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PROBIOTICS IN AUTOIMMUNE AND INFLAMMATORY DISORDERS

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Abstract: Probiotics have been used to ameliorate gastrointestinal symptoms since ancient times. Over the past 40 years, probiotics have been shown to exert major effects on the immune system, both *in vivo* and *in vitro*. This interaction is clearly linked to gut microbes, their polysaccharide antigens, and key metabolites produced by these bacteria. At least four metabolic pathways have been implicated in mechanistic studies of probiotics, based on carefully studied animal models. Microbial-immune system crosstalk has been linked to short chain fatty acid production and signaling, tryptophan metabolism and the activation of aryl hydrocarbon receptors, nucleoside signaling in the gut, and activation of the intestinal histamine-2 receptor. Several randomized controlled trials have now shown that microbial modification by probiotics may improve gastrointestinal symptoms and multi-organ inflammation in rheumatoid arthritis, ulcerative colitis, and multiple sclerosis. Future work will need to carefully assess safety issues, selection of optimal strains and combinations, and attempts to prolong the duration of colonization of beneficial microbes.

Keywords: Lactobacilli; bifidobacilli; arthritis; inflammatory bowel; microbiome; metabolomics; aryl hydrocarbon reductase; adenosine; histamine; short chain fatty acid

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1. HISTORY OF PROBIOTICS

Health benefits of bacteria were recognized throughout history. Fermented milk was consumed in the Middle East as early as 10,000 BC, followed by populations in Egypt (as evidenced by hieroglyphics), Greece and Italy [1]. Around 8,000 BC, Tibetan nomads living at altitudes > 4000 m maintained good health, despite the absence of fruits and vegetables in their diet, in part by consuming fermented yak milk and its products [2]. Only eight oz. of yak milk daily could provide > 200 billion lactobacilli, mainly *Lactobacillus fermentum* (*L. fermentum*) and *L. casei*! Yak milk also has been found to have free radical-scavenging and anti-inflammatory properties. In ancient Greece and Rome, around 400 BC, a condiment called *garum*, derived from fish intestines, which was (and still is) fermented for 12-18 months in clay pots, was consumed daily, with anti-powerful anti-oxidant properties and reported health benefits [3]. Nomadic Turks used “yogurtmak” to treat diarrhea, cramps, and sunburned skin, as evidenced by writings in the 11th century; and later Genghis Khan, the great Mogul conqueror, fed his army yogurt, because it reportedly “instilled bravery in them.” [4].

In 1905, Elie Metchnikoff of Russia probed the question of why Bulgarians lived so long. He concluded that their longevity was related to the heavy consumption of fermented yogurt, subsequently showing that a bacillus could be grown from the yogurt, which was identical to a bacillus found in their stools, later called *L. bulgaricus* [5]. At the same time, Henry Tissler of Paris isolated from an infant a y-shaped organism that he called *Bifidobacterium*. This bacterium was able *in vitro* to displace pathogenic bacteria. Healthy infants were colonized with the *Bifidobacterium*, whereas less healthy infants did not harbor the organism. Later, in World War 1, many soldiers were dying of diarrheal disease, and the German scientist Alfred Nissle isolated a strain of *E. coli* from a soldier that had *Shigella* in the stool but did not develop diarrhea [6]. The species, which he called “antagonistically strong”, was appropriately called *E. coli* Nissle 1917 and is still used as a probiotic today (called “Mutaflor”).

RECOGNIZED BENEFITS IN THE 1900s

The term probiotic was introduced in 1953 by the German Werner Kollath to mean “active substances essential for a healthy life” [5]. In the 1940’s, most research focused on culturing pathogenic bacteria and developing antimicrobial therapies. In line with this approach, after the 1950s, there was great interest in identifying probiotics that provided colonization resistance to pathogens, and research began to focus on lactobacilli and bifidobacilli to combat diarrheal disease. This research focused on the role of probiotics and “gut health” resulted in convincing evidence that probiotics can prevent and treat infections diarrhea (viral, salmonellosis, shigellosis, cholera) [7] and also facilitates peptic ulcer healing [1].

EXPANDED ROLE, IN INFANTS AND IN PATIENTS WITH GASTROINTESTINAL DISORDERS (2000-2015)

Between 2000 and 2017, there was an explosion of interest in probiotics, with an annual number of randomized controlled trials (RCTs) ranging between 144 and 194; in 2017, there were also 49 meta-analyses. Internationally recognized investigators has spent decades of their lives developing the field of probiotic research. In fact, a growing family of “Prolific Probiotic Proponents” (!) have

contributed more than 60 original high-impact scientific studies dating back to the 1990's; and their seminal contributions are listed in Figure 1.

Four significant conditions will be mentioned that have consistently been shown to respond to probiotics in humans in meta-analysis.

a. Necrotizing enterocolitis (NEC): Research was emerging around 2000 showing that probiotics could prevent necrotizing enterocolitis, a devastating disease of premature infants often resulting in bowel resection and short bowel syndrome. The first meta-analysis by Alfaleh and Bassler was published in 2008, showing benefit of probiotics in 9 trials [8]. By 2017, more than 23 studies in 7,325 infants showed that probiotics reduce the risk of developing NEC. This most recent meta-analysis by Thomas et al. showed that the risk of developing NEC was 3.9% if given probiotic and 6.6% if untreated with probiotic (relative risk of 0.57, 95% CI: 0.43-0.74, $P < 0.0001$) [9]. The problem with these studies was that there were many probiotics studied; and sometimes multiple-strain probiotics were tested; therefore, the optimal choice was not evident. One meta-analysis found that the benefit was restricted to multiple strain probiotics and to lactobacilli [10], while another meta-analysis (oppositely) found, that the benefit pertained only to bifidobacilli and multiple strain probiotics [11]. Both groups found that the yeast *Saccharomyces* was ineffective. Also of concern, the premature population is at high risk for septicemia, therefore safety concerns have until recently led to the U.S. Food and Drug Administration (FDA)'s caution in approving any probiotic RCTs in the United States. Paradoxically, probiotics have been consistently shown to reduce the risk of late-onset septicemia in breast-fed premature infants [12].

