. Indeed, mice reared in germ-free conditions have been demonstrated to display defective oral tolerance formation and atopic-type immune responsiveness and to regain the ability to establish oral tolerance if the intestinal microbiota are reconstituted with bifidobacteria at the neonatal age but not later.⁴²

A number of studies suggest that probiotics might provide a tool for stimulating these anti-inflammatory responses. The probiotic *Lactobacillus paracasei* inhibits proliferation of T cells and reduces the secretion of both T_H1 and T_H2 cytokines while inducing the development of a population of T cells producing anti-inflammatory TGF- β and IL-10 *in vitro*.⁴³ The genomic DNA from probiotic bifidobacteria has been reported to induce IL-10 secretion by PBMCs *in vitro*.⁴⁴ Lactobacilli inhibit allergen-specific T_H2 cytokine production by PBMCs obtained from allergic individuals,⁴⁵ and consistently with this *in vitro* observation, *Lactobacillus casei* suppresses IgE responses and systemic anaphylaxis in a murine model of food allergy.⁴⁶

Oral administration of *Lactobacillus* GG has been observed to result in increased serum IL-10 concentrations⁴⁷ and enhanced IFN- γ production by PBMCs,⁴⁸ demonstrating that systemic immunomodulation is

achieved with probiotic supplementation. Moreover, *Lactobacillus* GG administered to lactating mothers increased breast milk TGF- β concentrations, which suggests that intestinal microbial stimulation has effects on mucosal immune responses beyond the gut.⁴⁹

THE SHIFT OF EMPHASIS IN THE HYGIENE HYPOTHESIS: FROM INFECTIONS TO INTESTINAL MICROBIOTA

In 1976, Gerrard et al⁵⁰ found an inverse relationship between the incidence of infections and the prevalence of atopic disease and stated that "atopic disease is the price paid by some members of the white community [as opposed to native Americans in Saskatchewan, Canada] for their relative freedom from diseases due to viruses, bacteria and helminthes." In 1989, Strachan⁵¹ suggested in a classic article that infections acquired from older siblings in infancy or early childhood might confer protection against the development of atopic disease on the basis of epidemiologic findings, indicating an inverse correlation between family size and the prevalence of allergic rhinitis. Subsequently, a considerable amount of epidemiologic research has concentrated on elucidating the associations between childhood infections and atopic sensitization or atopic disease in general and, more specifically, the timing and precise nature of the infections that might carry protective potential.

Several microbes and infections, including mycobacteria, measles, *Helicobacter pylori*, the hepatitis A virus, *Toxoplasma gondii*, viral respiratory tract infections, and helminthic infections, have been suggested to be involved in causing the immune deviance proposed by the hygiene hypothesis, but only inconsistent or inconclusive data are available.⁵² The number of siblings and daycare attendance has recently been shown to protect against atopic disease in a recent cohort study of more than 24,000 mother-child pairs, but no protective effect can be attributed to early infections.⁵³ Consequently, it has been suggested that the principal microbial provocation resulting in nonatopic immune maturation in infancy might be derived not from pathogens but from the indigenous intestinal microbiota or environmental bacterial products.⁵⁴

The notion for a role of the indigenous intestinal microbiota in the hygiene hypothesis has stemmed from 2 directions. Experimental studies with germ-free animals have demonstrated the importance of intestinal microbiota in normal immunologic maturation. The clinical effects of probiotics have also suggested a role for intestinal microbes in eliciting effects that might confer protection against atopic disease. Subsequently, prospective epidemiologic studies investigating the association between early microbiota composition alterations and the development of atopic sensitization and atopic disease have been conducted. Infants with atopic sensitization have been shown to harbor more clostridia and a tendency toward less bifidobacteria in their stools at 3 weeks of age compared with infants without sensitization,⁵⁵ and infants

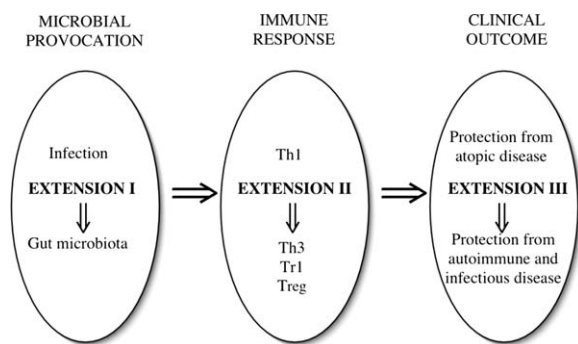


FIG 1. The extended hygiene hypothesis.

