Probiotics, Enteric and Diarrheal Diseases, and Global Health

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Introduction

Enteric and diarrheal diseases are a major worldwide cause of death among children under the age of 5. In this age group, diarrhea occurs 2.5 billion times per year [1] and causes 15% of childhood deaths.[2] Diarrheal diseases claim 59 million disability-adjusted life years (DALYs), nearly all from children in low- and middle-income countries.[3] Despite this enormous burden, these numbers fail to capture the full impact of enteric and diarrheal diseases. Early and frequent exposure to intestinal pathogens begins a cycle (Figure 1A) that affects digestion, nutrient absorption, growth, and immunity.[4] Repeated infections, with either overt diarrhea or subclinical enteropathy, produce acute and chronic undernutrition,[5] which leads to more frequent and severe infections.[6] Undernutrition contributes to 53% of childhood deaths [7] and is the leading risk factor for poor health outcomes in childhood;[8] survivors are at risk for developmental deficits in growth, fitness, and cognition that persist into adulthood with devastating consequences.[4] These consequences have a multiplicative effect on calculations of DALYs from diarrheal disease.[9]

Fortunately, there are strategies to break this cycle, although each approach has limitations. Sustainable access to potable water and improved sanitation reduces pathogen exposure; a $70 billion annual investment would only begin to reduce the number of people without these necessities (2.5 billion) by 2015.[10] Antimicrobial agents are effective against specific pathogens but are expensive and can exacerbate toxin-mediated diseases, disrupt the human microbiome, and induce antibiotic-associated diarrhea as well as drug resistance. Immunization with enteric vaccines can reduce the burden of severe diarrhea, but vaccines must be kept in the cold, only protect against specific pathogens, and are less effective in regions of high mortality. For example, the efficacy of the live, attenuated rotavirus vaccine against severe disease is only 48.3% in southeast Asia [11] and 39.3% in sub-Saharan Africa.[12] Zinc reduces the propensity to develop recurrent diarrhea and oral rehydration

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solution (ORS) attenuates overt symptoms of diarrhea and dehydration.[13] However, these approaches do not adequately address broader growth and developmental processes that could yield long-term benefits. Likewise, trials of therapeutics to reduce diarrhea severity, unplanned intravenous fluid administration, or duration of hospitalization fail to address longer-term, initially subclinical, consequences of recurrent infections. Complementary outcome measures, including measures of growth and biomarkers of acute intestinal inflammation, barrier disruption, and impaired immunity, would provide greater insight into underlying pathology and therapeutic efficacy. Use of these measures could reduce acute, overt, as well as chronic, often unrecognized, intestinal diseases (Figure 1B).

No single intervention is sufficient to eliminate the global burden of enteric and diarrheal diseases. Vaccines, for example, can protect against limited infectious agents but immunization can be overwhelmed by heavily contaminated water. Multiple interventions could work synergistically, such as the combination of improved water and sanitation, vaccines, micro- and macronutrient provision, and selectively-targeted antimicrobial therapy (e.g. single-dose albendazole for intestinal helminths). Do current global health strategies use the best available interventions?

One underexplored approach, probiotics, could combine favorable safety profiles with improved nutrition and microbiome function. Probiotics are live microorganisms that confer a health benefit on the host,[14] and have been used to treat multiple gastrointestinal (GI) diseases.[15] Microbes are inexpensive to grow, and have the potential for rapid global scale-up. Is there compelling evidence to recommend developing probiotics-based strategies to complement current approaches against enteric and diarrheal diseases for children in developing countries? If so, what steps must be taken before these therapies are ready for clinical impact in the global health arena?

