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## Probiotics in Disease Prevention and Treatment

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### Abstract

Few treatments for human diseases have received as much investigation in the past 20 years as probiotics. In 2017, English-language meta-analyses totaling 52 studies determined the effect of probiotics on conditions ranging from necrotizing enterocolitis and colic in infants to constipation, irritable bowel syndrome, and hepatic encephalopathy in adults. The strongest evidence in favor of probiotics lies in the prevention or treatment of 5 disorders: necrotizing enterocolitis, acute infectious diarrhea, acute respiratory tract infections, antibiotic-associated diarrhea, and infant colic. Probiotic mechanisms of action include the inhibition of bacterial adhesion; enhanced mucosal barrier function; modulation of the innate and adaptive immune systems (including induction of tolerogenic dendritic cells and regulatory T cells); secretion of bioactive metabolites; and regulation of the enteric and central nervous systems. Future research is needed to identify the optimal probiotic and dose for specific diseases, to address whether the addition of prebiotics (to form synbiotics) would enhance activity, and to determine if defined microbial communities would provide benefit exceeding that of single-species probiotics.

### Keywords

allergy; diarrhea; immunology; lactobacillus; microbiome; regulatory T cells

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Do I contradict myself?

Very well then I contradict myself

(I am large, I contain multitudes.)

Walt Whitman, in *Song of Myself*, 1892

Probiotics have been defined as “live organisms that when administered in adequate doses confer a health benefit to the host”<sup>1</sup> (FAO/WHO 2002) and are included in a number of fermentable foods, pills, powders, and liquid drops. Common probiotics are available in pharmacies, groceries, and online in the United States. They include but are not limited to *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Bacillus coagulans*, *Bacillus clausii*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Streptococcus thermophilus*, *Escherichia coli* strain Nissle

1917, and yeasts, including *Saccharomyces boulardii* and *Saccharomyces cerevisiae*. Many probiotics contain mixtures of 2 or more individual species. Prebiotics are defined as metabolic substrates that promote the growth and/or activity of beneficial microorganisms, usually in the gastrointestinal tract.<sup>2</sup> Although their definition has been debated and modified, the general consensus is that prebiotics are nondigestible by human gastrointestinal cells. Prebiotics include oligosaccharides, resistant starch, and soluble or insoluble fibers. Synbiotics are defined as mixtures of probiotics and prebiotics that beneficially affect the host by impacting the microbiome within the gastrointestinal tract.<sup>3</sup>

Each of the above definitions is loosely applied in most countries by the various food and pharmaceutical industries that produce these products, as they attempt to take advantage of the protean health advantages being documented by probiotic research. For example, many probiotic yogurts are marketed to promote human health. Live cultures are present in cheese, kimchee, kombucha tea, and miso soup. There are even probiotics designed to be administered to domestic pets. Prebiotics are added to formulas, cheese, and even juices. However, an improvement in health as a result of the selective stimulation of the growth of a defined population of intestinal bacteria—which is implied in the definition of prebiotics—is difficult to verify and is rarely done. Finally, prebiotics (typically fructooligosaccharides or inulin) are often added to probiotic foods at a low concentration to minimize gastrointestinal symptoms. Because of these low concentrations (<10%), there is most often no evidence to support an additive, synergistic, or even measurable effect of the product.<sup>3</sup> Strong legislation is needed in order to authorize health claims for only selected strains with health-proven benefits.

The human microbiome refers to our commensal microbiota (bacteria, fungi, archaea, viruses, and protozoans), their genes, and gene products. The microbiome is of great interest to all researchers in medicine, affecting each major organ system, including even the cardiovascular system and the central nervous system (the latter leading to the term *psychobiome*). Humans harbor  $10^{14}$  microbes, compared to  $10^{13}$  of our own cells, and there are 1000 to 1500 unique species that colonize the human colon, of which the average person has approximately 160 species, contributing to about 3% of the human body mass.<sup>4</sup> It seems ridiculous that one could consume a single probiotic at doses of  $10^8$  to  $10^{10}$  colony-forming units (CFU) daily and expect that it could produce an effect on a health outcome. Evidence, however, is provided herein that shows that, indeed, probiotics at these doses do have proven health benefits in humans, and we will provide the evidence and potential mechanisms.

## Evidence From Human Trials: Absent, Evolving, or Conclusive?

Few treatments for human diseases have received as much investigation in the past 20 years as probiotics. In the past single year, PubMed records 792 clinical trials of probiotics for human conditions. In examining the evidence for treatment efficacy, we are reminded that the strength of evidence (from weakest to strongest) is: case series → case control studies → cohort studies → randomized controlled trials (RCTs) → systematic reviews, and → (finally) meta-analysis. In 2017, English-language meta-analyses totaling 52 studies determined the effect of probiotics on conditions ranging from necrotizing enterocolitis and colic in infants to constipation, irritable bowel syndrome, and hepatic encephalopathy in

adults (Table 1). Other studies focused on serum lipid levels, late-onset sepsis in preterm infants, blood glucose and hemoglobin A<sub>1C</sub> levels in people with type 2 diabetes, and disease activity in ulcerative colitis. Each of the above studies demonstrated efficacy of the probiotic for the condition studied. Importantly, there were also a few negative meta-analyses. Probiotic efficacy was not demonstrated in studies investigating the prevention of urinary tract infection, reducing the risk of developing bronchopulmonary dysplasia or retinopathy of prematurity, or in helping to eradicate bacterial vaginosis.

The authors have summarized the strongest evidence in favor of probiotics in the prevention or treatment of 5 disorders: necrotizing enterocolitis, acute infectious diarrhea, acute respiratory tract infections, antibiotic-associated diarrhea, and infant colic. We will briefly discuss these entities.

### Netrotizing Enterocolitis (NEC)

NEC is the scourge of premature infants and neonatologists. It affects 5% to 10% of infants with birth weight between 500 and 1500 g, and approximately 50% of these infants require surgery.<sup>5</sup> Mortality rates range from 20% to 30%, and NEC is the leading cause of short bowel syndrome in children. In a multicenter prospective 4-year trial, Warner et al<sup>6</sup> studied the normal progression of microbial colonization in preterm infants and the disruption of this process prior to the onset of NEC. They found that during the first 60 days of life, infants not developing NEC experienced an expansion of fecal Negativicutes (composed largely of *Veillonellaceae*), whereas those developing NEC saw a reduction in Negativicutes and a more than doubling of the composition of Gammaproteobacteria (largely gram-negative aerobic bacilli) (Figure 1). Thus, NEC would appear to be a classical disease associated with gastrointestinal dysbiosis. (Dysbiosis is defined as the condition of having an abnormal microbial community, either in or on the body, as opposed to a disease caused by a single pathogen, such as *Salmonella* or *Helicobacter pylori*.)

There have been at least 3 meta-analyses showing that probiotics prevent NEC. In 2012, Wang et al reported a meta-analysis of 20 RCTs in which probiotics were given individually to prevent NEC in very preterm infants. There were approximately 3700 infants included in studies from 1997 to 2011, and there was little heterogeneity, even though various probiotics were studied. They found that the relative risk ratio of developing NEC was 0.33 (95% CI, 0.24-0.46), indicating a powerful preventative effect. In 2017, 2 larger meta-analyses were published,<sup>7,8</sup> both showing efficacy in preventing NEC. The largest analysis (n = 7345 infants) showed a similarly reduced odds ratio of developing NEC in probiotic-treated infants (0.36, 95% CI, 0.24-0.53). It also showed efficacy in preventing death of these infants in studies focusing on multiple strain probiotics, but not in those using a single strain.<sup>7</sup> One limitation has been that these studies of probiotics in preventing NEC investigated a number of different probiotic preparations, and the best strain is unknown. To our knowledge, no studies have been completed in the United States. Safety issues in this highly vulnerable population are still of concern to the US Food and Drug Administration (FDA), which categorizes probiotics in the same group as vaccines. Preclinical and clinical trials are needed because of critical host/bacterial crosstalk in the very early stages of life, at the time it may be possible to permanently modify the quality of potentially invasive bacteria by an

optimal local immune response. Looking at immunologic and microbiota effects of selected species in exclusively breastfed infants could be beneficial. *L. rhamnosus* GG and *L. reuteri* are among the best candidates. However, caution should be taken not to exceed doses of  $10^9$  CFU in premature neonates because of the risk of bacterial translocation.