Simultaneously with these clinical trials, animal research has powerfully confirmed efficacy of probiotics in preventing NEC, while also establishing possible mechanisms. Dvorak's group showed that *Bifidobacterium bifidum* stabilized the gut barrier via tight junction modification during experimental NEC [13]. Hackam's group showed that NEC is mediated by inflammatory signaling via epithelial cell pattern recognition receptor toll like receptor-4 (TLR4). TLR4 recognizes bacterial lipopolysaccharide and is expressed on gut epithelial cells and immune cells, such as T cells. Hackam et al. showed that the mitigating effects of *L. rhamnosus* HN001 are mediated by anti-inflammatory signaling via TLR9 [14]. Our group showed that protective effects of the probiotic *Lactobacillus reuteri* DSM 17938 (*L. reuteri* 17938) in a mouse model of NEC are mediated by a different Toll-like receptor, TLR2 [15], and its administration to newborn mice and rat pups results in an enhancement of local and peripheral levels of anti-inflammatory regulatory T cells (Tregs) [16].

b. Irritable bowel syndrome (IBS): IBS is defined as recurrent abdominal pain at least one day weekly for > 3 months, which is: (a) related to defecation; (b) associated with a change in stool form; or (c) related to a change in stool frequency [17]. Subjects with IBS have been found to harbor an altered fecal microbial population, with a shift toward reduced microbial diversity and reduced butyrate-producing bacteria. In addition, Pozuelo et al. showed that adults with IBS-C (constipation-predominant) differ from control individuals without IBS - and from those with IBS-D (diarrhea-predominant IBS) [18]. This finding was consistent with many studies of probiotics for patients with IBS. Meta-analyses have shown considerable heterogeneity, largely related to various

144 definitions of symptom severity in IBS and quality of life indicators. However, most meta-analyses
145 have shown efficacy of probiotics in treating IBS [19].
146 The most recent meta-analyses by Ford et al. [19] and Zhang Y et al. [20] showed a decrease in global
147 IBS symptoms of ~2-fold and an improvement in quality of life. Many studies showed improvement
148 in bloating and flatulence in those with IBS. Different probiotics have been studied, and the meta-
149 analyses have shown considerable heterogeneity. Therefore, the role of probiotic in IBS is best
150 described as “evolving but promising.”

151
152 c. Infant colic: Babies who cry and fuss for more than 3h daily have colic. The
153 condition generally starts at 3 weeks of age occurs more than 3 days/week, resolving after 3 months
154 of age (hence the “rule of 3’s” [21]. Infant colic previously was felt to be unresponsive to any
155 treatment. Microbial dysbiosis began to be linked to this condition and was confirmed by several
156 groups [22-24], and it was linked to gut inflammation [25]. Therefore, colic might represent a
157 condition for which probiotic treatment would be useful. Several meta-analyses have shown that
158 probiotic *L. reuteri*, isolated from a Peruvian mother’s breast milk, reduces crying time and irritability
159 in this condition [26-28].

160
161 d. Respiratory infections: Recently, lactobacillus- and bifidobacillus-containing
162 probiotics were found to improve outcomes in acute infectious diseases outside of the gastrointestinal
163 tract, such as upper and lower respiratory tract illnesses in infants and college students [29-32]. In
164 one moderately large multicenter study in Italy, addition of fermenting *L. paracasei* to milk or rice
165 milk resulted in reduce episodes of gastroenteritis, rhinitis, otitis, laryngitis, and tracheitis [33]. This
166 finding suggested that the benefits to the host extend beyond local interactions in the intestinal tract
167 between the gut organisms, enterocytes, and the immune system, perhaps involving microbial
168 metabolites and/or migrating dendritic cells that reach distant locations such as the spleen and lymph
169 nodes. Of additional benefit, probiotics stimulate IgA secretion in the respiratory epithelium in
170 animal models [34]. Currently, several over-the-counter products espouse the benefits of probiotics
171 in treating common upper respiratory ailments.

172
173 **2. EFFECTS OF PROBIOTICS IN HIGH-RISK POPULATIONS WITH IMMUNE**
174 **DYSREGULATION AND AUTOIMMUNE DISEASES**

175 In both animal trials and human trials, probiotics have been investigated for to determine potential
176 beneficial effects in prevention and treatment of a wide variety of systemic conditions. These
177 conditions include inflammatory and autoimmune diseases such as rheumatoid arthritis, ulcerative
178 colitis, multiple sclerosis and hepatic encephalopathy. Advantages of probiotics include regulation
179 of immune system function, which is often dependent on the strain of probiotic bacteria. Some
180 strains have demonstrated stimulation of the immune response, thereby being beneficial to patients
181 suffering from immune deficiencies. Other strains have been shown to inhibit the immune response,
182 thereby being beneficial for patients suffering from conditions with immune activation such as
183 rheumatoid arthritis (RA) [35,36].

184
185 a. Rheumatoid arthritis (RA). Rheumatoid arthritis is a systemic autoimmune disease
186 characterized by autoantibody formation leading to chronic inflammation of multiple joints. RA is

also known to affect other internal organs including the lungs, heart and kidneys [37]. Triggers leading to RA include HLA gene interaction and environmental factors. These environmental factors include smoking, infection and recently dysbiosis [35,38,39]. Early animal models have consistently demonstrated an influence between the gut microbiota and local/systemic immunity as well as activation of joint inflammation [40]. In earlier probiotic studies, investigators were not able to show a significant difference in activity of RA with the use of probiotics [41], but in a more recent study, Zamani et al. reported that probiotic supplementation resulted in improved disease activity scores (looking at 28 joints) in patients with RA, compared with placebo [42]. A study by Chen et al. evaluated the gut microbiota profile in 40 patients with RA and 32 healthy controls. They found decreased gut microbial diversity in RA compared to controls, which additionally correlated with disease duration and with levels of serum rheumatoid factor [43]. Alipour et al showed that *L. casei* 01 supplementation decreased serum high-sensitivity C-reactive protein (hs-CRP) levels, tender and swollen joint counts, and global health (GH) score ($P < 0.05$). A significant difference was also observed between the two groups with respect to circulating levels of IL-10, IL-12 and TNF- α , in favor of the probiotic group [44].

