

Microbial Components as Modulators of Mucosal Immunity

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The Human Microbiome

Beneficial microbes and probiotics may modulate mucosal immunity, and our evolving understanding of the human microbiome will provide fundamental insights into key aspects of pediatric nutrition. The human gastrointestinal tract contains complex microbial communities composed of bacteria, yeasts, and viruses. One study highlighted the fact that the human intestine contains between 800 and 1000 bacterial species, with >60% of DNA sequences representing previously unknown microbes, respectively.¹ Even the skin of the human forearm, a region of the body with a less complex microbiota, may contain more than 180 bacterial species.² In addition to compositional data, microbial communities may be structured in three-dimensional space. Published studies by Swidsinski et al^{3,4} highlighted the point that specific microbes are not randomly distributed in space, but instead, microbes are differentially distributed in different locations within the intestinal tract. Specifically, staining by fluorescence in situ hybridization of the human colon revealed that *Bacteroides fragilis*, *Eubacterium rectale*, and other bacteria inhabit discrete zones in successive layers within the intestinal lumen.⁴

Dynamic fluctuations and microbial population shifts within the human microbiome likely occur in the neonatal period and infancy, and in association with changes in nutritional status or exposure to antibiotics. Palmer et al⁵ demonstrated that the composition of intestinal microbial communities varied widely among infants during the first year of life, with complex communities existing by 1 week of age.⁵ Despite temporal variation, distinct features of each infant's microbial community were recognizable for months at a time. At the end of the first year

of life, each infant's gut bacterial population achieves adult-like complexity and a state of relative equilibrium. Thus, the first year of life provides a "window of opportunity" for directed manipulation of the composition and aggregate function of the human microbiome.

Beneficial Microbes and Probiotics

The probiotic concept was described in a treatise by Elie Metchnikoff, noted immunologist, microbiologist, and Nobel Laureate entitled *On the Prolongation of Life: Optimistic Studies* and published in 1907.⁶ However, this concept essentially lay dormant during the 20th century and throughout the "golden era" of antibiotics and vaccines for the treatment and prevention of infectious diseases. The rise in the number of multidrug-resistant pathogens and the recognition of the role that human microbial communities play in health and disease has generated a recent expansion of interest in probiotics. This phenomenon is apparent in both the numbers of probiotic products now marketed to consumers and the increased amount of scientific research occurring in probiotics. Evidence for the renewed interest in probiotics exists in the revival of the probiotic concept in the last 2 decades by Roy Fuller⁷ and a group of scientists working on behalf of the World Health Organization.⁸

Probiotics are living microbes that exert a variety of beneficial effects on the host when consumed in adequate amounts. Beneficial effects are broadly defined by design and may include antipathogenic effects, immunomodulatory features, regulation of cell proliferation, the ability to promote normal physiologic development of the mucosal epithelium, and enhancement of human nutrition. Commensal microbes may actively prevent gastrointestinal infections through production of antimicrobial factors, stimulation of the host immune system, or competition with pathogens for nutrients or host-binding sites (Fig 1).

[Fig 1]

Bacteria Modulate Immune Responses

Several studies have shown that bacteria can regulate innate and adaptive immunity. Studies from our own laboratory showed that secreted factors from lactobacilli could block cytokine production by mouse and human cells.⁹ Cytokines are key proteins that lead to the proliferation or migration of specific types of immune cells, and these proteins also may affect adaptive immunity by influencing antibody production. By developing laboratory assays that evaluate the relative abilities of candidate probiotics to suppress cytokine responses, our laboratory identified vast differences between microbes and their abilities to stimulate or block immune responses.¹⁰ These laboratory tests were used to select specific bacterial strains that were administered to mice in order to explore the abilities of candidate probiotics to regulate immune responses in animals.¹¹ The lessons that emerged from these studies included the recognition that *Lactobacilli* may suppress intestinal inflammation despite the presence of complex microbial communities in the intestine. Laboratory data indicated that the *Lactobacilli* secreted factors (possibly a combination of factors shed or secreted by bacteria) that regulated immune responses.

In addition to dampening cytokine production and suppressing inflammation, bacteria may stimulate immune responses. Several strains of *Lactobacilli* were found to stimulate signaling and cytokine production by immune cells. Furthermore, new studies in our laboratory have found that probiotics may enhance intestinal antibody responses and boost the immune defenses to infectious challenges. These effects by microbes may be due to secreted factors or substances associated with cell surfaces that ultimately stimulate immune responses.