With these safety considerations in mind, it nevertheless seems virtually certain at this point that a probiotic will be of major benefit in preventing NEC, especially when the infant is of very low birth weight (<1000 g). A quality improvement project in which a neonatal intensive care unit gave the probiotic *L. reuteri* to every premature infant admitted to the hospital reported significant and sustained improvements in the outcome of their babies after the new protocol was implemented, including most importantly a 6-fold lower risk of NEC.<sup>9</sup>

### Acute Infectious Diarrhea

Acute diarrhea is usually viral in origin, and acute diarrheal infections peak in children between the ages of 6 months and 2 years old. Incidence is directly related to water purity, sanitation, and hygiene. Infants with acute diarrhea represent a group at high risk of diarrheal dehydration, the second-leading cause of mortality in children worldwide, with approximately 700,000 deaths annually.<sup>10</sup>

Probiotics have been studied for more than 30 years for children with acute diarrhea. The most widely studied probiotics are *L. rhamnosus* GG and *L. reuteri*. Recently, Szajewska et al<sup>11</sup> summarized 15 studies of *L. rhamnosus* GG for acute diarrhea and concluded that *L. rhamnosus* GG reduces the severity of purging and the duration of diarrhea by approximately 1 day and is optimally effective at doses  $10^{10}$  CFU. Using meta-analysis, Szajewska et al<sup>12</sup> found that *L. reuteri* at a lower dose was effective in reducing the duration of diarrhea by approximately 1 day.

Deaths caused by diarrhea appear to be decreasing worldwide, most likely the result of improved hygiene, breastfeeding, and the rotavirus vaccine,<sup>10</sup> although recent refugee crises may negate these benefits. However, the ability to reduce the duration of a 3- to 4-day illness by 1 day, as well as the severity of purging, by feeding a probiotic represents a significant advance. In fact, 1 academic hospital (Cincinnati Children's Hospital) modified the hospital order set for children admitted with acute diarrhea to include *L. rhamnosus* GG as part of the treatment regimen; implementation of this quality improvement measure rose from approximately 1% to 100% of children admitted.<sup>13</sup> The impact on outcomes of such measures will be of interest to all.

### Upper Respiratory Infections

All probiotics induce an immune response, and probiotics increase immunoglobulin A (IgA)-secreting cells in respiratory and gastrointestinal mucosae.<sup>14</sup> Day care center studies showed that consuming a daily probiotic by healthy children resulted in an approximately 25% reduction in the number of days of school missed.<sup>15</sup> Systematic reviews of probiotics have shown that there is a reduction of the severity of symptoms associated with probiotics and a shorter duration of respiratory tract infection by approximately 1 day.<sup>16</sup>

### Antibiotic-Associated Diarrhea (AAD)

Antibiotics are the most often prescribed medicines for children, with more than 50% of all children <18 years old receiving at least 1 course.<sup>17</sup> The most widely prescribed antibiotics are amoxicillin, azithromycin, and amoxicillin/clavulanate. AAD is a very frequent side effect of antibiotic therapy, affecting approximately 11% of all children who receive antibiotics and 18% of those <2 years old.<sup>17</sup> Initially, a meta-analysis of AAD by Hempel et al included 63 RCTs and 11811 subjects. The majority used *Lactobacillus* as the study product. Results showed that the relative risk of developing AAD when taking a probiotic was 0.58 (95%CI, 0.50-0.68;  $P < .001$ ). However, there was heterogeneity in the pooled results. They concluded that probiotics are associated with a reduction in AAD; however, more research is needed to determine the best probiotic.

A more recent meta-analysis, extracted from 30 trials in China involving more than 7000 participants included 21 trials that focused on children. The probiotic always included *Bifidobacilli*, often in combination with other probiotic(s). Results confirmed the efficacy of probiotic prophylaxis, with an odds ratio of developing AAD of 0.34 (95%CI 0.23-0.43,  $P < 0.01$ ) in the children receiving a *Bifidobacillus*-containing preparation—a powerful impact.

### Irritable Bowel Syndrome (IBS)

Abdominal pain and IBS, collectively called functional bowel disorders, are the most common conditions leading to consultation by the pediatric gastroenterologist, and they affect about 10% of all school-aged children, similar to the prevalence in adults. There may be some regional variability; for example, IBS has been identified in 13% of children in China, 10% in the United States, and 6% in Sri Lanka.<sup>18</sup> In adults, the prevalence of IBS is 18% in Latin America, 8% to 10% in North America and Asia, and 6% in Africa and the Middle East.<sup>19</sup> IBS is defined by the Rome Criteria: recurrent abdominal pain at least 1 day weekly for >3 months that is (1) related to defecation, (2) a change in stool form, and/or (3) a change in stool frequency.

The etiology of IBS is multifactorial and includes a genetic predisposition, a reaction to life stresses, and/or the presence of an environmental trigger (such as infectious diarrhea). Several groups have identified an altered microbial community, as an additional and potentially central factor, in the feces of patients with IBS. The most notable patient population was those individuals with IBS characterized by diarrhea<sup>20,21</sup> (Figure 2). These patients clearly have a distinct microbial population, characterized by reduced microbial diversity, reduced butyrate-producing organisms (a metabolite that enhances gut barrier function), and reduced methane producers (which dispose of gas).<sup>21</sup> These observations make IBS a notable disease for targeted microbial manipulation.

Many RCTs have now been performed to determine if probiotic(s) are effective in patients with IBS. Ford et al<sup>22</sup> from the United States and Canada identified 43 RCTs involving >3000 volunteers and showed that the relative risk (RR) of having persistent IBS symptoms was 0.79 (95%CI, 0.70-0.89) while taking probiotic. A recent update from China looking at 21 RCTs showed similarly an association between probiotic ingestion and an improvement in IBS symptoms, including an improvement in quality of life.<sup>23</sup> The 2 studies differed in

that one concluded that there were better results with a single probiotic, whereas the other found similar results with single- and multiple-organism probiotics.

Recently, there have been a number of RCTs that analyzed individual probiotics and showed efficacy in subjects with IBS, allowing meta-analysis. These probiotics included *S. cerevisiae* CNCM1-3856<sup>20</sup> and *B. infantis* 35624.<sup>24</sup> The latter probiotic was effective only when used as a member of a multiorganism probiotic. In summary, individuals with IBS were found to respond to probiotics with a reduction in some symptoms, such as flatulence, abdominal pain, and constipation, but without change in other symptoms, such as bloating or urgency. *L. reuteri* benefitted children with functional abdominal pain, with a reduction in frequency of episodes and intensity of pain.<sup>25</sup> Probiotics can benefit children with IBS, but with a lesser “effect size” compared to other treatments, such as low-dose antidepressants.<sup>26</sup> There are important remaining questions, such as magnitude of effect, optimal dose, safety in vulnerable populations, and the most effective species and strain of probiotic.

### Infant Colic

Defined as crying plus fussing for more than 3 hours daily, infants usually develop this condition between 3 weeks and 3 months of age.<sup>27</sup> Colic affects up to 10% of normal infants. Crying occurs throughout the day but peaks in the hours between 5 and 11 PM<sup>27</sup>; this crying can be quantified by a “Barr diary,”<sup>28</sup> which often shows increased crying after feedings. Colic may be a factor in child abuse and infanticide.<sup>29,30</sup> In one investigation of 112 cases of abusive head trauma to infants, forensic interrogation revealed that shaking of the infant was violent and repetitive in most cases. The parent, usually a father, reported that he shook the infant in order to stop the baby from crying in 63% of cases, not intending to hurt the baby.<sup>31</sup>

Theories to account for the etiology of colic include parental psychological stress and inadequate resources to help them cope, cow milk protein allergy, the fourth-trimester theory (which postulates that the infant would prefer to stay in the protected “warm environment” of the amniotic fluid), and the inflammation-dysbiosis theory. Whereas there is some information to support each of these theories in a subset of patients, our data and the data of others suggests that dysbiosis and gut inflammation are reproducible findings. Savino et al,<sup>32,33</sup> from Torino, Italy, found increased *Escherichia coli* and reduced lactobacilli; Rhoads et al<sup>34</sup> identified increased *Klebsiella* and reduced microbial diversity in infants with colic in Houston, Texas; while Partty et al<sup>35</sup> reported increased *Bifidobacterium breve* in infants with colic in Turku, Finland. Using a microarray technique and prospectively following a defined newborn population in Nijmegen, Holland, de Weerth et al<sup>36</sup> found that the “top 10% of crybabies” harbored increased Proteobacteria, a group that would encompass both *Klebsiella* and *E. coli*. Two studies showed reduced lactobacilli,<sup>32,35</sup> and 2 studies (1 our own) have shown elevated fecal calprotectin, a marker of gut inflammation.<sup>34,37</sup>