In a recent meta-analysis, Mohammed et al showed that pro-inflammatory cytokine IL-6 was significantly lower in rheumatoid arthritis volunteers treated with probiotics compared to their placebo-treated controls. However, this study did not show an overall difference in clinical symptoms between probiotics and placebo group [36]. Another study by Liu et al., aimed to investigate the human fecal lactobacillus community and its relationship to RA. In comparing quantitative PCR in fecal samples of 15 RA patients and 15 healthy controls, the authors reported increased absolute numbers of *Lactobacillus salivarius*, *Lactobacillus iners* and *Lactobacillus reuteri* in untreated RA patients and suggested a potential relationship between the lactobacillus community and development of RA [45]. Thus, evolving evidence suggests a relationship between altered intestinal microbiota and rheumatoid arthritis, and we anticipate that further studies will be needed to delineate the microbiota profiles, which might contribute to RA and the potential for treatment with adjuvant probiotics.

b. Systemic lupus erythematosus (SLE). SLE is an autoimmune disease involving multiple organs, including skin, joints, kidneys, and central nervous system and is characterized by the formation of high levels of antibodies against double-stranded DNA. SLE is influenced by genetics and environmental factors that is characterized by immune intolerance to self-antigens [46]. In a classic hypothesis regarding the etiology of lupus in 1964, Kingsley Stevens pointed out that polysaccharide-containing antigens were 60-fold more effective stimulators of plasma cell proliferation and antibody formation than were the protein antigens present in vaccines [47]. He went on to propose that “the causative agent in SLE” is bacterial polysaccharide, which must be present in the oropharynx, vagina, or gut.

In humans with SLE, elevated interferon-gamma has been found to be proportional to the fecal firmicutes/bacteroides level, giving credence to Stevens’ hypothesis [48]. In this study, several strains of probiotics were helpful in modulation of excessive inflammatory responses *in vitro*. Both experimental and clinical trials have revealed that selective strains of probiotics (*B. bifidum*, *Ruminococcus obeum*, *Blautia coccoides*, and *L. casei* Shirota) can reduce inflammation and restore tolerance in SLE animal models [49]. There are several mouse models of SLE, for example, the

MRL/lpr mouse that spontaneously develops nephritis. MRL/lpr mice suffer from endotoxemia and increased gut paracellular permeability [50]. Using MRL/lpr mice, researchers found that combinations of lactobacilli or *L. reuteri* alone when given enterally skewed Treg-Th17 balance toward Treg cell dominance, reduced endotoxemia, reduced levels of double-stranded DNA-IgG, improved proteinuria, and better survival. These results were associated with a change in gut microbiota, with expansion of Clostridiales, Lactobacilli, and Desulfovibrionales. In the NZB/W F1 mouse, systemic lupus-like inflammation is characterized by oxidative stress and reduced levels of circulating regulatory (anti-inflammatory) Tregs [51]. Treatment with *L. reuteri* GMNL-263 reduced levels of cytokines and restored Tregs in this model, as well.

At this time, we are unaware of any randomized controlled trials of a probiotic for patients with lupus, but there is evidence that the gastrointestinal tract may be an avenue for disease modification. *In vitro*, probiotic lactobacilli when cultured with immature dendritic cells from lupus patients reduce the expression of costimulatory molecules and increases levels of interleukin-10 and indoleamine 2, 3 dioxygenase (anti-inflammatory molecules), suggesting that they could promote immune tolerance [52]. A pilot study by Frech et al. in a related autoimmune disorder, progressive systemic sclerosis, suggested that probiotics significantly improved esophageal reflux, distention and bloating, and total gastrointestinal symptom scales [53].

c. Inflammatory bowel disease (IBD). IBD, including ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic inflammation in the gastrointestinal tract influenced by several factors, including genetics, epigenetics, gut microbiota and the host immune system [54]. There have been many RCTs evaluating the effects of probiotics in IBD, associated with ample evidence suggesting that altered gut microbiota contribute to the initiation and progression of IBD. It has been well established that VSL #3, an 8-strain probiotic, which includes Lactobacilli, Bifidobacilli, and Streptococcus thermophilus, is effective in UC; however, this and other probiotics were not effective in CD [55]. In 2017, Derwa et al. showed VSL#3 to be effective in inducing remission in active UC and suggested that probiotics may be as effective as 5-ASAs in preventing relapse of quiescent UC [56,57]. In a recent meta-analysis of 27 trials, Ganji-Arejanaki et al confirmed that VSL #3 was effective in UC and showed that probiotics *S. boulardii*, *Lactobacillus* (*L.rhamnosus*, *L. johnsonii*) and VSL #3 were effective in patients with CD who also used corticosteroids [58]. The authors suggested that VSL #3 and *Lactobacillus johnsonii* after surgery for CD might be efficacious if the duration of treatment under study were longer.