Mechanisms of Immunomodulation by Probiotics

New insights are emerging from various studies that suggest that very small organic molecules are secreted by a variety of beneficial microbes and probiotics. In our own studies, we have uncovered folate derivatives that seem to confer anti-inflammatory features. Other candidate compounds include fatty acids that appear to be secreted by probiotics and are associated with anti-inflammatory effects. Other laboratories have documented that supernatants from bacterial cultures may have potent immunoregulatory effects,¹² and small molecules from different bacterial strains confer immunosuppressive or anti-inflammatory effects. Work from Brent Polk's laboratory at Vanderbilt has documented the production of specific proteins by *Lactobacilli* that regulate the proliferation and programmed cell death of intestinal epithelial cells.¹³ A different group found that cell-wall components (lipoteichoic acids) from *Lactobacillus plantarum* have immunoregulatory effects.¹⁴ Probiotics and commensal bacteria also produce vitamins (eg, vitamin B₁₂ and vitamin K) and essential nutrients (amino acids and folates) that may have important functional consequences for immune function. A variety of microbial compounds have emerged as candidate immunoregulatory molecules that may contribute to the role of the microbiome in the development and maintenance of a healthy immune system.

Bacteria Regulate Key Immune Signaling Pathways

Beneficial microbes and probiotics can regulate key signaling pathways in different types of immune cells. In our own studies and in studies from other labs, bacteria have been shown to block the activation of transcription factors that may regulate key immune response genes such as cytokine genes. Probiotics secrete or shed organic factors that somehow transmit signals to immune cells (intestinal epithelial cells, myeloid cells, and lymphocytes), and these signals regulate pathways inside cells that ultimately result in reprogramming gene expression. Recent

papers from our laboratory highlight the ability of probiotics to block different immune signaling pathways depending on the nature of incoming immune signals (Fig 2).^{15,16}

[Fig 2]

Our latest model suggests that intestinal bacteria provide signals that stimulate immune responses, and probiotics can suppress these signals by effectively diminishing cytokine gene expression. Some cytokines are produced, and these cytokines provide signals to other immune cells. However, probiotics also may block the signals from these human proteins, thereby further regulating immune responses via a two-step mechanism. So beneficial microbes and probiotics have evolved patterns of immunoregulation by production of immunomodulatory factors. These factors may be identified as key microbial components of new functional nutrition strategies in infants and children.

Conclusion

The human microbiome includes many microbes that together may have a fundamental impact on the development and healthy functioning of mucosal and systemic immunity. As the intestine contains a complex immune system with different types of immune cells, directed manipulation of the human microbiome with probiotics and their secreted components may influence immune function in infants and children. Microbes may stimulate immunity and enhance the body's defense mechanisms against infectious and dietary challenges. In addition, microbes may suppress inflammation and limit the impact of allergies and chronic inflammatory disorders. Microbe: host interactions in the gastrointestinal tract provide opportunities for directed

manipulation of immunity by nutritional strategies that affect the composition and function of the human microbiome.