With these considerations in mind, at least 5 published studies have now investigated the role of a single probiotic, *L. reuteri* to alter the course in infants with colic. The preparation was originally isolated from a Peruvian mother’s breast milk, cured of an antibiotic-resistant plasmid, and is now provided as liquid drops in sunflower oil. Two meta-analyses concluded that in breastfed infants with colic, quantified “crying + fussing time” was reduced by

approximately 1 hour per day within 2 weeks of administering the probiotic,<sup>38,39</sup> with no significant side effects. However, 2 studies in formula-fed infants failed to show an advantage of probiotic over placebo.<sup>40,41</sup> The negative findings could be related to a different probiotic that was studied, the “moving target effect” with the crying time decreasing with age naturally, a lack of optimal prebiotic in formula (eg, human milk contains oligosaccharides, which are prebiotics), or to an inadequate sample size. Currently, infants with colic are typically treated with acid blockers, which are ineffective,<sup>42</sup> overused,<sup>43</sup> over-dosed, and may contribute to cause small intestinal bacterial overgrowth.<sup>44</sup> A new and safe treatment, such as *L. reuteri*, represents a welcome addition to the armamentarium of the pediatrician.

### Allergic Diseases

The primary purpose of the immune system is to protect the host from the ever-changing microbes. In doing so, it has to balance a double-edged sword between defense against emerging organisms and tolerance to nonpathogenic antigens/allergens. This duty is challenging, and the immune system must recruit alliances in this never-ending battle. An obvious ally would be probiotics to help in the regulation between inflammation and allergic responses. The important role of microbes in regulating allergic development is supported by the hygiene hypothesis, and epidemiological data shows less susceptibility to allergic diseases in people living in rural and developing countries.<sup>45,46</sup> Because of these data, there has been great interest in harnessing probiotics to prevent or treat allergic diseases such as asthma, eczema, and food allergy.

With the exception of atopic dermatitis, data from meta-analyses have revealed insufficient evidence for probiotics in preventing the development of asthma, allergic rhinitis, or food allergy.<sup>47,48</sup> With regard to preventing or reducing the severity of atopic dermatitis, there is evidence suggesting a benefit of probiotics, but this effect is variable and not consistent between studies.<sup>49–51</sup> In one recent meta-analysis examining 17 studies, results showed that when mothers were treated along with their infants with a probiotic, the infant had a significant reduction in RR for developing eczema compared to controls (RR, 0.78 [95% CI, 0.69-0.89];  $P < 0.001$ ), particularly those supplemented with a mixture of probiotics (RR, 0.54 [95% CI, 0.43-0.68];  $P < 0.001$ ).<sup>52</sup>

Thus far, the most intriguing benefit of probiotics is in their potential adjuvant effect for oral immunotherapy (OIT) of food allergy.<sup>53</sup> In a recent double-blind, placebo-controlled randomized trial of the probiotic *Lactobacillus rhamnosus* CG MCC 1.3724 in conjunction with peanut oral immunotherapy in 56 children (1-10 years old) with peanut allergy, 82% achieved a sustained unresponsiveness to peanut challenge, versus 3.6% that were given placebo ( $P < .001$ ).<sup>54</sup> However, a definitive conclusion on the beneficial effect of the probiotic cannot be determined, because the trial lacked a control group receiving oral immunotherapy without the probiotic. Unfortunately, probiotics have failed to produce significant therapeutic efficacy for the treatment of allergic rhinitis and asthma in RCTs.<sup>55,56</sup>

Although current data from clinical trials indicate some beneficial effects of probiotics in the prevention and treatment of allergic diseases, the results are far from being robust enough to warrant a universal recommendation for their therapeutic usage. Clearly, there may be

benefit in the prevention of atopic dermatitis (eczema). The outcomes from clinical trials with probiotics can be confounded by the different strains tested. Their activities and potency might vary among patients and their microbiota. It is conceivable that a specific strain of probiotic might be more viable and functional in the intestinal microbiome of one person and less so in another. There are many factors besides the interplay between different microbes that can impact the functionality of probiotics, such as the patient's diet, immune system, mucosa, and emotional makeup. Another important consideration is the route of administration. Instead of ingestion, the probiotics might be more effective if delivered via nasal spray for allergic rhinitis, inhalation for asthma, or topically for atopic dermatitis. It is clear that more research is needed. The key is in deciphering the different mechanisms of how probiotics contribute to immunoregulation and homeostasis at the mucosal level.

## Preclinical Trials and Mechanism of Action in Inflammatory Diseases

A number of mechanisms whereby probiotics regulate inflammation have been identified using animal models of inflammatory diseases (Figure 3). Important evidence attesting to the essential function of beneficial microbes come from studies of germ-free mice. Germ-free mice have reduced intestinal surface area, thin villi, increased cell-cycle time, a poorly developed intestinal microvasculature, and impaired peristalsis.<sup>57</sup> Therefore, one must conclude that the resident microbial population contributes significant nutritional and local benefits to the host.

### Probiotics Inhibit Pathogenic Bacterial Adhesion

Adhesion of pathogenic bacteria to mucosal surfaces mediated by the interaction between bacterial adhesins and specific mucosal receptors is considered to be the first step of intestinal infection.<sup>58</sup> Some probiotics have the property of competitive exclusion, by which probiotics adhere to the intestinal mucosa to prevent the subsequent attachment of pathogens.<sup>59</sup> For examples, specific *Lactobacillus* strains were found to inhibit the adhesion of pathogens such as enterotoxigenic *E. coli* to porcine enterocytes,<sup>60</sup> diarrheagenic *E. coli* to human intestinal epithelium Caco-2 cell lines,<sup>59</sup> and *Salmonella typhimurium* to intestinal mucus.<sup>61</sup>

### Probiotics Enhance Intestinal Barrier Function

A hyperpermeable epithelial barrier in the gastrointestinal tract is proposed to be a major cause of chronic inflammation. Probiotics enhance the structure and function of intestinal epithelial barriers, including increasing mucin production, enhancing tight junctions, and modulating signaling pathways that affect cell proliferation and survival.<sup>62,63</sup> Under normal physiological conditions, goblet cells continually produce mucins to replenish and maintain the mucus barrier; however, goblet cell function can be disrupted by various factors (such as microbes, microbial toxins, and cytokines) that can affect the integrity of the mucus barrier. This occurs in various pathological conditions such as chronic inflammatory diseases.<sup>64</sup>

*L. plantarum* (strain 299v) has the capacity to enhance the production and secretion of mucins (MUC2 and MUC3) from human intestinal (HT-29) epithelial cells.<sup>65</sup> Probiotic mixtures also increase MUC2 gene expression and mucin protein secretion in rat colon.<sup>66</sup> *L.*



*rhamnosus* GG–derived soluble proteins (p40 and p70) ameliorate intestinal injury and inflammation by inhibiting epithelial cell apoptosis<sup>67</sup> and increasing mucin production through transactivation of the epidermal growth factor receptor.<sup>68</sup> Some probiotics have been demonstrated to protect tight junctions by changes in tight junction–related proteins, such as zonulin-1, occludins, and claudins, and by enhancing the electrical resistance of tight junctions contained in the apical junction complexes between adjacent polarized epithelia.<sup>69</sup> Feeding *Lactobacillus rhamnosus* GG early in life to newborn mice enhanced epithelial cell proliferation, differentiation, tight junction formation, and mucosal IgA production.<sup>70</sup> Other cellular and molecular mechanisms such as the release of metabolites and bioactive molecules, suppression of oxidative stress, interference with inflammatory pathways, and augmentation of the levels of mucosal IgA help protect and repair epithelial barriers.<sup>71</sup>

### Probiotics Modulate Both Innate and Adaptive Immune System

One of the most powerful effects of probiotics is to modulate the immune system. Probiotics strengthen both innate and adaptive immune responses through bacterial-epithelial-immune cell crosstalk.

