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Bioengineered probiotics, a strategic approach to control enteric infections

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Enteric infections account for high morbidity and mortality and are considered to be the fifth leading cause of death at all ages worldwide. Seventy percent of all enteric infections are foodborne. Thus significant efforts have been directed toward the detection, control and prevention of foodborne diseases. Many antimicrobials including antibiotics have been used for their control and prevention. However, probiotics offer a potential alternative intervention strategy owing to their general health beneficial properties and inhibitory effects against foodborne pathogens. Often, antimicrobial probiotic action is non-specific and non-discriminatory or may be ineffective. In such cases, bioengineered probiotics expressing foreign gene products to achieve specific function is highly desirable. In this review we summarize the strategic development of recombinant bioengineered probiotics to control enteric infections, and to examine how scientific advancements in the human microbiome and their immunomodulatory effects help develop such novel and safe bioengineered probiotics.

Introduction

Enteric infections account for about 1.5 billion episodes of diarrheal diseases with 2.2 million deaths (mostly children) annually and are the fifth leading cause of death at all ages worldwide.[1] Children under 5 y of age are most susceptible and the disease burden is the greatest in developing countries.[2] Consequences of childhood enteric infections are impaired physical growth and cognitive development.[3] Enteric infections may be caused by bacterial, viral, parasitic, or fungal agents, which disrupt intestinal function with or without causing dehydrating diarrhea. Seventy percent of all microbial diarrheal diseases are foodborne,[4] and foodborne illnesses are a serious public health concern (**Table 1**). The global burden of foodborne illness is currently unknown; however, the World Health Organization (WHO) reported that 1.8 million people died from diarrheal diseases in 2005, largely due to contaminated food and water.[5,6] In the US, the Centers for Diseases Control and Prevention (CDC) estimates that each year there are about 48 million cases of foodborne infections with 128 000 hospitalizations and 3000 deaths.[7] There are over 200 known microbial, chemical, or physical agents that can cause foodborne illness.[6] CDC estimates that of all the foodborne infections, 44% of the hospitalizations and deaths are attributed to 31 known pathogens.[7] In light of this serious public health crisis, efforts have been directed toward the detection, control, and prevention of food-borne pathogens and diseases. It is estimated that a reduction in foodborne illness by 10% would keep about 5 million Americans from getting sick each year.[7] With increasing trend in consumer preference for safe and wholesome food, probiotics offer an effective and alternative intervention strategy to control foodborne illnesses. Among the microbial etiologies responsible for enteric infections, WHO has prioritized around 22 infectious agents for surveillance based on their higher prevalence, morbidity and mortality. These include *Brucella* spp., *Campylobacter* spp., *Clostridium botulinum*, enteroaggregative *E. coli* (EAggEC), Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC), *Helicobacter pylori*, hepatitis A virus, hepatitis E virus, *Listeria monocytogenes*, *Mycobacterium bovis*, *Vibrio cholerae* O1/O139, non-cholera *Vibrio* spp., norovirus, rotavirus, prions, *Salmonella* spp. (non-typhoidal), *Salmonella enterica* serovar Typhi, *Shigella* spp., and *Yersinia* spp., and toxins from *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus.*

Various strategies have been employed to control enteric pathogens in foods, food producing animals, and humans. Antibiotics are used in meat animal production as prophylactic to control disease and improve growth rate and efficiency.[8] However, increasing concerns about antibiotic resistance has led to research efforts to use naturally occurring antimicrobials as alternatives. Antimicrobials may include organic acids, essential oils and plant extracts, bacteriocins,[9] probiotics,[10] and bacteriophages.[11-13] Organic acids (acetic, lactic, and citric acids) are commonly used to rinse animal carcasses, fruits, and vegetables.[14] To enhance antimicrobial efficacies, acids are also used in combination with oxidizing agents such as hydrogen peroxide. In addition, thermal (ionizing radiations and heating) and non-thermal treatments such as high hydrostatic pressure, high-intensity pulsed electric fields, oscillating magnetic fields, intense light pulse, photosensitization, or a combination of above (hurdle approach) are also effective.[15]