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Figure 1

Beneficial Roles of Microbes in the Mammalian Intestine

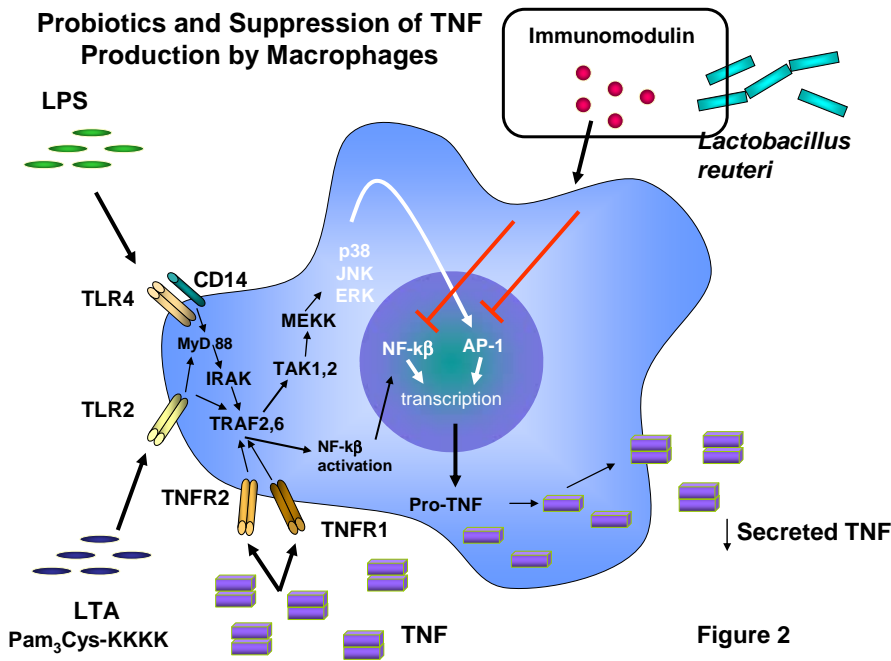
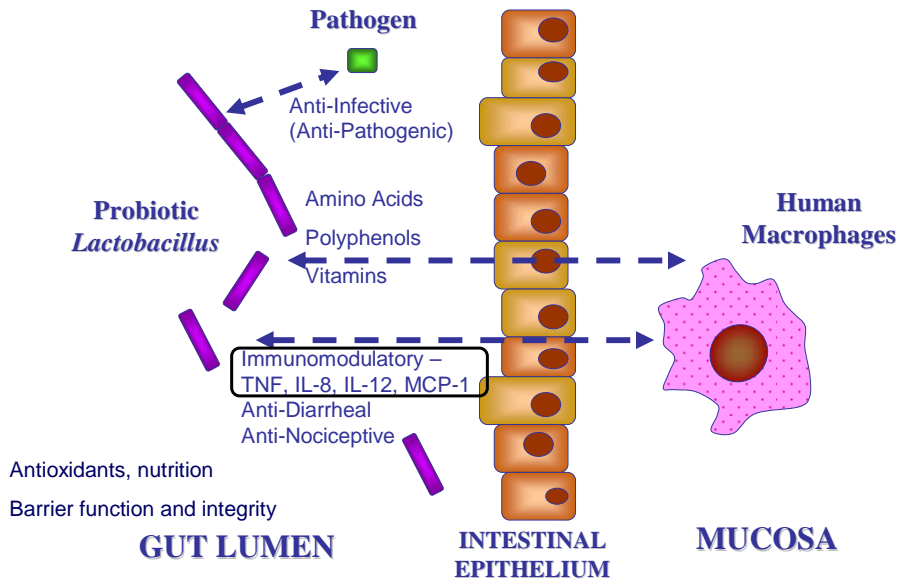


Figure 2

Q & A

Q: I was intrigued by the result of injecting the probiotic. Did you compare the effect of injection to that of oral administration?

Dr Versalovic: No. I just brought up the point that investigators have explored the impact of different routes of administration on systemic immunity. In our mice studies, we used only orogastric gavage.

Q: But there are data on the effect of injecting probiotics, and I am intrigued by whether injection reproduces the results you get with oral administration. Can you comment on that?

Dr Versalovic: Clearly, the route of administration may have an effect. The oral route of administration has enabled us to see important effects on mucosal immunity, but these effects often are localized to the mucosal immune system. In our rotavirus model using orogastric gavage, for instance, we do not see the induction of rotavirus specific IgA systemically. Other routes of administration may affect systemic immunity more broadly. A number of studies have been done using subcutaneous routes.

Q: Dr Versalovic, you demonstrated that *L reuteri* interfere directly with the signal pathway in inflammatory responses, which involve NF-kappaB in terms of kinases and so on. Can we infer that, in the present, these bacteria, which can suppress inflammatory responses, may interfere with immune responses?

Dr Versalovic: If you are asking whether the presence of certain bacterial strains present early in life may suppress the development of immunity, that may be true. Looking ahead, I think we may find important differences in the composition of that microbial community very early in life that may be partly determined by the mother's genotype, the breast milk her infant gets, or by a combination of the infant formula and the mother's genetic makeup. We know the host's

genotype has a major impact on bacterial composition in the gut. We knew that when we studied IL-10-deficient animals. We saw a complete deficiency in *L reuteri* in animals that simply lacked the function of a single gene, albeit an important one, in the immune system. The isogenic animals that were IL-10-intact had plenty of *L reuteri*. So the question is, is it the deficiency of the microbe that predisposes the animals to disease? That is likely, but we also know from a variety of studies that the host genotype has an impact. Some infants are less capable of fostering the proliferation of certain classes of bacteria; in effect, their immune system does not develop as well as that of other infants. As we begin to do metagenomics and community analyses, we may be able to introduce certain organisms early on in an infant's life to compensate for deficiencies. However, we have to be careful which probiotic strain we select. Roughly 10% of the strains we examined had potent activity. If we use a very potent anti-inflammatory probiotic, we may cause more harm than good.