**Toll-Like Receptor Signaling.**—Probiotics act as ligands for innate immune system receptors such as Toll-like receptors (TLRs) expressed on the intestinal epithelial cells and mucosal immune cells to influence important signaling pathways including nuclear transcription factor nuclear factor-kappa B (NF- $\kappa$ B), mitogen-activated protein kinases, phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), and peroxisome proliferator–activated receptor- $\gamma$  (PPAR- $\gamma$ ) pathways.<sup>62,63</sup> Studies of our group demonstrated that human-derived *L. reuteri* DSM 17938 significantly increased survival and decreased the incidence and severity of experimental NEC via inhibition of TLR4 and NF- $\kappa$ B signaling in the intestine of newborn rats. This probiotic effect resulted in decreased gut secretion of tumor necrosis factor- $\alpha$  and interleukin 1 $\beta$  (IL-1 $\beta$ ).<sup>72</sup>

We further observed that the beneficial regulation of *L. reuteri* DSM 17938 in intestinal inflammation during NEC is mediated by TLR2, which recognizes cell wall components of gram-positive bacteria. Recent studies reported that certain lactobacilli can protect from experimental colitis via mechanisms involving TLR2 and cyclooxygenase 2 (*L. rhamnosus* GG),<sup>73</sup> TLR2-dependent induction of regulatory T cells (Tregs) (*L. casei* Lbs2),<sup>74</sup> and TLR2-dependent inhibition of NF- $\kappa$ B signaling by macrophages (*L. paracasei*).<sup>75</sup> Other studies using models of IBD also showed that probiotics can prevent activation of the NF- $\kappa$ B, resulting in decreased secretion of the chemokine IL-8, which is a potent neutrophil chemoattractant.<sup>63,76</sup>

The effects of various probiotics on the host are strain specific. Some probiotics (eg, *Bifidobacterium lactis* BB12 and *Bacteroides vulgatus*) are able to activate NF- $\kappa$ B and increase levels of the proinflammatory cytokine IL-6,<sup>63</sup> but this could also be beneficial, as suggested by current evidence indicating that a low level of inflammation could have physiologic benefits, including maintenance of the epithelial barrier and priming of immune responses. Immunostimulatory phenotypes of probiotics or commensal bacteria might serve the important purpose of promoting host defense against pathogens.

**Immune Cells.**—Dendritic cells (DCs) are embedded within the epithelium (Figure 3), and they contain projections reaching into the lumen to sample bacteria. These cells can travel to mesenteric lymph nodes and beyond. Probiotics modulate DCs, which, in turn, influence T-cell populations. Various microbial factors interact with different DC surface pattern recognition receptors (such as TLRs) to determine DC maturation and subsequent DC-regulated differentiation of naive T cells into T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 or Tregs.<sup>77</sup> Intestinal DCs are central to maintaining immune tolerance in the gut, ultimately by generation of tolerogenic T-cell responses toward food antigens and the commensal microbiota, preventing unnecessary inflammation and hypersensitivity.<sup>63,78</sup>

It is known that a probiotic can be taken up by DCs, facilitating the maturation of DCs and the induction of Tregs.<sup>63,79</sup> Tregs are critical for establishment of immune homeostasis and maintenance of tolerance,<sup>80</sup> which is supported by evidence that, in humans, Treg deficiency due to mutations in the *Foxp3* gene results in a rare condition called *immune dysregulation, polyendocrinopathy, and enteropathy, with X-linked inheritance* (IPEX syndrome). IPEX syndrome is associated with eczema, severe enteropathy, type 1 diabetes, thyroiditis, and inflammation in multiple organs.<sup>81</sup> A critical role of Tregs in preventing colitis and IBD has also been supported by published murine studies.<sup>82,83</sup> Weitkamp et al<sup>84</sup> demonstrated that the proportion of Tregs was significantly decreased in premature infants with NEC. We studied an experimental NEC model and demonstrated that adoptive transfer of Tregs attenuated the severity of NEC, suggesting a role in controlling excessive inflammation.<sup>85</sup> Our studies subsequently showed that oral administration of the probiotic *L. reuteri* DSM 17938 significantly increased the percentage of gut-derived CD103<sup>+</sup>DCs and Foxp3<sup>+</sup>Tregs while diminishing the percentage of inflammatory T-effector cells (including T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17) in the intestinal mucosa of newborn mice during NEC.<sup>86,87</sup>

Intestinal CD103<sup>+</sup>DCs are critical for gut homeostasis. Acting in concert with conditioned mucosal CD103<sup>+</sup>DCs are dietary components (vitamin A), lipid ligands for PPAR- $\gamma$  and aryl hydrocarbon receptor (AhR), intestinal epithelial cell-produced transforming growth factor- $\beta$  (TGF- $\beta$ ); thymic stromal lymphopoietin (TSLP); retinoic acid; and enteroendocrine cell-produced neurotransmitters (vasoactive intestinal peptide).<sup>88</sup> These conditioned CD103<sup>+</sup>DCs are subsequently capable of inducing Foxp3<sup>+</sup>Treg cell differentiation. The CD103<sup>+</sup> receptor on DCs helps to modify its own paradigm in the sense of tolerance through an upregulation of different cytoplasmic proteins such as indoleamine 2,3-dioxygenase. As a consequence of this upregulation, the DC in close contact with the naive CD4<sup>+</sup>T cell will costimulate its cytotoxic T-lymphocyte-associated protein 4 receptor to promote Treg induction.<sup>89</sup> CD103<sup>+</sup>DCs also drive the induction of  $\alpha$ 47 integrin and CCR9 on newly generated Tregs, which makes them home to gut-associated lymphoid tissue.<sup>90</sup>

In Treg deficiency-induced autoimmune disease, called the scurfy mouse (a mouse model of human IPEX syndrome), effector T cells proliferate out of control, resulting in T<sub>H</sub>1- and T<sub>H</sub>2-driven autoimmune disorders. In the mouse, as in the human, there is early-onset dermatitis, progressive multiorgan inflammation, and death within the first month of life caused by a lymphoproliferative syndrome.<sup>91</sup> Oral administration of *L. reuteri* DSM 17938 markedly prolonged survival and reduced symptoms; treatment also reduced multiorgan inflammation by inhibiting T<sub>H</sub>1 and T<sub>H</sub>2 cells and their associated cytokines.<sup>92</sup>

Different probiotics influence the development and survival of different types of Tregs including T<sub>H</sub>3, Tr1, CD4<sup>+</sup>CD25<sup>+</sup>, CD8<sup>+</sup> suppressor, and  $\gamma\delta$ T cells,<sup>63</sup> and assist the secretion of immunomodulatory cytokines including IL-10 (which can strongly suppress T<sub>H</sub>1-driving inflammation) and transforming growth factor- $\beta$  (which can promote development of Tregs) by other cells.<sup>93</sup> These observations suggest that probiotics have a widespread role in maintaining intestinal homeostasis and the balance between tolerance and reactivity to ingested food antigens and commensal microbes.

In addition to the immunomodulation of probiotics involving DCs and T cells, some probiotic strains have the ability to promote the differentiation of B cells into plasma cells and increase the production of secretory IgA.<sup>94</sup> Secretory IgA provides a defense against pathogens by limiting bacterial association with the epithelium and preventing penetration of host tissue.<sup>95</sup>

### **Probiotics Produce/Promote Bioactive Metabolites That Have Anti-Inflammatory Properties**

Specific probiotic and/or other gut bacteria modulated by probiotics are being found to produce multiple bioactive metabolites with anti-inflammatory properties. We will discuss 4 of these: reuterin, histamine, butyrate, and inosine.

Many human-derived *L. reuteri* strains produce the antimicrobial 3-carbon aldehyde reuterin with broad-spectrum in vitro antimicrobial activity against enteric pathogens and other intestinal bacteria.<sup>96</sup> The vitamin B<sub>12</sub>-dependent production of reuterin occurs when *L. reuteri* ferments the substrate glycerol. Reuterin does not typically interfere with the growth of commensal lactic acid bacteria,<sup>96</sup> but in the setting of *Clostridium difficile* infection (CDI), this compound changes in the composition and function of the microbial community that preferentially targets *C. difficile* outgrowth and toxicity.<sup>97</sup>

Some human-derived *L. reuteri* strains, for example, *L. reuteri* strain ATCC PTA 6475, synthesize histamine, which can suppress inflammation via type 2 histamine receptor activation in the mammalian intestine, resulting in suppression of chronic intestinal inflammation and colorectal tumorigenesis.<sup>98,99</sup>

Probiotics have the ability to ferment certain types of fibers, thus increasing the production of short-chain fatty acids (SCFAs), such as propionate, acetic acid, and butyrate. Short-chain fatty acids play multiple critical roles in host defense and immunity, including anticancer, anti-inflammation, and antioxidant activities, as well as outcompetition of enteric bacterial pathogens.<sup>100</sup> Butyrate is particularly important as an inflammatory modulator, with anti-inflammatory effects on the intestinal epithelial cells, macrophages, and leukocytes. Butyrate interferes with inflammatory signal pathways to regulate cytokine production, inhibits histone deacetylase to regulate expression of numerous proinflammatory genes, and induces the differentiation and expansion of Tregs. Coming from the lumen of the colon, butyrate serves as the primary fuel source for colonocytes and facilitates the maintenance of the epithelial barrier.