Clinical Evidence

In 1907, Russian Nobel laureate Elie Metchnikoff suggested that ingestion of microbes could benefit human health.[16] As we learned about multidrug-resistant pathogens and the role of the human microbiome in health and disease, numerous trials showed the safety of probiotics[17] and their beneficial outcomes in patients with various illnesses.[18] A systematic review that included 12 randomized, controlled trials (RCTs) in the Cochrane database (the majority from affluent countries) concluded that probiotics reduced the mean duration of acute diarrhea in children by 29.2 hours in a fixed-effects model and by 30.48 hours in a random-effects model.[19] Two meta-analyses that evaluated similar studies found statistically significant but modest reductions of diarrhea duration.[20,21] Although combination analyses of trials with microbes of different genera, species, strains, and doses provide limited information about specific therapeutic interventions, it is clear that many probiotics reduce the duration of acute diarrhea.

There are studies of probiotics for enteric and diarrheal diseases targeting children in developing regions (Table S1). The majority of RCTs studied acute gastroenteritis and reported modest reductions in diarrhea duration. However, the effects of probiotics were statistically equivalent to those of placebo in 25% of trials. Some of the negative results might be attributable to small sample size or administration of insufficient doses;[22-24] each trial must be viewed in the context of the specific disease and probiotic strain analyzed. Two RCTs evaluated probiotics for children with persistent diarrhea and reported dramatic reductions in diarrhea duration—4.8 and 3.9 days in Argentina[25] and India,[26] respectively. Two trials evaluated probiotics for diarrhea prevention; children in Peru had 13% fewer diarrheal episodes after 15 months of *Lactobacillus rhamnosus*,[27] whereas

diarrhea frequency was reduced by 14% among children in India who received daily doses of *Lactobacillus casei* for 12 weeks, with a 12-week follow-up period.[28]

Few studies have examined markers of acute or chronic immunity or underlying intestinal function. Administration of *Bifidobacterium bifidum* and *Streptococcus thermophilus* increased numbers of CD4+ T cells in HIV-infected Brazilian children.[29] A similar effect was observed following administration of *L. rhamnosus* to HIV-infected adults in Tanzania. [30] Tropical enteropathy was studied in Malawian children using urinary carbohydrate excretion; *L. rhamnosus* failed to improve ratios of lactulose:mannitol excreted,[31] despite evidence that probiotics ameliorated GI permeability defects in children with atopic dermatitis in Germany.[32] Probiotics improved growth among healthy children in Thailand[33] and Estonia,[34] but not among HIV-exposed infants in South Africa.[35] In Malawi, probiotics failed to improve nutrition status in severely malnourished children who received inpatient nutritional rehabilitation. Despite the negative primary outcome, there was a trend toward decreased mortality among children treated with probiotics on an outpatient basis.[36] Probiotics also reduced the duration of rotavirus shedding in India.[37] Strategies to reduce fecal shedding of pathogens are important for the billions of people who live without adequate sanitation.

Probiotics could also have a role in immunization programs. *L. rhamnosus* increased the virus-specific antibody response in children with acute rotaviral gatroenteritis,[38] so immunostimulatory probiotics might help children's immune systems increase the memory responses to vaccines. Based on initial data from studies in industrialized regions, *Bifidobacterium longum* and *L. rhamnosus*, administered during the first 6 months of life, increased vaccine-specific antibody production following vaccination against hepatitis B. [39] Infants that were given *Lactobacillus paracasei* from 4 to 13 months of age had increased titers of antibodies to *Haemophilus influenzae* type B (Hib) capsular polysaccharide, diphtheria toxin, and tetanus toxoid. [40] Concentrations of antibodies against Hib increased among infants when women were given daily doses of probiotics during the final month of pregnancy; therapy continued for infants during their first 6 months of life.[41] Taking probiotics during pregnancy and lactation appears to be safe[42] and might yield post-natal benefits. Intriguingly, maternal consumption of *L. rhamnosus* or *B. lactis* increased the amount of immunoglobulin (Ig)A detected in breast milk.[43] Increased IgA levels in breast milk might protect infants from enteric pathogens and serve as a biomarker for studies of probiosis in lactating women. It is a challenge to assimilate and analyze all the clinical evidence of the effects of probiotics. Study quality varies, randomization and blinding methods are rarely reported, and appropriate placebos are not always used. Exclusion criteria are numerous, limiting the generalization of findings to children that are very ill. Probiotic strain designations, bacterial growth phase, and variations in administration (in fermented dairy products, infant formula, solid food, ORS, water, juice, capsules) are often unreported. It is important that studies report these parameters, so findings can be reproduced—proteins and metabolites synthesized by live microorganisms are strain-specific and vary with growth conditions. Many probiotics have shown beneficial effects, but improving our knowledge of the mechanisms that mediate these effects would facilitate identification of more potent probiotics for specific applications.