Q: You mentioned that some of these strains were isolated from breast milk. I am a bit skeptical. Do you know how this was done? Was the strain actually from breast milk or from skin contaminants?

Dr Versalovic: We know breast milk is not sterile. A number of studies published in the past few years have documented that breast milk does have a bacterial component. *Bifidobacterium* is more common than *Lactobacillus*. Currently, we are not performing microbiome studies on breast milk, although that is on the table. There may be some skin and surface contamination in such studies, although these groups of organisms are not commonly found on the skin. A recent interesting paper in *Pediatrics* suggested that commensal bacteria may translocate through the intestine through a leaky barrier in the third trimester of pregnancy. Also, the mouse model suggests that there may be a leaky barrier late in pregnancy, and certain commensal microbes

may attach to leukocytes and get into the breast milk. This is controversial, but populations of some organisms have been cultured directly from breast milk.

Q: I am reassured to hear that the strains that you have isolated in your lab are stable and do not change. However, do you know whether several kinds of strains appear in breast milk, or just one or two in each woman?

Dr Versalovic: We do not know enough about this yet. We are just beginning to understand the nature of the microbiome and glycome in breast milk. New prebiotics will be characterized, and at this point the stability of these bacterial populations is just conjecture.

Q: Were the samples you use isolated by you and your colleagues?

Dr Versalovic: No. The ones I alluded to were isolated by Ivan Casas and his group in Peru. He also did microbiology in this field with a group in Finland.

Q: Were these bacteria cultivated before or after nursing was started? Contamination from the mouth to the infant is an important issue.

Dr Versalovic: That is a good point. There is always some potential surface contamination. I think we are getting a greater appreciation of the fact that microbial communities are in breast milk, and the fact that *Lactobacillus* and *Bifidobacterium* are there suggests that infants are getting these beneficial microbes from mother's breast milk.

Q: Dr Versalovic, you mentioned Andy Neish's work looking at NF-kappaB suppression with non-pathogenic strains of *Salmonella*, and you said that your work showed similar data using monocytic macrophage cell lines. Which cell population do you think is being targeted? Is it important that you target epithelial cells and macrophages? Has anyone looked at dendritic cells?

Dr Versalovic: Dendritic cell data are sparse. Some studies have examined effects of probiotics on dendritic cells with respect to different patterns of cytokine production. Probiotics may have

targeted effects on macrophages. Epithelial cells have been studied in more depth. Clearly, epithelial cells are a primary target. We have examined cytokine production, and we are beginning to explore signaling pathways in epithelial cells in more detail. These microbes are producing very small molecules, which I think are targeting multiple cell types. I think that the molecules get through the epithelial barrier fairly easily; they are small products. We have other “candidates” I did not share today, such as very small fatty acids. Other small organic compounds that we picked up by mass spectrometry are specifically found in organisms that suppress NF-kappaB signaling.

Many small factors affect epithelial cells, but they also get through the tight epithelial barrier and may affect dendritic cells. I think these factors get through the wall and affect macrophages and lymphocytes in the lamina propria. In that way, commensal bacteria are directly regulating immune responses, beyond just affecting epithelial cells.

Q: Do you anticipate that these immunomodulants or these polyglutamates are actually produced by commensal flora other than the *L reuteri*?

Dr Versalovic: Yes. We do think that a variety of bacteria produce them. Ultimately, we are interested in metabonomics, so we are interested in the metabolites that these communities produce, which could include a variety of other compounds. So in the end, we may be interested in, not just the metabolites, but the metagenome and the functions that are encoded by that metagenome.

Q: In the experiments in which *L reuteri* increased IgA antibody, did the course of diarrhea change, such as a decrease in rotavirus?

Dr Versalovic: Yes, we saw a reduction in duration of the diarrhea, whether the mouse pups got either strain—TNF inhibitory or TNF stimulatory. These findings were obtained strictly with the

murine rotavirus model. We have not studied other etiologies of acute gastroenteritis in the mouse model.