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Table 1. Diseases caused by foodborne pathogens

Disease or clinical symptoms	Pathogens/toxins involved
Vomiting, diarrhea, dysentery	*Staphylococcus, Bacillus, Cronobacter, Salmonella, Shigella, Vibrio,* norovirus, rotavirus, *Entamoeba; Cryptosporidium; Cyclospora; Giardia; Isospora; Taenia*
Arthritis (reactive arthritis, Reiter syndrome, rheumatoid arthritis)	*Campylobacter, Salmonella, Shigella, Yersinia*
Hemorrhagic uremic syndrome (HUS) kidney disease	Shiga-toxin producing *E. coli* (STEC); *Shigella* spp.
Hepatitis and jaundice	Hepatitis A virus (HAV), hepatitis E virus (HEV)
Guillain Barre syndrome (GBS)	*Campylobacter*
CNS/meningitis/encephalitis	*Listeria*, bovine spongiform encephalopathy (BSE)
Miscarriage, stillbirth, neonatal infection	*Listeria*, *Toxoplasma*
Paralysis	*Clostridium botulinum*, seafood toxin, *Campylobacter*
Malignancies and auto-immune diseases	Mycotoxin
Allergic response	Seafood toxin

Probiotics

The word "probiotic" is derived from the Greek word meaning "for life." Probiotics are live nonpathogenic microorganisms that are administered to maintain and improve intestinal microbial balance and protect host from infective agents. Physiologically, these microbes are endowed with certain characteristics that enable them to survive in the gut environment and colonize mucosal surfaces. The rationale for the use of probiotics in the prevention of enteric infections and treatment of diarrhea are associated with three major factors: (1) maintenance of the epithelial gut barrier, (2) modulation of innate and acquired immunity, and (3) inhibition of pathogen growth by producing bacteriocins, hydrogen peroxide and other antimicrobials.[16] Besides, probiotics also help prevent chronic enteric infection associated with stunted growth, abnormal low body mass indices, and impairment of cognitive function in children.[16]

The use of probiotics, prebiotics, and synbiotics (combination of prebiotics and probiotics) has also gained increased interest in recent years. The use of microflora to reduce pathogen load in the gut is termed as a probiotic strategy.[17] Probiotic techniques involve the introduction of a normal microbial population into the gut to provide a nutrient (prebiotic) that is limiting and allows the growth of a specific subset of the gut microflora. The goal of this approach is to fill all the niches available in the gut so as to exclude the establishment of pathogenic microbes.[18,19] Due to increased concern about the emergence of antibiotic resistance, use of probiotics provides an effective alternative to combat foodborne illnesses.[20]

Beneficial attributes of probiotics are broad and well documented (**Table 2**). These include lactose metabolism, improved digestion, increased nutritional value, production of antimicrobial factors, antimycotic effects, anti-carcinogenic properties, immunologic enhancement, production of short-chain fatty acids, antiatherogenic, and cholesterol-lowering attributes, regulatory role in allergy, protection against vaginal or urinary tract infections, maintenance of epithelial integrity and barrier, stimulation of repair mechanism in cells, and maintenance and reestablishment of a well-balanced indigenous intestinal, respiratory, and urogenital microbial communities.[10,21-24]

Prevention and Control of Enteric Infections Using Wild Type Probiotics

Enteric viral infections. Probiotics have been used to control viral infections. Rotavirus is responsible for 20–25% of the diarrheal diseases worldwide. Gnotobiotic pigs fed with *Lactobacillus acidophilus* and *L. reuteri* enhanced IFNγ and IL-4 levels in serum and decreased rotavirus infection.[25] Probiotics are also effective against Norovirus, which is responsible for 58% of foodborne illnesses.[26,27] Probiotic fermented milk containing *L. casei* Shirota strain was effective in controlling norovirus gastroenteritis in a health service facility.[28] A controlled double-blind study using a probiotic formulation (VSL#3) was shown to significantly reduce stool frequency and requirement for oral rehydration in children.[29]