Probiotic Mechanisms

There are 3 general classes of probiotic anti-pathogenic mechanisms: direct antagonism, immunomodulation, and exclusion (Figure 2).

Direct antagonism

Many probiotics secrete small molecules or bioactive peptides that have anti-microbial activities. *Lactobacillus salivarius* UCC118 protects mice against infection with *Listeria monocytogenes*; UCC118 produces a broad-spectrum bacteriocin (antimicrobial peptide) that kills *Listeria* in the lumen of the GI tract, preventing translocation and systemic spread of infection.[44] Mice were not protected when they were fed a derivative of UCC118 that no longer produced the bacteriocin or when they were infected with an engineered strain of *Listeria* that was resistant to the bacteriocin. *Listeria* pathogens were eliminated within 30 minutes following oral administration of UCC118, indicating a direct effect of the probiotic on the pathogen. UCC118 also protects mice against *Salmonella* infection, but the bacteriocin is not involved. Thus, a single probiotic can protect mammals against different pathogens via multiple mechanisms.

Several in vitro studies have reported down-regulation of virulence factors in pathogens exposed to probiotics or their cell-free supernatants.[45-47] *Lactobacillus acidophilus* LA-5 suppresses transcription of *Escherichia coli* O157:H7 genes involved in adherence; this corresponds with reduced colonization in mice.[48] Probiotics can also interfere with toxin production or directly antagonize enterotoxins. *Saccharomyces cerevisiae* var. boulardii (*S. boulardii*), which reduces *Clostridium difficile* -associated diarrhea,[49] secretes a 54 kDa serine protease that hydrolyzes toxin A (a *C. difficile* virulence factor) and its receptor, which is present in the intestinal brush border.[50,51]

Immunomodulation

Probiotics elicit a variety of responses from immune cells in vitro and in vivo, through mostly unknown mechanisms.[52] The responses of specific immune cells to particular microbes result from complex interactions between surface-bound and secreted ligands (e.g. pathogen-associated molecular patterns) and host Toll-like receptors (TLRs). [53] Reductionist approaches to studying these interactions, such as analyses of knockout or transgenic mice, have provided limited information about probiotic immunomodulation. In one successful example, the presence of D-alanines on teichoic acids in the cell wall of *Lactobacillus plantarum* elicited production of pro-inflammatory cytokines by peripheral blood mononuclear cells.[54] Co-culture of dendritic cells with polysaccharide A (PSA) derived from *Bacteroides fragilis* induced naïve T cells to generate an IL-10-producing regulatory T cell population.[55] Purified PSA prevents intestinal inflammation in multiple mouse models.[56]

The immunomodulatory effects of probiotics can be species-[57] and strain-specific [58,59] and involve multiple mammalian signaling pathways that affect immune cell phenotypes. Some probiotic *Lactobacillus* strains increase production of tumor necrosis factor (TNF) through activation of the transcription factors NF-κB and STAT, [60] whereas others suppress TNF production by inactivating NF-κB [61,62] or mitogen-activated protein kinase and c-Jun signaling.[58] Differential immune regulation might prime the immune system to limit infections, inflammation, and pathogen-mediated damage. Probiotic signaling molecules that regulate immunity have not been identified.