who had atopic dermatitis, became sensitized, or both were less often colonized by enterococci at the age of 1 month or by bifidobacteria at the age of 12 months than those who remained healthy.⁵⁶ Markedly, these alterations in intestinal microbiota composition precede the development of atopy, suggesting a possible causal mechanism.

In the first double-blind, placebo-controlled clinical trial evaluating the effect of probiotics in primary prevention of atopic disease, administration of *Lactobacillus* GG to mothers of high-risk infants before birth and to the lactating mother or the infant during the first 6 months of life resulted in a significant reduction in the prevalence of atopic eczema up to the age of 4 years.^{57,58} However, no protective effects were observed on other manifestations of atopic disease, such as food allergy or asthma, nor was there an effect on atopic sensitization. The effects of probiotics in the treatment of atopic disease have been investigated in a number of clinical studies. Both a significant improvement of the skin condition and a decrease of systemic markers of inflammation have been reported after probiotic supplementation in infants with atopic eczema,^{20,59} and there are data indicating alleviation of both symptoms and intestinal inflammation in cow's milk allergy with probiotics.⁶⁰ Still, no improvement of symptoms was detected after ingestion of *Lactobacillus* GG in adults with birch pollen allergy.⁶¹ Probiotics thus appear to be effective in the prevention and treatment of atopic disease in infancy and childhood but not in later life. This might be explained by arguing that immune responses to bacteria early in life are distinct from those later in life,⁶² and perhaps probiotics exert their effects during the development of atopic responsiveness but fail to influence fully established atopic disease.

In immunologic terms the hygiene hypothesis effect was originally conceived as immune deviance from the neonatal T_H2 responsiveness by T_H1 -type responses elicited against infectious agents. However, the rigid T_H1/T_H2 paradigm appears to fall short of providing a satisfactory immunologic grid for understanding the development of atopic disease. Although T_H2 responses are undisputedly active in fully established atopic disease, also T_H1 -type responsiveness is encountered in chronic atopic inflammation characteristic of atopic eczema and asthma, and the immune responses in infants with and

without atopic disease appear not to follow a strict T_H1/T_H2 dichotomy. Moreover, autoimmune diseases characterized by T_H1 -type inflammation often coexist with atopic disease both on the individual and population levels.⁵⁴ An alternative to the immune deviance by T_H1 interpretation of the hygiene hypothesis effect conceives anti-inflammatory immune responses to be of fundamental importance in the development of tolerance. These suppressive mechanisms are orchestrated by intestinal regulatory T cells that control inflammation of both T_H1 and T_H2 types and are thus implicated in protection against both atopic and autoimmune disease. It has recently been suggested that the defective generation of regulatory T cells in the intestinal immune system, resulting in development of atopic responder phenotype, might be caused by a lack of sufficient stimulation of the innate immune system by intestinal microbes.³⁷

Consequently, an extended version of the hygiene hypothesis of atopic disease has been introduced.⁵⁴ The original conception of infectious diseases providing protection from atopic disease by causing immune deviance in the form of T_H1 -driving signals that downregulate atopic T_H2 responsiveness is revised in 3 interrelated respects (Fig 1). First, the role of the indigenous intestinal microbiota might outweigh that of infections in providing maturational signals to the infant's immune system. Second, the role of the innate immune system and anti-inflammatory adaptive responses elicited by TGF- β -secreting T_H3 cells, IL-10-secreting T_R1 cells, and $CD4^+CD25^+$ regulatory T cells is to be appreciated. Third, the development of appropriate and adequate immune competence as a result of contact with microbes might be essential not only in establishing a nonatopic immune responder phenotype but also in protection against infectious and autoimmune disease. The extended hygiene hypothesis provides a sound rationale for the use of probiotics in supporting immunologic maturation and reducing the risk of disease during a critical period of immunologic development.⁵⁴

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