Ganji-Arejanaki et al. additionally concluded that in children aged 2-21 with IBD (both CD and UC), lactobacilli (*L. reuteri* ATCC 55730, *L. rhamnosus* strain GG, and VSL #3) confer a significant advantage. The role of probiotics in patients with persistent GI complaints when inflammation cannot be demonstrated, resembling irritable bowel syndrome, remains to be determined. Overall, in inflammatory bowel disease probiotics appear to be safe and promising as adjuvants to standard therapy [56].

d. Multiple sclerosis (MS). MS is a chronic relapsing or progressive disease of the brain and spinal cord characterized by onset in early to middle adulthood with severe, relapsing neurologic deterioration. Many individuals with MS develop sensory loss, weakness, visual difficulties, severe fatigue, and paraesthesia. Key pathological features of MS include axonal loss,

demyelination, gliosis, and a progressive inflammatory reaction of the brain and spinal cord [59,60]. During the course of MS, activated autoreactive T cells have been proposed to differentiate into interferon- γ -producing T helper 1 (Th1) cells) and/or interleukin (IL)-17-producing Th17 cells, which are distributed throughout the CNS and spinal cord [61]. Growing evidence from both rodent and human studies suggests that microbiota within the intestine contribute to the pathogenesis in this disease [62-65]. In a rodent model of MS called experimental autoimmune encephalomyelitis (EAE), two studies showed that alteration of the gut microbiota by oral antibiotic administration reduced the severity of EAE [66,67]. Human studies of MS patients recently showed that the relative abundance of the families *Prevotella* and *Lactobacilli* are decreased compared to healthy controls [63,64]. Similarly, we found in the EAE model evidence for fecal microbial dysbiosis and reduction of *Prevotella* during the disease. We also found that *L. reuteri* improved clinical severity of EAE, shifted the microbial beta diversity, and reduced Th1 and Th17 cytokine levels in the serum and gut [68]. There is one human study suggesting that *L. reuteri* improves symptoms and quality of life in human MS [69]. Thus, evidence from MS in humans and mice provides further evidence of a strong connection between the human brain and gut, with microbes and their products being key mediators of disease severity, while beneficial microbes represent key candidates for disease modification.

3. MECHANISM OF ACTION OF PROBIOTICS

Probiotics have been found to affect every compartment of the gut, including the luminal microbiome, the mucus barrier, the microbe- and cell-free “kill zone,” the epithelium, the lymphocyte- and plasma cell-rich lamina propria, the vascular and neural elements of the lamina propria, underlying smooth muscles, which control motility, and the mesenteric lymph nodes that communicate with the systemic immune system. Probiotic-modulated local and systemic metabolites have been identified which may modify autoimmune diseases (Figure 2).

a. Short-chain fatty acids (SCFAs) in colon. SCFAs, specifically acetate, propionate, and butyrate, are produced by commensal bacteria (such as *Facecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, and *Ruminococcus bromii*) and by many probiotics. Lactobacilli produce SCFAs and pyruvate by fermentation of carbohydrates and heterofermentative processes [70]. Bifidobacteria also use the fermentation to produce SCFA, mainly acetate and formate, during growth when carbohydrates are limited. Bifidobacteria alternatively produce acetate and lactate when carbohydrates are in excess [71]. Various dietary carbohydrates (called prebiotics) can selectively stimulate microbial growth and metabolic activity. A combination of probiotics and prebiotics (called a symbiotic) is powerfully able to shift the predominant bacteria and production of SCFAs. For examples, *L. rhamnosus* GG (LGG) with a mixture of prebiotics produces SCFAs. *Lactobacillus acidophilus* CRL 1014 was also recently shown to increase SCFAs (acetate/butyrate/propionate) when studied in a reactor called SHIME (*Simulatory of Human Microbial Ecosystem*) [72]. Bifidobacteria such as *B. longum* SP 07/03, and *B. bifidum* MF 20/5 produce and release propionate and acetate but not butyrate [73]. SCFAs may have beneficial effects on gut health through various mechanisms. SCFAs play an important role in maintaining metabolic homeostasis in colonocytes, and they protect colonocytes from external harm. SCFAs, especially butyrate, confer protection against the development of

colorectal cancer (CRC) [74,75]. Butyrate promotes colon motility, reduces inflammation, induces apoptosis by inhibition of histone deacetylation, and inhibits tumor cell progression. Evidence points toward SCFA receptors in the colon, which includes both free fatty acid receptors (FFARs) and G-protein-coupled receptors (GPRs). FFAR3 (GPR41) and FFAR2 (GPR43) on colonocytes control motility [76]. SCFAs are able to bind and activate FFAR2 and/or FFAR3 located on intestinal epithelia, inducing glucagon-like protein-1 (GLP-1) and peptide tyrosine tyrosine (PYY) release into the basolateral milieu. Released GLP-1 and PYY activate enteric or primary afferent neurons in pelvic and vagal networks. Neural information travels to the central nervous system (CNS), affecting host metabolic energy expenditure [77]. SCFAs reduce neutrophil cytokine production [78], while reducing macrophage nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling [79], resulting in anti-inflammatory actions. Most importantly, butyrate has the ability to induce the differentiation of Tregs, which control intestinal inflammation [80]. However, the understanding of the underlying molecular mechanisms remains incomplete, mainly due to the lack of data on actual uptake fluxes of SCFAs under different conditions i.e. with different dietary substrates, microbiota, and disease models. Most studies report concentrations of metabolites or transcript levels, but these do not necessarily reflect SCFA flux changes [73].

b. Tryptophan metabolism-Aryl hydrocarbon receptor. L-Tryptophan (Trp) plays crucial roles in the balance between intestinal immune tolerance and activation [81]. Recent studies have underscored those changes in the gut microbiota that modulate the host immune system by modulating Trp metabolism. Trp metabolites include host-derived Trp metabolites, such as kynurenines, serotonin and melatonin, but also bacterially produced Trp metabolites, including indole, indolic acid, skatole, and tryptamine [82].

Trp metabolites are ligands of the aryl hydrocarbon receptor (AhR) [83]. AhR is a cytosolic ligand-activated transcription factor in dendritic cells and T cells. AhR plays a critical role in maintaining gut immune tolerance and barrier function, as evidenced by the finding that AhR-null mice exhibit severe symptoms and mortality in animal models of DSS-induced colitis [84]. AhR^{-/-} mice are more susceptible to intestinal challenge with toxins [85] and pathogens [86]. Studies have identified a critical mechanism of AhR in immune tolerance involving anti-inflammatory IL-22 production which tolerizes intraepithelial T lymphocytes and innate lymphoid cells (ILCs) [87]. Host and bacterial Trp metabolites stimulate AhR and AhR-dependent gene expression including IL-6, IL22, prostaglandin G/H synthase 2 (PTGS2), vascular endothelial growth factor A (VEGFA), cytochrome P450 1A1 (CYP1A1), and mucin 2 (Muc2) in the intestine. These products individually and additively modulate intestinal homeostasis [82].