Exclusion

Exclusion is used as a "catch-all" term for probiotic mechanisms that make the GI environment less hospitable for pathogens. These mechanisms include altering the resident microbiota, decreasing luminal pH, improving epithelial barrier function, interfering with pathogen binding by down-regulating specific host receptors, and stimulating production of defense-associated factors, including mucins and defensins. Multiple probiotics have been

implicated in each of these functions, but clear links between individual bacterial compounds and specific responses have been difficult to establish.[63]

Rats that consumed the probiotic mixture VSL#3 increased their luminal mucin content by 60% through an unidentified, heat-resistant secreted soluble compound.[64] Some bacterial products, including short-chain fatty acids produced by fermentation, can stimulate epithelial cell differentiation and improve barrier function[65,66]—this protects against pathogens that cause disease through loss of tight junction integrity, increased paracellular transport, fluid loss, and invasion of the submucosa.[67,68] Indole, an aromatic compound secreted by commensal *E. coli* and detected in human feces, increases expression of genes whose products regulate production of mucins and organization of the cytoskeleton, tight junctions, and adherens junctions. Indole increases transepithelial resistance in enterocyte cultures.[69] The quorum-sensing molecule CSF, a 3 kDa heat-stabile, pepsin-sensitive pentapeptide from the probiotic *Bacillus subtilis*, activates the heat shock protein (Hsp27) after CSF is internalized by the enterocyte oligopeptide transporter OCTN2; Hsp27 activation protects epithelial cells from oxidant-induced stress.[70] The in vivo roles of these molecules in preventing infections have not been established.

Probiotics can also stimulate defensins, cationic antimicrobial peptides produced by cells of the intestinal epithelium.[71] The probiotic *E. coli* Nissle 1917 increases synthesis of human β-defensin 2 by activating NF-κB and AP-1[72] via secretion of flagellin.[73] Increased β-defensin 2 levels were detected in stool samples from healthy volunteers 9 weeks after administration of nonpathogenic *E. coli*.[74] Resistance to host-derived antimicrobials may be another important probiotic property. [75] Finally, some probiotics promote class switching to increase IgA production, by inducing enterocytes to secrete B-cell stimulatory factors such as APRIL. Lipopolysaccharide- and flagellin-stimulated secretion of APRIL occurs in human enterocytes via TLR4 and TLR5 signaling.[76]

In addition to anti-microbial mechanisms, probiotics benefit host physiology, nutrition, and the ability to counteract pathogenesis. In mouse models, weight gain and adiposity are influenced by the intestinal microbiome. [77-79] Metabolism could be regulated by individual microbes; for example, *S.boulardii* increases activities of brush border enzymes including sucrase, maltase, trehalase, lactase, aminopeptidase, and alkaline phosphatase.[80] Intestinal bacteria also synthesize niacin, pantothenic acid, biotin, folic acid, and vitamins K, C, and B12.[81] These functions of probiotics have not been correlated with pathogen resistance.

Probiotics may also interact with the enteric nervous system to attenuate secretory diarrhea. [82] In mice, *Lactobacillus* inhibited post-infective intestinal hypercontractility through an unidentified, heat-labile fermentation product.[83] In rats, lactobacilli reduced hypercontractility by blocking calcium-dependent potassium channels.[84,85] Lactobacilli can also blunt visceral pain responses by increasing expression of enterocyte opioid and cannabinoid receptors[86] or by inhibiting sodium channels.[87] Further studies are needed to identify microbe molecular signatures associated with specific responses against pathogens.

Using Probiotics Worldwide

Step 1: Identify Molecular Mechanisms of Probiosis and New Therapeutics

The first step to realizing the full potential of probiotics is to define the specific microbial genes, small molecules, and host–microbe interactions that mediate their beneficial functions. Basic scientists must identify, isolate, and characterize bacterial fermentation products, immunomodulating factors, antimicrobial agents, and cell-wall components that

produce discrete physiological effects through specific host interactions. These types of studies will improve our understanding of probiotic function and allow microbial libraries to be screened to identify new probiotics.