Bacterial enteric infection. Among enteric pathogens that cause diarrhea, *Campylobacter jejuni* is responsible for about 400 million cases every year in both industrialized and developing countries.[30] Several probiotic strains have been evaluated for their efficacy in controlling *Campylobacter* infection. *Lactobacilli* and *Bifidobacteria* were shown to enhance colonization resistance in mice that were infected by *C. jejuni* or *Salmonella*. Probiotics also increased proliferation of lymphocytes against *Salmonella* antigens and reversed pathogen-induced immunosuppressive activity.[31] Synbiotics consisting of prebiotic galacto-oligosaccharide and probiotic *Bifidobacterium longum* significantly reduced *C. jejuni* load in poultry feces.[32] *Vibrio cholera* causes acute dehydrating watery diarrhea with 1.8 million cases and 27 000 deaths annually.[2] Experimental administration of *L. acidophilus* BKM B-2020 orally in mice and suckling rabbits prior to infection prevented cholera. Probiotic *L. plantarum* AS1 attached efficiently to cultured cell lines (HT-29) and reduced *V. parahemolyticus* attachment by competitive exclusion and displacement.[33] Probiotics are also found to be effective against diarrhea causing *E. coli* including STEC and ETEC. *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, and *Enterococcus faecium* significantly reduced *E. coli* O157:H7 shedding by sheep.[34] Bifidobacteria caused reduced Shiga toxin production by STEC in mice and protected against *E. coli* O157:H7 infection.[35] Nonpathogenic probiotic *E. coli*

Table 2. Health benefits of probiotic bacteria and their proposed mechanisms

Health benefits	Proposed mechanism
Resistance to enteric pathogens	Antagonism
	Increased antibody production
	Colonization resistance
	Limiting access of enteric pathogens (pH, bacteriocins, antimicrobial peptides, lactic acid production)
Aid in lactose metabolism	Bacterial lactase hydrolyzes lactose in the small intestine
Small bowel bacterial overgrowth	Decrease toxic metabolite production
	Normalize small bowel flora
	Antibacterial characteristics
Immune system modulation	Strengthening of non-specific and antigen-specific defense
	Regulate/influence Th1/Th2 cell activation
	Production of anti-inflammatory cytokines
Anticolon cancer effect	Antimutagenic and anticarcinogenic activity
	Detoxification of carcinogenic metabolites
	Stimulation of immune function
Decreased detoxification/excretion of toxic microbial metabolites	Increased bifidobacterial cell counts and shift from a preferable protein-to carbohydrate-metabolizing microbial community
Anti-Allergic activity (eczema or atopic dermatitis, asthma)	Prevention of antigen translocation into blood stream
	Prevent excessive immunologic responses to increased amount of antigen
Blood lipids, heart disease	Assimilation of cholestrol by bacterial cell
	Alteration in the activity of bile salt hydrolase (BSH)
Urogenital infections	Adhesion to urinary and vaginal tract cells
	Competitive exclusion
Necrotizing enterocolitis	Decrease in TLRs and signaling molecules and increase in negative regulations
	Reduction in IL-8 response
Rotavirus gastroenteritis	Increased IgA response to the virus
Inflammatory bowel disease	Enhancement of mucosal barrier function
Crohn disease	Reduction in proinflammatory cytokines production