The effects of indolic acid derivatives from Trp by gut bacteria and probiotics have earned recognition as major metabolic products in this process. Metabolites such as indole-3-acetic acid (IAA), indole-3-aldehyde (IAld), indole acryloyl glycine (IAcrGly), indole lactic acid (ILA), indole acrylic acid (IAcrA), and indolyl propionic acid (IPA) all can impact intestinal homeostasis. For examples, *Clostridium sporogenes* can convert Trp into IPA, which protects mice from DSS-induced colitis [88]. IPA significantly enhances anti-inflammatory cytokine IL-10 production after LPS stimulation and reduces TNF- α production. Probiotic *Bifidobacteria infantis* when given enterally attenuates proinflammatory immune responses by elevating plasma Trp and kynurenic acid levels in rats [89]. Probiotic *Lactobacillus reuteri* in the presence of luminal Trp produces IAld, which is able to activate

ILC3s to produce IL-22 via AhR, contributing to antifungal resistance and mucosal protection from inflammation [90].

In summary, as a therapeutic strategy, probiotic treatment in combination with Trp metabolism can alter the intestinal microbiota, increase the generation of AhR ligands, and ultimately protect the host from intestinal inflammation.

c. TGF- β . Transforming growth factor-beta (TGF- β) is a multifunctional polypeptide with profound regulatory effects, which affect many developmental and physiological processes. TGF- β in the intestinal mucosa is a key immunoregulatory molecule, shown to induce Tregs and to promote B-cell IgA production. One TGF- β signaling pathway activates the transcriptional factors SMAD2 and SMAD3 [91]. SMAD3 is a crucial transcription factor enhancing Foxp3 expression in Tregs. TGF- β induces Foxp3 gene transcription in thymic Treg precursors, and also converts naïve T cells into inducible Treg (iTregs), while protecting Tregs against from apoptosis [92].

Probiotic bacteria have been shown to generate a Foxp3⁺ Treg response in the small intestine. Our study of experimental NEC models demonstrated that orally feeding *L. reuteri* 17938 increases the frequency of Foxp3⁺Tregs in the intestinal mucosa to prevent the development of NEC [16,93]. *Lactobacillus gasseri* SBT2055 induces TGF- β expression in dendritic cells and activates TLR2 signaling to produce IgA in the small intestine [94].

Probiotic VSL#3-induced TGF- β also ameliorates food allergy inflammation in a mouse model of peanut sensitization through the induction of Tregs in the gut mucosa [95]. The administration of *B. breve* to preterm infants also can up-regulate TGF- β 1 signaling and may possibly be beneficial in attenuating inflammatory and allergic reactions in infants [96]. In the setting of infectious enteritis, *L. acidophilus* attenuates *Salmonella typhimurium*-induced gut inflammation via TGF- β 1/MIR21 signaling [97].

d. Nucleosides (Adenosine Signaling). We have identified a novel mechanism of *L. reuteri* 17938 in regulating multiorgan inflammation. *L. reuteri* modifies the microbiota-inosine-adenosine receptor 2A (A_{2A}) axis, which in turn inhibits T_H1 and T_H2 cell differentiation to reduce inflammation in liver, lung, gut, and skin [98,99]. This mechanism was identified in the “scurfy” mouse model in which genetic Treg deficiency induces autoimmune total body inflammation. Foxp3⁺Treg cell deficiency in these mice results in gut microbial dysbiosis and autoimmunity over their entire lifespan. A severe autoimmune disease named IPEX syndrome (immunodysregulation, polyendocrinopathy, and enteropathy, with X-linked inheritance) is the parallel syndrome in humans [100].

Remodeling gut microbiota with *L. reuteri* 17938 markedly prolonged survival and reduced multi-organ inflammation in sf mice. We found that *L. reuteri* 17938 changed the metabolomic profile disrupted by Treg-deficiency; and the predominant change was to restore serum levels of the purine metabolite *inosine*, alongside downstream products xanthine and hypoxanthine. One of the key mechanisms of Tregs is to control inflammatory effector T cells (Tems). Tems include T_H1, T_H2 and T_H17 subsets of T cells; these pro-inflammatory families of T cells are controlled via the interaction of adenosine (produced by Tregs) and the receptor A_{2A}, which is highly expressed on T cells. In the absence of Tregs, the adenosine metabolite inosine at high doses may replace the effect of adenosine to interact with A_{2A} receptor and inhibit T_H cell differentiation. When we fed inosine itself to Treg-

deficient scurfy mice, we observed that inosine prolonged lifespan and inhibited multi-organ inflammation by reducing T_H1/T_H2 cells and their associated cytokines. Mechanically, the inhibition by *L. reuteri* and inosine of the differentiation of T_H1 and T_H2 cells depended on the A_{2A} receptor, which was confirmed by using an A_{2A} antagonist to block A_{2A} receptors [98] and by genetic knockout of the A_{2A} receptor in sf mice [99].

e. Histamine. It is interesting that the tolerogenic effects of Lactobacilli are very strain- and metabolite-dependent. For example, a *L. rhamnosus* strain that secretes low levels of histamine is immunosuppressive [101,102], whereas a *L. saerimneri* strain secreting high histamine levels induces gut inflammation [103]. *L. reuteri* ATCC PTA 6475 (*L. reuteri* 6475), differs from the sister strain *L. reuteri* 17938, in that it has histidine decarboxylase (HDC), enabling it to produce histamine and suppress $TNF-\alpha$ production *in vitro* [104]. Gao et al. showed that *L. reuteri* 6475 has anti-inflammatory effects in the trinitrobenzoate (TNBS) model of colitis via a mechanism dependent on intestinal histamine-2 receptor signaling [105]. A mutant *L. reuteri* 6475 strain lacking histamine conversion genes did not suppress TNBS-induced colitis in mice; furthermore, the anti-inflammatory effect of *L. reuteri* 6475 was dependent on the histamine H-2 receptor [106]. This HDC-dependent gene effect may have bearing on colorectal carcinoma, which is more prevalent in humans deficient in HDC, inasmuch as *L. reuteri* 6475 when administered in the *Hdc*^{-/-} mouse model of colon cancer suppressed tumor size and number [106].