The Human Microbiome Project (HMP), MetaHit, and the International Human Microbiome Consortium (IHMC) published a partial catalog of microbial reference genomes to help identify new probiotic species; [88] studies to associate changes in microbial populations[89] or microbial gene content [90] with states of health and disease are underway. For example, the anti-inflammatory effects of *Faecalibacterium prausnitzii* were identified after reductions in this bacterium were associated with recurrence of ileal Crohn disease.[91] However, HMP and IHMC studies have been limited to subjects in affluent, developed countries. Diarrhea disrupts the microbiota,[92] and environmental and lifestyle variations yield microbiomes that are specific to geographic regions, cultures, or ethnic groups.[93,94] The composition and function of GI microbiomes of undernourished children in disease-endemic regions must be studied separately from the microbiomes of people in developed regions.

Step 2: Develop New Biomarkers for Acute and Chronic Intestinal Disease

To more accurately assess intestinal pathology and therapeutic efficacy, new host and microbial biomarkers must be validated. Fecal samples are easily obtained, but their microbial composition primarily reflects that of the large bowel, a self-regulating community that can resist introduction of probiotics by virtue of niche exclusion.[95] Lactobacilli, administered daily, comprise only 0.001% of the fecal microbiota and quickly disappear once they are no longer ingested—they have only a minor presence in the large bowel biome.[96,97] Many enteric pathogens infect the small bowel, overgrowth of which is a significant feature of tropical enteropathy.[98] Thus, probiotics are likely to mediate their greatest effects against enteric and diarrheal diseases in the small bowel, where they comprise a substantial proportion of the biomass and functionally alter the proximal GI tract. [99] Ideal biomarkers would reflect the health status of these sites.

Metabolomics is a systematic, quantitative analysis of changes in the complete set of low molecular weight metabolites produced by cells in response to environmental or cellular changes.[100] Bacterial products are absorbed from the bowel lumen into lymph and blood circulations; body fluids therefore contain many bacterial and host metabolites that could serve as biomarkers of relationships between food, bacteria and host cells and indicate health or disease.[101] Infection of mice with the nematode *Schistosoma mansoni* can be diagnosed based on alterations to the urinary metabolome, which reflect disruption of the bowel ecosystem by the intestinal parasite.[102] Urine, saliva or bowel fluid, which are abundant and easily collected, could be sources of biomarkers for small bowel function.

Analysis of metabolomic profiles lags behind advances in detection methods, and new pattern recognition systems must be developed. Comprehensive reference sets of identified metabolites must be assembled to improve yield from metabolomic comparisons.[103] Linking metabolomics to probiotics research could lead to new ways to identify biomarkers and have practical applications in developing countries. Initial studies should compare metabolomes between healthy children and those with defined enteric infections, controlling for ethnicity, age, sex, socioeconomic status, and nutritional state. Biomarkers are likely to be identified that are associated with overt and subclinical intestinal disease.

Step 3: Optimize Therapeutic Regimens for Specific Populations

High-impact interventions must be developed for target populations—namely, children under 2 years of age in disease-endemic areas. Core and variable components of their

intestinal microbiomes should be catalogued. Children should be characterized with respect to overall health status, disease susceptibility, nutritional (macro and micro) status, and common enteric pathogens. Dietary evaluations must consider microbiomes and glycomes of breast milk and seek to identify natural substrates for probiotics that optimize their metabolic activities. This detailed picture of the microbiome and its interactions will guide selection of specific probiotic-based therapies for the pathogens relevant to each population.

Prospective RCTs should aim to reduce short-term pathologies associated with acute diarrhea, prevent long-term morbidities from recurrent or persistent infections, and increase vaccine efficacy. Specific strain and dose recommendations should be made, with the long-term goals of improving survival, growth, and development during childhood. With trials in multiple geographic locations and ethnic groups, patterns will emerge to guide selection of specific microbial-based therapies for specific regions of the world.