In the Treg-deficient scurfy mouse, we showed that *L. reuteri* DSM 17938 prolongs survival and reduces inflammation in multiple organs (Figure 4). *L. reuteri* DSM 17938 restores

levels of the purine metabolite inosine to inhibit Treg-deficiency-induced autoimmunity via adenosine 2A receptors (A<sub>2A</sub>). A key to Treg suppression of T<sub>H</sub>1/T<sub>H</sub>2 cells is an interaction between adenosine produced by Tregs (mediated by a CD39-CD73 pathway) and the A<sub>2A</sub> receptor predominantly expressed on neighboring T<sub>H</sub>1/T<sub>H</sub>2 cells in lymphoid organs such as the gastrointestinal tract. During Treg-deficiency, T<sub>H</sub>1 and T<sub>H</sub>2 cells lose their regulation by adenosine-A<sub>2A</sub>-mediated signaling, resulting in T<sub>H</sub>1 and T<sub>H</sub>2 cell-induced pathology. Inosine, a metabolite of adenosine, is increased by *L. reuteri* DSM 17938, and interacts with the A<sub>2A</sub> receptor to inhibit T<sub>H</sub>1 and T<sub>H</sub>2 development and differentiation (Figure 5). The direct mechanism of this change in inosine level has not been elucidated, but indirect evidence indicates that *L. reuteri* DSM 17938 may promote inosine absorption in the intestine, by improving villus length and increasing the gut levels of equilibrative nucleoside transporters that absorb adenosine and inosine. In addition, the genome of *L. reuteri* DSM 17938 contains the transfer RNA-specific adenosine deaminase gene; adenosine deaminase is required to synthesize inosine.

### Probiotics Impact the Enteric and Central Nervous System

Many studies now provide evidence that probiotics have the capacity to activate specific opioid and cannabinoid receptors in the gut, which could reduce visceral pain by which the strains could be beneficial for patients with IBS and chronic IBD.<sup>101</sup>

### Myths and Mysteries

Probiotics are the *plat du jour* of the 2010s. Sales of probiotic supplements exceeded US\$1.3 billion in 2015, while in Asia the total was the equivalent of \$US15 billion. Whereas a number of health claims are suggested by studies such as those mentioned above, some web sites claim much more. [Probiotics.org](http://Probiotics.org) cites >100 distinct benefits, including improved mental health; reduced dental caries; and beneficial effects in the treatment of lung cancer, pneumonia, and pleural effusion. It also cites reduced risk of cirrhosis, increased burn-healing rate, an antitumor effect in gastric cancer, and a reduced risk of cervical cancer. On the opposite side of these health claims is the FDA, which regulates probiotics as if they were vaccines. In 2016, the FDA stated: “To date, the U.S. Food and Drug Administration has not approved health claims for any probiotic.”<sup>102</sup>

The FDA and the European Medicines Agency have been concerned about safety and quality control, stating: “The current literature is not well equipped to answer questions on the safety of probiotic interventions with confidence.”<sup>102</sup> There is justification for this concern. Safety concerns especially pertain to the newborn. The intestinal microbiota at birth commences when the neonate is colonized with myriad of strains originating from his or her own mother. These microbes aid in correcting the antenatal physiologic immune imbalance, allowing the fetus to be tolerated by the mother. The overall quality of the first colonization by invasive bacteria depends also on environmental influences (mode of delivery, where the birth takes place, perinatal medications, and early source of nutrition). Furthermore, the more invasive strains have a dramatic impact on postnatal immune development, with an optimal response against pathogens but also with an induced immune tolerance to successive selection of commensal microbes and environmental antigens, including dietary antigens.

These very complex immune adaptations between the host and its developing microbiome evolve and appear to reach a stable equilibrium sometime between 2 and 3 years of age.<sup>103</sup> The intestinal physiologic resistance to further microbial colonization is effective at that time. Later in life, probiotics will further help to degrade dietary macromolecules and to modulate host immune responses but only when they are permanently given. As a consequence, probiotics may have more potency in affecting the immune system and ultimate microbiome when given in the early stage. However, absolute safety of giving probiotics at this time of immune immaturity—especially in premature neonates who are clearly immunocompromised—needs to be demonstrated. Safety and efficacy are both notably strain and dose dependent.

Probiotic adverse events include diarrhea, sepsis, subacute bacterial endocarditis, and meningitis, although these are exceedingly rare and not likely to be more frequent after probiotic than after a placebo.<sup>104</sup> Contamination of a marketed product is of great concern, exemplified by the infection of an infant who was given a probiotic (ABC Dophilus) that contained both live bacteria and a *Rhizopus* fungus, the latter of which caused invasive gastrointestinal mucormycosis and death.<sup>105</sup> One probiotic for children, *S. boulardii* (SB), is a yeast preparation isolated from the bark of the lychee fruit. SB has been extensively administered to immunocompromised patients, including premature infants, human immunodeficiency virus–infected adults, and individuals with Crohn disease. SB treatment has been associated with immunological benefits, and SB reduces the severity of infant diarrhea.<sup>106,107</sup> However, SB is not a normal inhabitant of the human gut and will not become a stable member of the microbial community. This strain has been historically registered and marketed based on experimental data from animal studies. By now, there are numerous reports of fungemia (often with SB isolated) in critically ill or elderly patients. 108–110

Below, we describe 6 of the myths and mysteries surrounding the probiotic “lore.”

### 1. All probiotics are similar.

In many of the meta-analyses cited above, different probiotics and combinations were effective, but careful scrutiny in preclinical randomized controlled trials often showed marked differences, even among different strains of the same species. This is likely true because different strains have different mechanisms. For example, as mentioned above, studies in the lab of Versalovic showed that *L. reuteri* strain ATCC PTA 6475 produces vitamin B<sub>12</sub> and histamine; the probiotic reduced tumor necrosis factor- $\alpha$  production via a protein kinase A–dependent mechanism.<sup>111</sup> Subsequently, in our lab, a different strain of *L. reuteri* DSM 17938, which does not produce vitamin B<sub>12</sub> or increase histamine levels, did not reduce gut inflammation in rat intestine *ex vivo*, but prevented NEC<sup>87</sup> and markedly attenuated multiorgan inflammation in the scurfy mouse.<sup>92</sup> The mechanism involved, in contrast, was the activation of an adenosine A<sub>2A</sub> receptor.

Even though mechanisms may differ, it is notable that in all of the meta-analyses noted above, probiotics were either effective in treating the conditions targeted or not deleterious. In none of the studies did the condition worsen. It is possible but seems unlikely that this is

because of publication bias, given the thousands of RTCs that have been reported and funnel-plot analyses showing no bias.

## 2. Probiotics affect diseases only via a placebo effect.

The placebo effect is always worth considering, but the authors note that all the above-cited studies were placebo controlled.

## 3. Probiotics have an enduring effect on symptoms via long-term colonization.

In most trials, the administered probiotic cannot be detected in the stool weeks after discontinuing the probiotic. In some trials, it was difficult to isolate the probiotic even while it is being consumed! In our safety trial of *L. reuteri* in adult volunteers, for example, only low levels of the probiotic could be detected in stool by PCR *during* treatment, and at 1 and 4 months after treatment, there was no more detectable *L. reuteri*.<sup>112</sup> In a study in which we investigated a formula with or without *L. rhamnosus* GG, we could detect an increase in *L. rhamnosus* in the stool with a median value of 4% of total bacterial sequences after 2 weeks of consumption of *L. rhamnosus* GG (using 16S ribosomal DNA sequencing). However, while the infants were continuing to drink the formula, at 42 and 90 days, the population of *L. rhamnosus* decreased to <1%. We suspect that in both studies the probiotics were colonizing the small intestine and/or being outcompeted in the colon. Similarly, with minimal evidence of fecal excretion, daily oral *L. reuteri* evoked observable effects on fecal calprotectin,<sup>112</sup> and in infants *L. reuteri* had potentially beneficial effects on the low neutrophil counts in peripheral blood that were seen in approximately 50% of the infants with colic.<sup>113</sup> There may be probiotics that are able to establish long-term colonization under optimal conditions. For example, Panigrahi et al<sup>114</sup> showed that in term infants in India, supplementation with *L. plantarum* and fructooligosaccharides daily for 7 days resulted in 100% colonization by 2 months, but fewer of these infants (32%) remained colonized by 6 months. It is unclear if these infants remained colonized later in life.