4. “POLARIZATION” WITHIN THE MEDIAL COMMUNITY REGARDING THE USE OF PROBIOTICS

The medical community has not yet endorsed the use of probiotics. In fact, the U.S. Food and Drug Administration has not yet approved any probiotics for preventing or treating any health problem. Despite the numerous evidence-based reviews and meta-analyses cited herein, there are legitimate reasons for caution. Some experts have warned that the rapid growth in marketing of probiotics may have outpaced scientific research for many of their proposed uses and benefits [107]. More concerning is that there have been rare reports of bacteremia with cultures positive for the probiotic administered, probiotic-associated endocarditis, and even death. One notable case involved an infant who developed invasive mucormycosis, leading to intestinal perforation and death, resulting from a probiotic (ABD-Dophilus) which was contaminated with a fungus *Rhizopus oryzae* [108]. However, overall, probiotic groups compared with matched placebo-treated controls have often shown a reduction in sepsis rates –in preterm infants [109,110] and in adults following gastrointestinal surgery [111].

There are other concerns among skeptics.

a. Numerical Skepticism. The argument is sometimes raised, “How can 1-50 billion cfu’s of a probiotic outweigh the effects of 10-75 trillion commensals in the gut?”, noting a 1:1000 ratio of probiotic to commensal bacteria [112]. This numerical consideration is based on an assumption that a probiotic needs to establish itself (colonize) and differentiate in the large intestine. Consider the following: An infective dose of *E. coli* i0157:H7 of only 50 cfu’s is sufficient to cause a potentially lethal bloody diarrhea in humans, leading to the potentially lethal hemolytic uremic syndrome [113]. It is actually remarkable that the previously mentioned body of research does show significant effects

of probiotics in light of the sheer numbers of normal commensal microorganisms. However, the meta-analyses above show strong evidence of probiotic efficacy without significant colonic colonization.

Most studies can show limited recovery of probiotics in the stool [114], but the number of colony forming units (cfu's) for *L. reuteri* are on the order of 1:1,000 of the dose administered and for *L. rhamnosus* GG are only 1:10,000 of the dose administered [115]. Another study showed fecal recovery of orally administered probiotic *L. fermentum*, but as in most studies, there was only a low level of the probiotic in the stool [116]. We have not consistently been able to identify by PCR significant numbers of probiotic in the stools--even while patients are actively on treatment [117,118]. Nevertheless, in our studies of *L. reuteri* we have consistently found significant evidence of recognition by the host of the probiotic, for example a mild elevation in the fecal level of antimicrobial calprotectin (within the normal range) [119], a shift in microbial community composition, and an increase in circulating neutrophil count in infants with colic [120]. We believe a possible explanation lies in the observation most lactobacilli and bifidobacilli are primarily small bowel colonizers, where they exert their immunologic effects.

b. Publication Bias. It is generally recognized that clinical trials with negative findings are hard to publish. For this reason, meta-analyses will often contain a funnel plot, asymmetry of which is a way of determining publication bias [121]. Funnel plots for probiotic studies have generally shown no publication for probiotics in most of the conditions described, such as NEC prevention [122], IBS improvement [123], *H. pylori* eradication [124], and amelioration of infant colic [125].

c. Generalizability of Findings. Some have argued that probiotic may be effective only in a well-defined, narrow population. For example, is there greater efficacy in children vs. adults? Children less than 3-6 years old have an incompletely developed microbiome and may be more responsive to microbial manipulation. The strongest effect size for probiotics has been shown in pediatric studies, for example the effect of probiotics in reducing the incidence of NEC (in the latest systematic reviews, RR 0.55, 95% CI 0.43 to 0.70) or in shortening the course of acute infectious diarrhea (0.67 days, 95% CI, -0.95 to -0.38) [126]. Another concern is whether probiotics may be more or less in different geographical locations, where populations have different dietary habits and differences in microbial exposure owing to differences in hygiene and food storage. This concern is reasonable, and broader meta-analyses including studies from different countries are indicated.

d. Safety in Immunodeficiency States. Finally, there is concern about giving chemotherapeutic agents or immunomodulators along with live microorganisms to patients who are immunocompromised. Children and adults with autoimmune diseases, such as lupus, ulcerative colitis, and rheumatoid arthritis are often on immunosuppressive medications, biologics or corticosteroids. Is it safe to give probiotics in these individuals? Our opinion is that it is safe and indicated. In fact, may the question may be better phrased *Is it safer to give probiotics than not to withhold them*, in view of the deleterious effects of patient exposure to multiple systemic antibiotics, resulting changes in microbiome, and alterations in barrier function of intestinal and other epithelial surfaces in these patients. Certainly, clinicians are quick to administer antimicrobial and/or antiviral agents to these individuals.

There are numerous RCTs in the literature describing adults and children with cancer and immunodeficiency who have been treated with probiotics or placebo [127-130]. The most comprehensive review to date examined safety in immunocompromised adults using Common Terminology Adverse Event Reporting. There were 57 studies in 4,914 individuals, 2,506 of whom received probiotic or synbiotic (probiotic plus prebiotic). These included critically ill “intensive care unit” subjects, those with cancer, HIV-infected individuals, and those with arthritis, inflammatory bowel disease, or recent gastrointestinal surgery [131]. The authors concluded that probiotics were safe and, overall, associated with *fewer* adverse events compared to the control group. However, there were flaws in precise reporting in most of the cited studies. That report was in 2014, and it is likely that there will be upcoming reports and systematic reviews of probiotics in immunocompromised individuals.