This plan has risks—although probiotics are assumed to be safe, undernourished children with immune and GI permeability defects could be more prone to bacterial translocation and sepsis. Safety monitoring will be critical. Immunostimulatory probiotics might not affect children whose immune systems have been highly stimulated by contaminated environments; in this case, other mechanistic bases of probiosis must be pursued. Finally, global application of probiotic therapies requires development of technologies to make freeze-drying, or other preparative methods of preserving probiotic viability, feasible under challenging conditions. Strategies must also be developed to deliver probiotics through local distribution networks, and the products must be acceptable to diverse cultures.

Will Probiotics Be Ready for Worldwide Use in the Near Future?

Beyond the acute effects of severe diarrhea and dehydration, repeated and persistent infections yield devastating long-term consequences. For children in less-developed settings, many probiotics are effective for acute gastroenteritis, persistent diarrhea, and diarrhea prevention; their potential roles in growth, immunity, and vaccine efficacy must be further evaluated. Mechanisms that mediate their beneficial effects are being elucidated. Characterization of specific molecular interactions between probiotics and the host or microbiome will enable selection of more potent therapeutic microbes. Biomarkers of intestinal pathology must be developed to determine therapeutic efficacy of existing and new probiotics. Ultimately, clinical studies that test specific therapies in well-defined populations must be performed to determine the overt and hidden consequences of enteric infections.