Perhaps a useful analogy for how a small number of probiotics could affect the microbiome of a host is one that gardeners would appreciate. A probiotic is not like a rapidly spreading, colorful, perennial flower, such as *Impatiens walleriana*. Probiotics are more like simple garden clover (*Trifolium*). Clover prevents weeds from forming, retains moisture for the other flowers, fixes nitrogen into the soil, and improves soil tilth (softness).<sup>115</sup>

## 4. Probiotics are inexpensive.

The cost of probiotics is remarkably variable. In the United States, a single product (*L. rhamnosus* GG) costs from \$0.37 to \$1.00 per capsule (*Culturelle*<sup>TM</sup>; i-Health, Inc., Cromwell, Connecticut). A combination of *L. acidophilus* and *B. longum* BB-12 (*Trubiotic*<sup>TM</sup>; Bayer, Leverkusen, Germany) ranged from \$0.30 to \$0.67 per capsule online. A multiorganism probiotic (*Ultimate Flora*<sup>TM</sup>; Renew Life, Palm Harbor, Florida) is sold at concentrations ranging from 25 to 200 billion CFU per pill; online cost ranges from \$0.46 to \$1.40, in proportion to the number of CFU. [Note that the authors are not endorsing these specific products.] Probiotics are not covered by insurance companies or federal medical assistance plans. Thus, it is of concern that, at present, probiotics can be viewed at some level as a *concierge medication*.

## 5. Probiotics are dangerous in patients with severe diseases or in those with immune deficiencies.

Probiotics were originally felt to benefit mild digestive illnesses and were subsequently found to be beneficial in reducing infections in children in day care centers.<sup>116</sup> However, now they have now been found to be beneficial in a number of severe conditions such as ulcerative colitis,<sup>117</sup> hepatic encephalopathy,<sup>118</sup> and rheumatoid arthritis.<sup>119</sup> They are being actively studied in HIV-infected individuals as a benefit to standard highly active antiretroviral therapy.<sup>120–122</sup> No severe adverse events have been identified in these studies that would be attributable to the probiotic. We believe probiotics may be more efficacious in children and adults with severe disorders.

In summary, evidence-based, mechanistic research on probiotics reveals that cultured microorganisms, when given in adequate quantities for sufficient periods of time, are beneficial in many human disease conditions and safer than most pharmaceuticals. These conditions include infantile colic, acute infectious diarrhea, and IBS. Probiotics also can prevent antibiotic-associated diarrhea and NEC. Much more research is needed, and clearly probiotics do have potential risks that need to be monitored. Future investigations are needed to identify the optimal probiotic and dose for specific diseases, first in animal models comparing various strains, and second in RCTs. Animal trials should use multiple “omics” to investigate not only the organisms but their effects on the global fecal community and their key metabolites, establishing mechanism of action. Their impact on the immune system is key to the understanding of how to therapeutically approach the globally increasing incidence of autoimmune diseases, including arthritis and other rheumatologic disorders, IBD, eosinophilic diseases, and multiple sclerosis.

These studies must also address whether the addition of a prebiotic would enhance the therapeutic effect of a probiotic and to determine if the administration of human-derived or synthetic “defined microbial communities” (as opposed to single organism probiotics) would provide additional benefit. Safety considerations will almost certainly favor a probiotic approach over the administration of fecal transplantation, because of lower risks to the host, greater ease of administration, and lower cost of production.