5. THE FUTURE OF PROBIOTICS

Henri Poincare in *The Foundations of Science* said, "It is far better to foresee even without certainty than not to foresee at all." Based on the collective evidence, the authors suggest the following events are likely to take place in the near future.

a. *Probiotics are likely to be used in autoimmune diseases as a component of various treatment regimens. One size will not fit all.* The choice of optimal probiotic or multispecies strains will evolve for each disease entity studied.

b. *The present “third party” insurance reimbursement problem will change.* Currently, insurance plans in the U.S. cover antibiotics but not probiotics; but (as discussed) a body of evidence is evolving that clinical outcomes will be improved with probiotics. Once safety issues in vulnerable populations are adequately addressed by properly controlled and regulated trials, we expect widespread use in children and adults with autoimmune disorders and (we hope for) coverage by insurance plans.

c. *Quality improvement efforts by medical institutions will likely reward treatments with the best outcome.* An example of this is the protocol for treatment of infants admitted to hospital with diarrheal dehydration at Cincinnati Children’s Hospital. An international working group selected care protocols for children with acute diarrhea, using systematic reviews, Delphi methodology, and external peer review. They decided that oral rehydration and probiotics were the only treatments recommended for infants presenting with acute diarrhea [132]. At Cincinnati Children’s, investigators placed in the electronic order set an entry for the administration of *Lactobacillus rhamnosus* GG. After implementation of this initiative, the prescribing of this probiotic increased from 1% to 100% [133]. However, a retrospective study of 145 U.S. hospitals, assessing ~ 1,900,000-hospital discharge showed that only in 2.6% of all hospitalizations were probiotics administered [134].

d. *Novel delivery systems will facilitate probiotic delivery and efficacy.* “Designer probiotics” is a term that has been given to probiotics with genetic engineering to facilitate delivery to the small intestine, enhance competitiveness within the gastrointestinal tract, and improve outcomes in certain disease states (reviewed in [135]. To overcome thermal and osmotic stress, probiotics have been

suspended in high osmolarity solutes such as betaine. Additionally, expression cloning of solute uptake genes for the betaine transporter *BetL* by *Bifidobacterium breve* resulted higher fecal levels of the probiotic in murine stool, probably because of improved survival in the hyperosmotic upper small intestinal lumen. Recently, an *E. coli* strain was engineered to secrete HIV gp41-hemolysinA hybrid peptides. These peptides block HIV entry into target cells. There are 2 other studies demonstrating potential use of designer probiotics in protecting from HIV infection [135].

Another interesting way to magnify probiotic retention and clinical impact is to administer the organism with agents that promote biofilm formation. Recently Olsen et al administered *L. reuteri* grown as a biofilm on the surface of dextranomer microspheres (DM) loaded with mannitol and sucrose. A single dose administered to newborn rat pups was sufficient to reduce the severity of necrotizing enterocolitis [136].

e. *Probiotic products may in some cases replace the probiotics themselves.* Metabolites may be identified that can be given instead of or along with live microorganisms. Mechanistic studies have begun to unravel the secrets of probiotic effects. Metabolites mentioned above, including short chain fatty acids, growth factors, bacteriocins, tryptophan metabolites, and adenosine derivatives could be beneficial. If the optimal, most potent metabolite were identified for a given disease, it may be possible to achieve the probiotic effect without the inherent risks of live cultures. However, it is possible that sustained luminal levels may not be attained with such an approach or that the effect of probiotic requires synthesis of metabolites by microbial consortia.

f. *The scientific community may begin to refer to probiotics as evidence-based, rather than “alternative” medicine.*



Seppo Salminen: University of Turku, Finland. Probiotic safety, adhesion to intestinal cells and mucus. Probiotics effective in milk hypersensitivity in infants. Probiotics reduce incidence of upper respiratory and middle ear infections in infancy.



Eamonn Quigley: University College, Cork, Ireland, currently Methodist Hospital, Houston, Texas, USA. Probiotics beneficial in irritable bowel syndrome, ulcerative colitis, chronic fatigue syndrome, and psoriasis.



Gregor Reid: Lawson Health Research Institute, London, Ontario, Canada. Probiotics effective in urogenital infections and for individuals with human immunodeficiency virus. Dietary probiotic supplementation in rural Tanzania improved dysbiosis.



Fergus Shanahan: University College, Cork, Ireland. Probiotic reduces severity of experimental colitis in IL-10 knockout mouse. Investigated various probiotics with respect to internalization and translocation across intestinal microfold (M) cells. Subcutaneous probiotic effective in arthritis model. Symbiotic effective in adult constipation. Showed that lactobacilli (not bifidobacilli) degrade oxalate.



Erika Isolauri: University of Turku, Finland. Probiotics improve symptoms in children with atopic dermatitis, infant colic, rhinovirus infections and acute gastroenteritis. Prenatal probiotics result in infant gut toll like receptor expression, reduce infant allergy development, and may reduce neurodevelopmental disorders. Maternal probiotic/prebiotic affects breast milk composition: fatty acid composition and cytokine levels.



Riitta Korpela: University of Helsinki and Valio Research Centre, Helsinki, Finland. Probiotics prevent day care center infections, reduce severity of infant eczema, and enhance babies' growth in the first 6 months of life. Reduced number of candidial infections in the elderly with probiotic treatment.



Hania Szajewska: Medical University of Warsaw, Poland. Meta-analyses showing that probiotics can prevent antibiotic-associated diarrhea and are effective in treating acute infectious diarrhea, upper respiratory infections, infant colic, and (in combination with antibiotics) *H. pylori* gastritis.



R. Paul Ross: University College, Cork, Ireland. Probiotics reduce cholesterol and atherogenesis in Apo E knockout mouse. Probiotics produce gamma-amino butyric acid (GABA) and bacteriocins; engineered probiotic producing conjugated linoleic acid reduces liver and adipose tissue.