Basic scientists, clinical researchers, and industrial leaders have the opportunity to work together against one of the most pressing health problems facing the world today. If we accept the challenge, probiotic-based therapies might be incorporated into global health strategies, to reduce the burden of enteric and diarrheal diseases borne by millions of children worldwide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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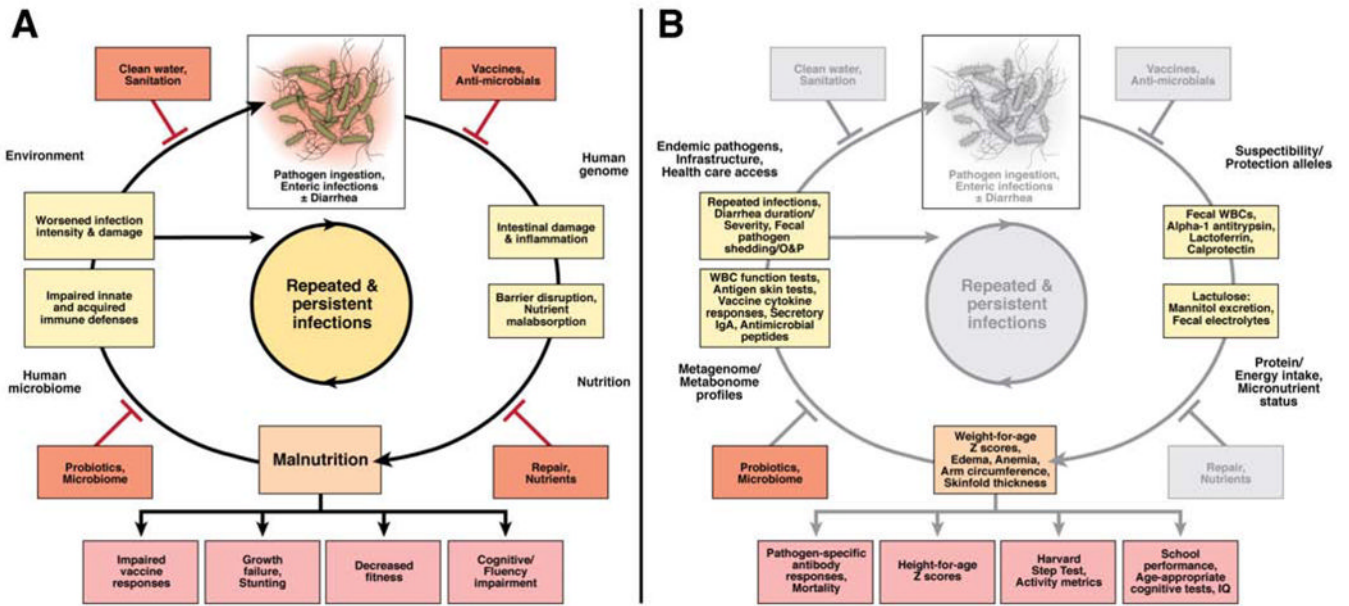
Figure 1. The vicious cycle of diarrhea and undernutrition in susceptible children
(A) The devastating synergy between enteric infections and undernutrition is influenced by the environment, the human genome, host nutrition, and the human microbiome. Various interventions (red boxes) may inhibit progression to the next step in the cycle, minimizing both acute and chronic morbidities. (B) Employing a spectrum of disease outcome measures would lend greater insight into the pathology underlying enteric and diarrheal diseases, while providing a more complete understanding of interventions targeting basic steps of enteric and diarrheal disease pathogenesis. Adapted with permission from Wiley: *Nutrition Reviews*,[4] copyright 2008.
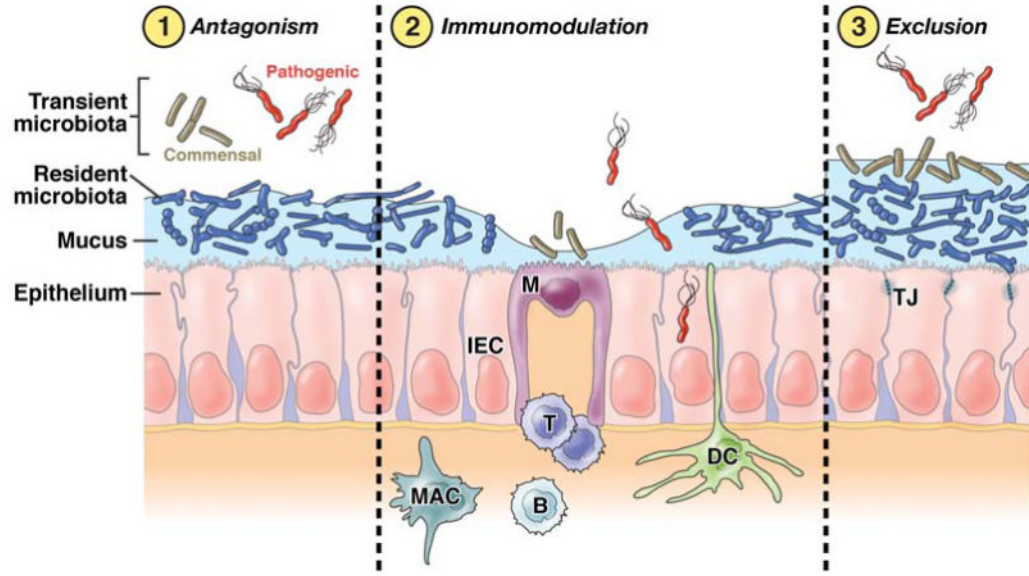http://www.http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1753-4887.

Figure 2. Three general mechanisms of probiosis for enteric infections
In direct antagonism, probiotics kill or inhibit the pathogen to limit infection, or they down-regulate the expression of virulence factors, such as adhesins or toxins, required for pathogenesis. Probiotics can also interact with the immune system (immunomodulation) to enhance the functionality of innate and/or adaptive immunity, or to limit the ability of the pathogen to initiate or facilitate an immune response. Through "exclusion," probiotics can alter the microenvironment to prevent pathogens from gaining access to appropriate receptors, to limit pathogen attachment, entry, or translocation, or to improve barrier function. A beneficial microbe may use a combination of these mechanisms, and may employ different mechanisms against different pathogens. B, B cell; DC, dendritic cell; IEC, intestinal epithelial cell; M, M cell; MAC, macrophage; T, T cell; TJ, tight junction.