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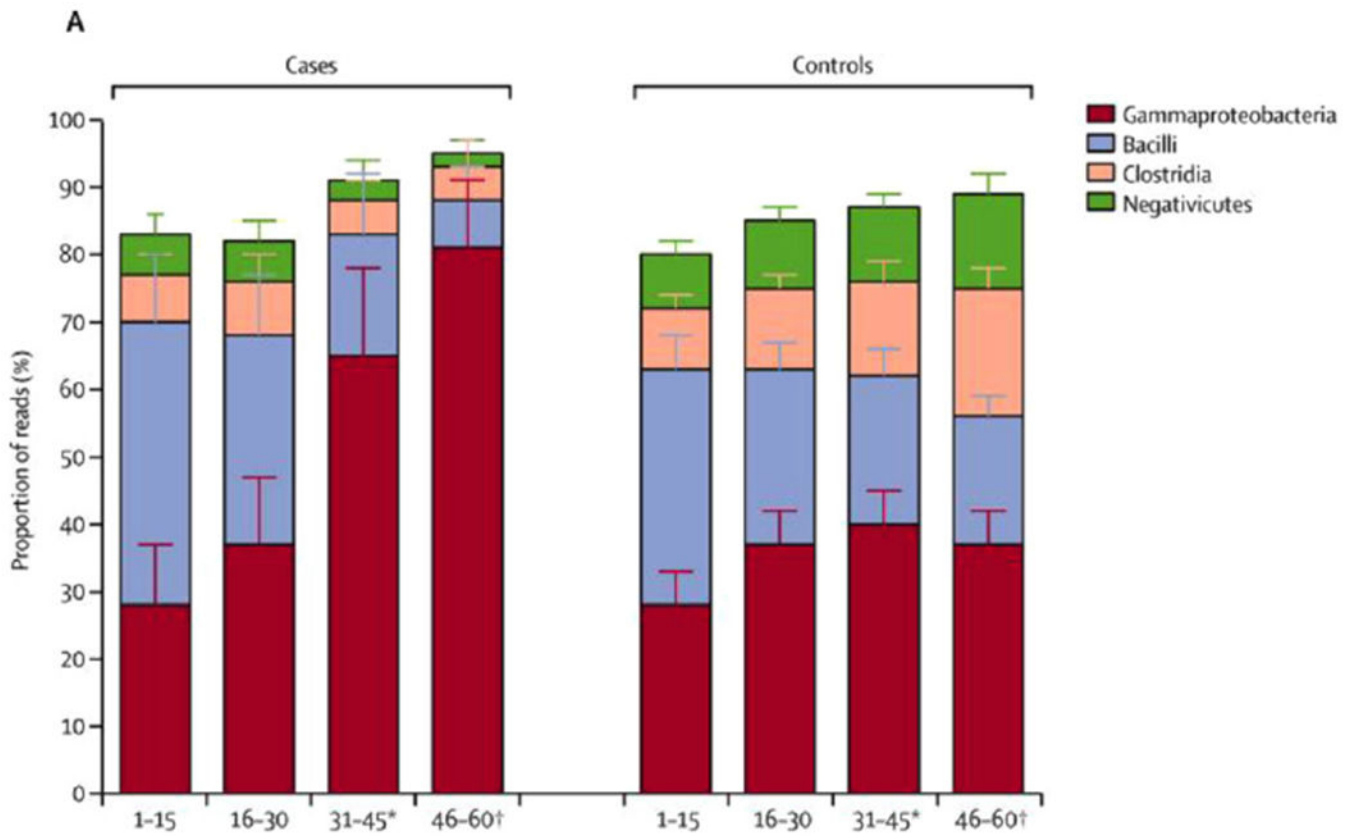
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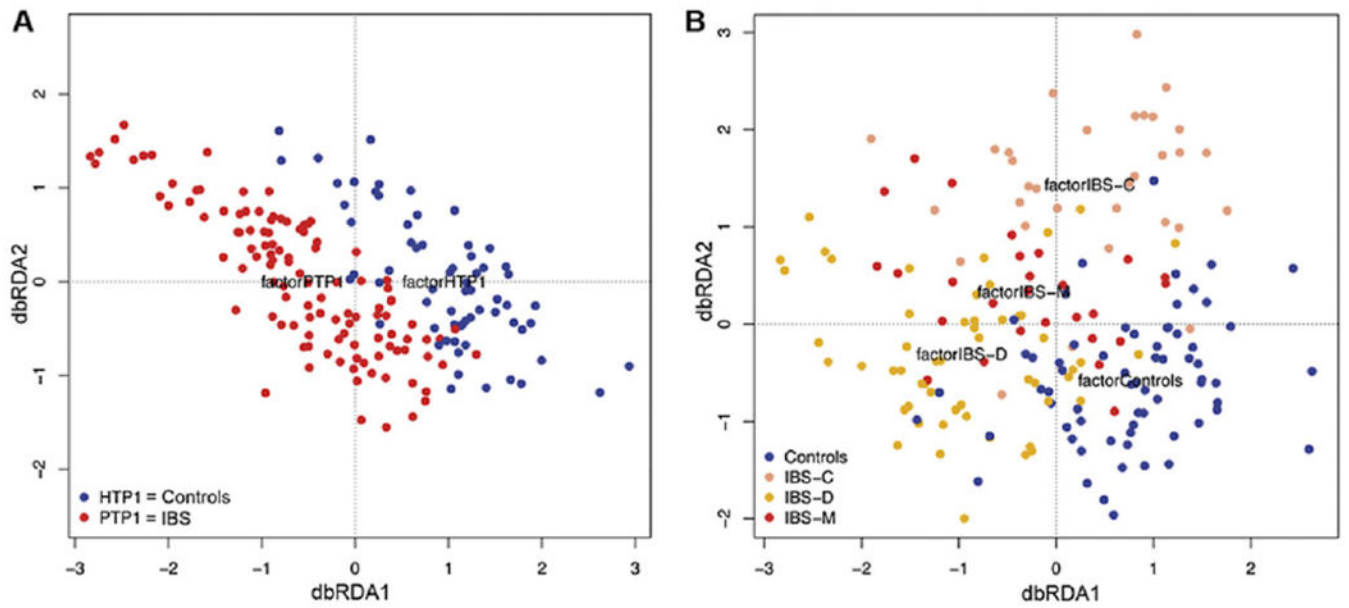
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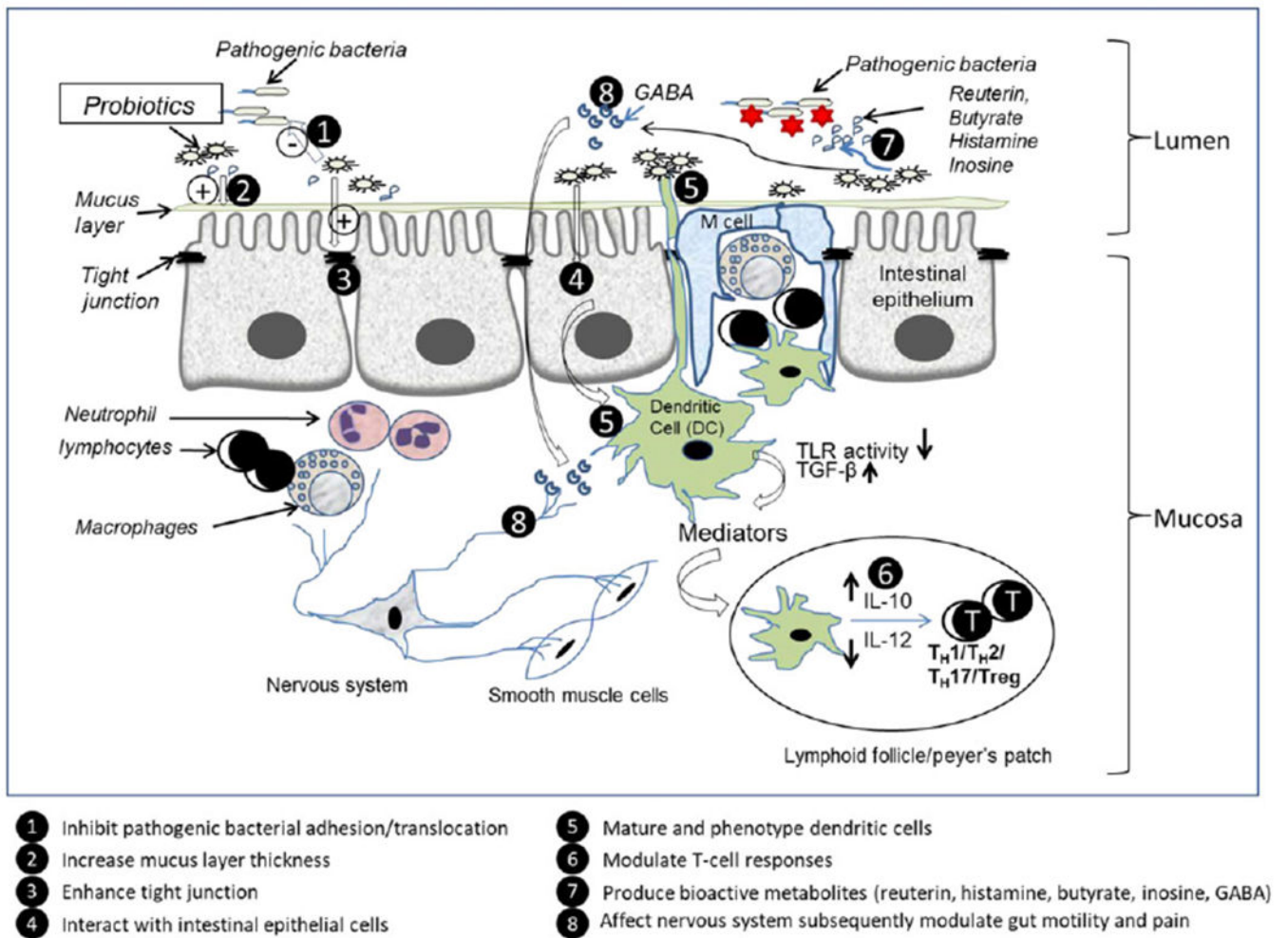


**Figure 1.** Major phyla in prospective multicenter study of microbiota of premature neonates during the first 60 days of life. Bar graphs show evolution toward increased negativicutes in control group, as contrasted to increased gamma proteobacteria in those developing NEC. From Warner et al.<sup>6</sup>