Arthur Ouwehand: University of Turku, currently Danisco Institute, Wilmington, Delaware, USA. Glycoprotein adhesion of probiotic facilitates pathogen exclusion. Probiotics reduce winter febrile illnesses and rhinorrhea. Probiotics increase regulatory T cells and ameliorate experimental T cell transfer colitis. Probiotics and customized yogurts improve symptoms of constipation and irritable bowel syndrome.



Mary Ellen Sanders: International Scientific Association for Probiotics and Prebiotics, Centennial, Colorado, USA. Probiotic safety and regulatory expert. Fermented foods help to prevent day care and school-acquired infections.



Glenn Gibson: University of Reading, U.K. Coined term prebiotic. Studies probiotics in the elderly. Pectins facilitate growth of bifidobacilli. Bile acid-tolerant probiotics are more effective colonizers. Probiotics and synbiotics can reduce cholesterol levels.

Figure 1. “Prolific Probiotic Proponents.” These investigators have been studying probiotics and their mechanisms for 3-4 decades. Many have seminal findings, as indicated. All have > 50 publications in high impact journals.

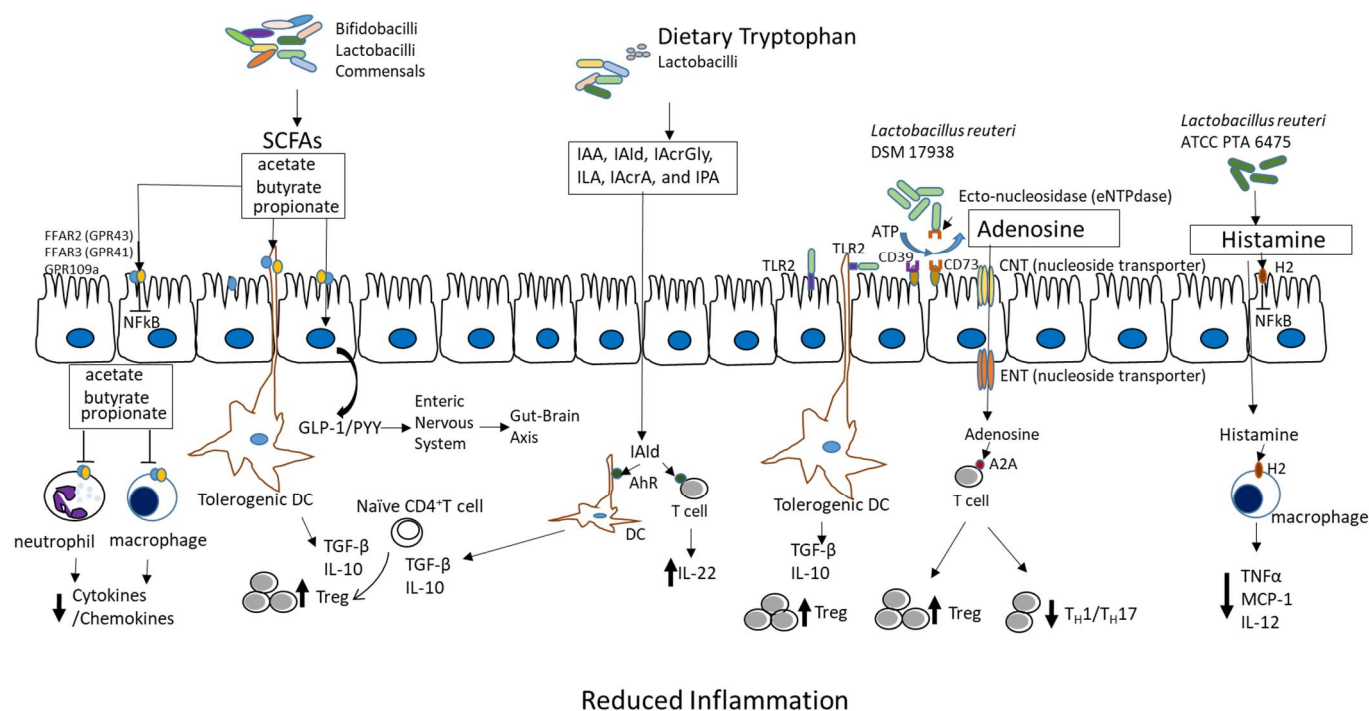


Figure 2. Critical metabolites produced by probiotics, which have anti-inflammatory function. SCFAs (acetate, butyrate, and propionate) produced by bifidobacilli, lactobacilli and commensals, bind and activate receptors (FFAR2, FFAR3 or GPR109a) on intestinal epithelial cells to inhibit the NF-κB pathway to prevent inflammation. They also may release GLP1/PYY to act on the enteric nervous system and the CNS to affect energy homeostasis and gut motility. SCFAs also induce tolerogenic DC, which educate naïve CD4⁺T cell to differentiate into Tregs. These actions inhibit cytokine production by neutrophils and macrophages via interaction with receptors. Dietary tryptophan and probiotic produced-indole derivatives interact with AhR expressed on immune cells to produce anti-inflammatory effects. *L. reuteri* 17938 promotes adenosine generation, most likely by an ecto-nuclease present on the probiotic itself and on intestinal epithelial cells. Adenosine and its derivative inosine interact with adenosine receptor-2A located on T cells to promote Treg functions and inhibit inflammatory TH1 and TH17 subsets. Histamine produced by *L. reuteri* 6475 interacts with H2 presented on intestinal epithelial cells and macrophages to reduce levels of pro-inflammatory cytokines (TNF-α, MCP-1, and IL-12). In summary, the critical metabolites produced by probiotics generate anti-inflammatory effects during diseases (Illustration by Yuying Liu).

Abbreviations: SCFAs: short chain fatty acids; FFARs: free fatty acid receptors; GPRs: G-binding protein receptors; NF- B: nuclear factor kappa-light-chain-enhancer of activated B cells; GLP1: glucagon-like protein-1; PYY: peptide tyrosine tyrosine; CNS: central nervous system; AhR: aryl hydrocarbon receptor; TH1 and TH17: T helper cells; H2: histamine receptor 2; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein-1; IL-12: interleukin-12.

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