Adapted from Nagpal et al.[23]

strains 1307 and Nissle also inhibited STEC growth and Shiga toxin production.[36] Furthermore, pre-exposure to *L. paracasei* resulted in an upregulation of dendritic cells, activation of helper T cells and antibody production, and downregulation of proinflammatory cytokines resulting in enhanced intestinal integrity and protection against enteric infection.[37] Probiotics have been widely tested to control *S. enterica* colonization and infection. Administration of one or several probiotic strains in broiler chicks inhibited *Salmonella* contamination.[38] A commercial probiotic cocktail significantly reduced *Salmonella* counts in the tonsils and ceca of chickens and poults.[39] Furthermore, administration of reuterin producing *L. reutri* strain significantly reduced *Salmonella* populations and increased the survival rate in chicks.[40] In vivo study using a mouse model demonstrated that continued administration of *L. casei* CRL diminished *Salmonella* counts in the intestine and extraintestinal dissemination.[41] *L. casei* Shirota strain also protected mice against lethal infection with multi-drug resistant *S.* Typhimurium DT104.[42] Besides the antimicrobial effects, probiotics also increased the performance and feed conversion in chickens and turkey poults. Probiotics were also effective against other enteric pathogens such as *Shigella sonnei*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*.[43] A bacteriocin (Microcin S) producing probiotic *Escherichia coli* G3/10 also suppressed EPEC adherence and pathogenesis.[44]

Recombinant Bioengineered Probiotics

As discussed above probiotics can be effective in the prevention and treatment of intestinal diseases. However, probiotic action is non-specific and non-discriminatory or ineffective in certain hosts.[45] This is in part due to broad mode of action and strain variability (**Table 2**). Probiotics differ from one another, therefore, the beneficial attributes of one strain or a cocktail of strains may not be reproducible and may vary from person to person.[46] Additionally, the probiotic strain, dose, route of administration, and the formulation of probiotic preparation can also affect the efficacy of a probiotic.[47] Furthermore, the manufacturing process

and probiotic delivery system have been shown to modify exopolysaccharide production by the probiotics and thereby modify their efficacy.[48,49] Recent studies on the gut microbiome diversity have revealed that the variability in the indigenous flora among different populations may also affect probiotic efficacy.[50] These limitations reinforce the need for novel and innovative approaches to design and create genetically modified probiotic strains to exclusively target a specific pathogen or toxin to be used either as a vaccine or for drug delivery.[51,52]

Over the last decade recombinant probiotics have been generated for mucosal delivery of therapeutic and prophylactic molecules including DNA, peptides, single-chain variable fragments, cytokines, enzymes, and allergens.[53,54] The major advantages of probiotic bacteria as delivery system are their (1) ability to colonize mucosal surface, (2) tolerance to gastric acid and bile salts enabling survival and transit through the gastrointestinal tract (GIT), and (3) sustained colonization and prolonged protection against pathogen.[53,55] Furthermore, oral recombinant probiotics offer several advantages: direct delivery of active molecule to the mucosal surface without the need for bio-separation of the active molecules, increased shelf-life and stability, low delivery costs, and ease of technology transfer following prototype development. This led to the concept of "biodrug" that is based on the oral administration of live recombinant microorganisms for the prevention and treatment of various diseases.[56]

In order to create therapeutically effective bioengineered recombinant probiotics, certain physiologic attributes are essential: (1) tolerance to stressors encountered during product manufacturing and storage, and during oral delivery, (2) strong mucosal colonization, (3) expression of target antigen under the gastrointestinal environment, and (4) potent antipathogenic action.

Bioengineering of Probiotics to Improve Stress Tolerance

Probiotics encounter stress during manufacturing, storage, and passage through the host GIT, namely temperature, acidity, salts, and water activity.[57] Physiologically, accumulation of compatible solutes helps stabilize protein function at low temperatures and prevent plasmolysis under low water activity. To improve stress tolerance in probiotic strains, the betaine transporter gene (*betL*) from *Listeria monocytogenes* was cloned into *Lactobacillus salivarius* under the control of the nisin inducible promoter.[58] Thus accumulation of betaine in recombinant *L. salivarius* enabled it to be osmotolerant (7% NaCl) and cryo- and baro-tolerant. Similarly, cloning of the trehalose synthesis gene (*ostAB*) from *E. coli* into *Lactococcus lactis* protected recombinant bacteria from freeze-drying, bile toxicity, and resistance to gastric acid.[59] Furthermore, cloning of *betL* into *Bifidobacterium breve* UCC2003 significantly improved its survival in gastric juice thus improving its therapeutic attributes.[60]