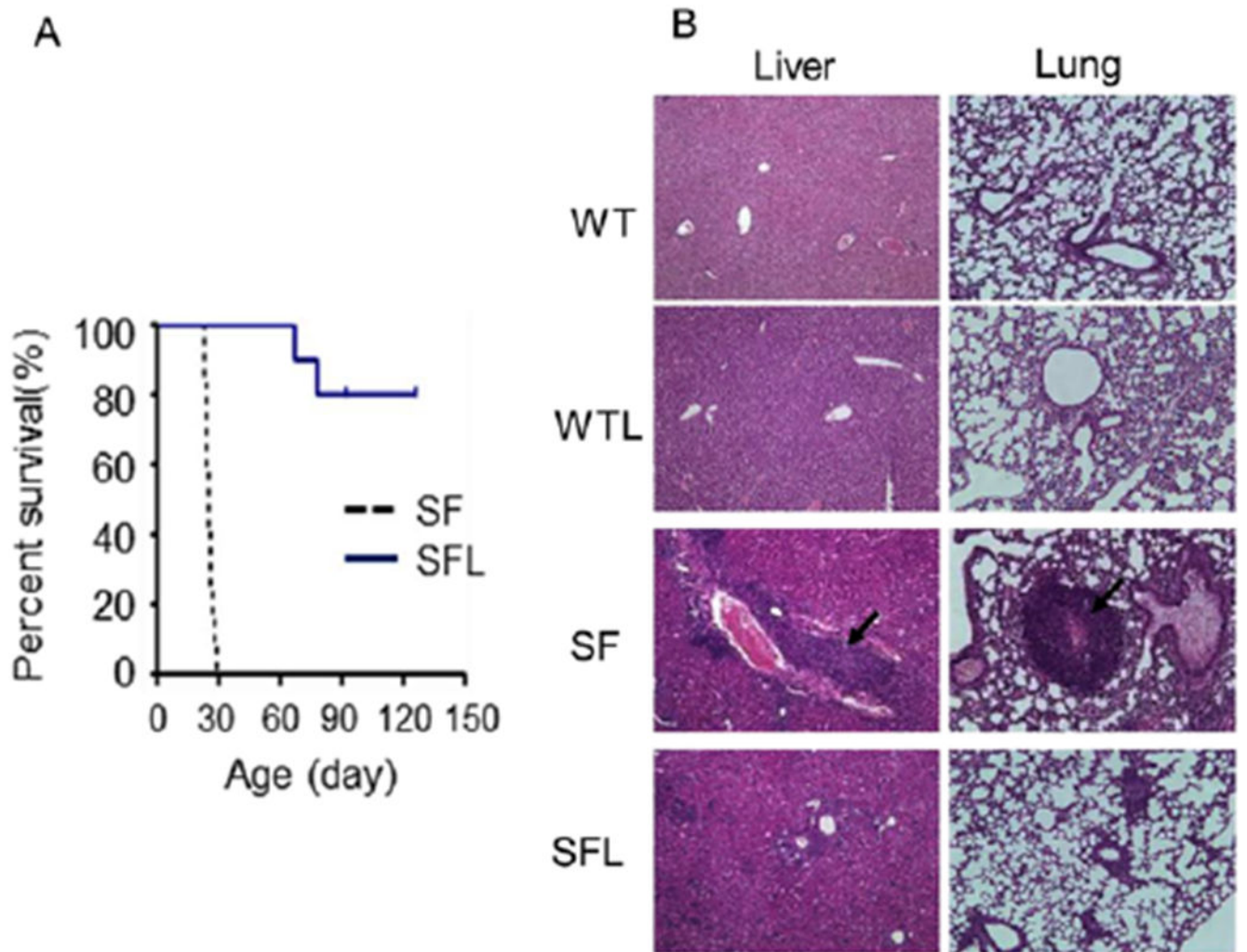


**Figure 2.** Two-dimensional principal components. Microbiota in IBS (A), divided by subgroup (B) in which C refers to constipation, D refers to diarrhea, and M refers to mixed diarrhea and constipation. From Pozuelo et al.<sup>21</sup>

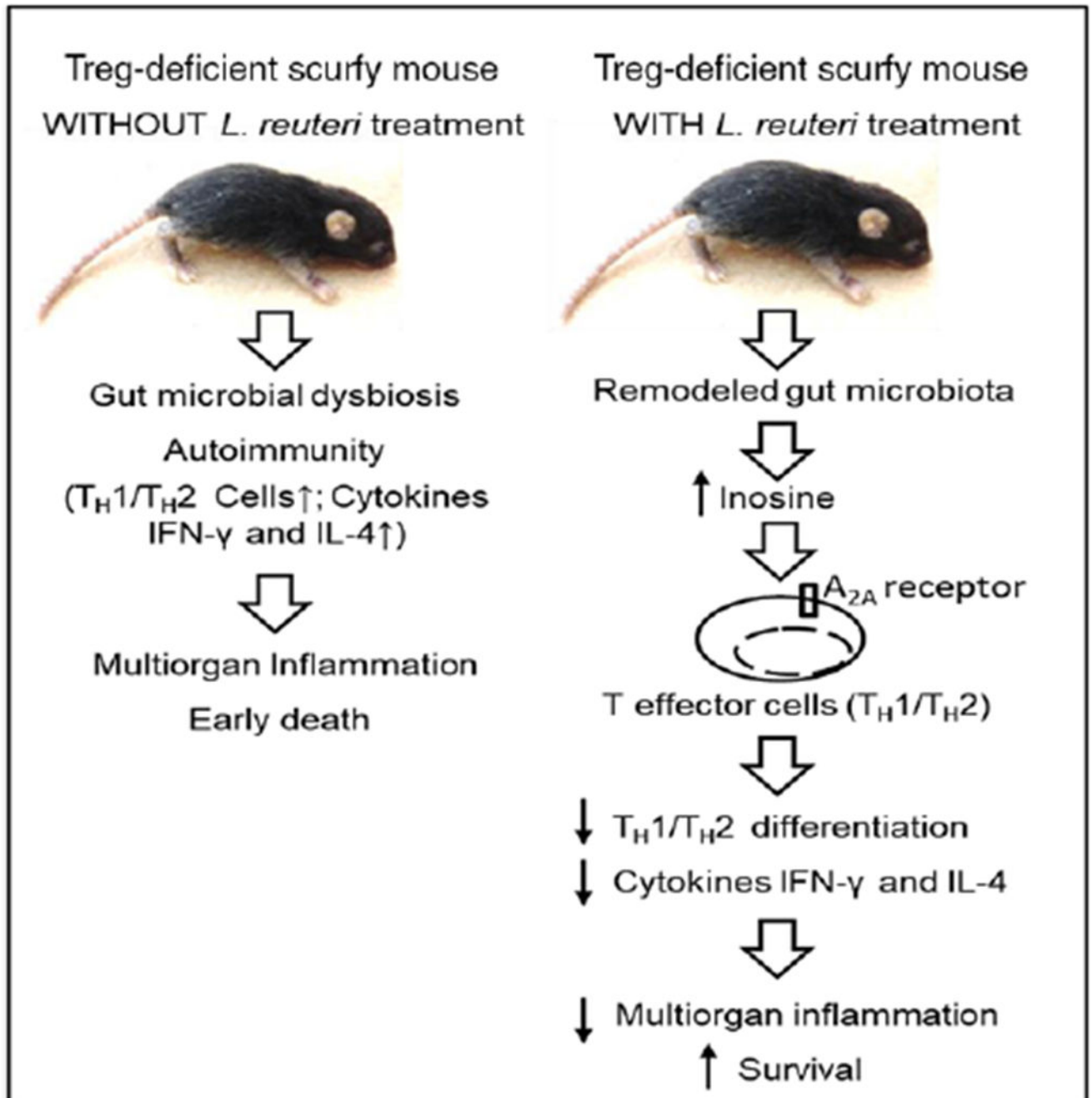




**Figure 3.** Documented mechanisms of action of probiotics. Probiotics may inhibit pathogenic bacterial adhesion, enhance barrier function, and interact with TLRs expressed on the intestinal epithelial cells and dendritic cells to produce cytokines/chemokines to further modulate T cells. Probiotics also can produce bioactive metabolites and affect nervous system subsequently modulate gut motility, reduce pain, and involve in gut-brain function (Illustration by Liu, Y.).



**Figure 4.** *Lactobacillus reuteri* DSM 17938 treatment increases survival (A) and reduces inflammation (B) in organs of SF mice (arrows indicate lymphocyte infiltration). From He et al.<sup>92</sup>



**Figure 5.** Mechanisms of *Lactobacillus reuteri* DSM 17938 protection against Treg-deficiency-mediated autoimmunity. Treg deficiency shapes gut microbiota and induces autoimmunity resulting in multi-organ inflammation and early death (left). *Lactobacillus reuteri* DSM 17938 remodels gut microbiota, alters the metabolites (inosine) and protects against Treg-deficiency-induced autoimmunity by suppressing T<sub>H</sub>1/T<sub>H</sub>2 cells via inosine-adenosine A<sub>2A</sub> interaction (right). From He et al.<sup>92</sup>

**Table 1.**

## Meta-Analyses Reporting Probiotic Efficacy in 2017

Condition	Probiotic	Reference
Abdominal pain in children	Various <sup>a</sup>	123
Antibiotic-associated diarrhea (children)	5 <i>Bifidobacterium</i> preparations	124
Blood glucose and A <sub>1c</sub> levels in type II diabetes mellitus	Various	125
Constipation	<i>Lactobacillus</i> , <i>Bifidobacillus</i> preparations	126
Halitosis	<i>Lactobacillus</i>	127
<i>Helicobacter pylori</i> eradication	Various	128–130
Hepatic encephalopathy	Various <sup>a</sup>	118
Infant colic	<i>Lactobacillus reuteri</i>	131
Infection risk in the critically ill	Various	132
Irritable bowel syndrome	<i>Saccharomyces cerevisiae</i> , <i>Bifidobacterium infantis</i> , combinations	23,24,133
Late-onset sepsis in preterm infants	Various	134,135
Prevention of <i>C. difficile</i> infections in hospitalized patients	Various	136
Prevention of necrotizing enterocolitis	Various	7,8
Prevention of radiation-associated diarrhea	<i>Lactobacillus</i> ± <i>Bifidobacterium bifidum</i>	137
Small intestinal bacterial overgrowth	Various	138
Surgical site infections	Synbiotic	139,140
Total cholesterol and LDL-cholesterol lowering	<i>Lactobacillus reuteri</i> , <i>Lactobacillus plantarum</i>	141,142
Ulcerative colitis	VSL#3, various others	117

LDL indicates low-density lipoprotein.

<sup>a</sup> Cochrane review.