Antimicrobial Action of Bioengineered Probiotics

Receptor mimicry system and toxin neutralization. To achieve pathogen and/or toxin-specific activity, several strategies were employed to create bioengineered probiotics. Paton and colleagues[61] cloned and expressed toxin-specific host cell receptor on probiotic *E. coli* thus creating a competitive environment for toxin binding to host cells. They cloned glycosyltransferase genes from either *Nisseria mennigitidis* or *C. jejuni* on the surface of non-pathogenic probiotic *E. coli* to express chimeric lipopolysaccharide that mimics host cell receptor (ganglioside) for cholera toxin or ETEC heat labile toxin, LT. During infection enterotoxins are sequestered by the probiotic *E. coli* thus protecting host against diarrheal infection. In another study, *L. reuteri* was engineered to express ETEC heat stable (ST) and heat labile (LT) enterotoxins under the nisin inducible promoter. This recombinant probiotic successfully bound to the enterotoxins and prevented enterotoxicity in a mouse model. Furthermore, orally immunized mice with the toxin secreting recombinant *L. reuteri* increased serum IgG and mucosal IgA levels and protected animals from ETEC infection.[62]

Prevention of colonization. Cloning and expression of adhesins, toxins, or secretory systems of pathogens may serve as potential targets for the development of therapeutics to prevent infection.[63] Several strategies were employed to enhance probiotic adhesion to mucosal surface using gene products of target pathogen to create a competitive environment for pathogen colonization. Probiotics expressing adhesion factor LAP (*Listeria* adhesion protein) from *L. monocytogenes* was able to exclude pathogen colonization and prevented pathogen induced cell damage.[64] LAP is an adhesion factor in *L. monocytogenes* that interacts with the host cell receptor, heat shock protein 60 (Hsp60),[65-67] and promotes listerial adhesion and transepithelial translocation during intestinal phase of infection.[68,69] Pre-exposure of intestinal monolayers to the recombinant probiotic *Lactobacillus paracasei* expressing LAP followed by *L. monocytogenes* infection led to a reduction in adhesion, invasion and transepithelial translocation by 44, 45, and 46%, respectively[64] (**Fig. 1**). The recombinant probiotic also protected the epithelial monolayers from *L. monocytogenes* mediated cytotoxicity and tight junction compromise.

Similarly, *S. enterica* attachment was inhibited by using recombinant probiotic bacteria. *Lactococcus lactis* expressing flagellin of a probiotic strain of *Bacillus cereus* CH, adhered strongly to mucin-coated polystyrene plates in an in vitro experiment and competitively inhibited the adhesion of pathogenic *E. coli* and *S. enterica* to the same molecule.[70]

A recombinant *L. acidophilus* strain carrying the K99 fimbriae from ETEC was able to reduce the attachment of ETEC to porcine intestinal brush border in a dose dependent manner.[71] Similarly, *L. casei* was bioengineered to express ETEC adhesins K99 or K88[72] and the efficacy of the recombinant probiotic to protect host from ETEC infection was verified in a mouse model. Oral vaccination of mice with the recombinant strain resulted in high levels of mucosal IgA in bronchioalveolar lavage and intestinal fluids and systemic IgG response. The recombinant probiotic protected more than 80% of the vaccinated mice after challenge with a lethal dose of ETEC.[72] Likewise, *L. casei* expressing adhesion protein (intimin of EPEC) induced systemic and mucosal antibodies in mice and the antibodies inhibited the adhesion of EPEC in an in vitro epithelial cell culture model.[73] Employing

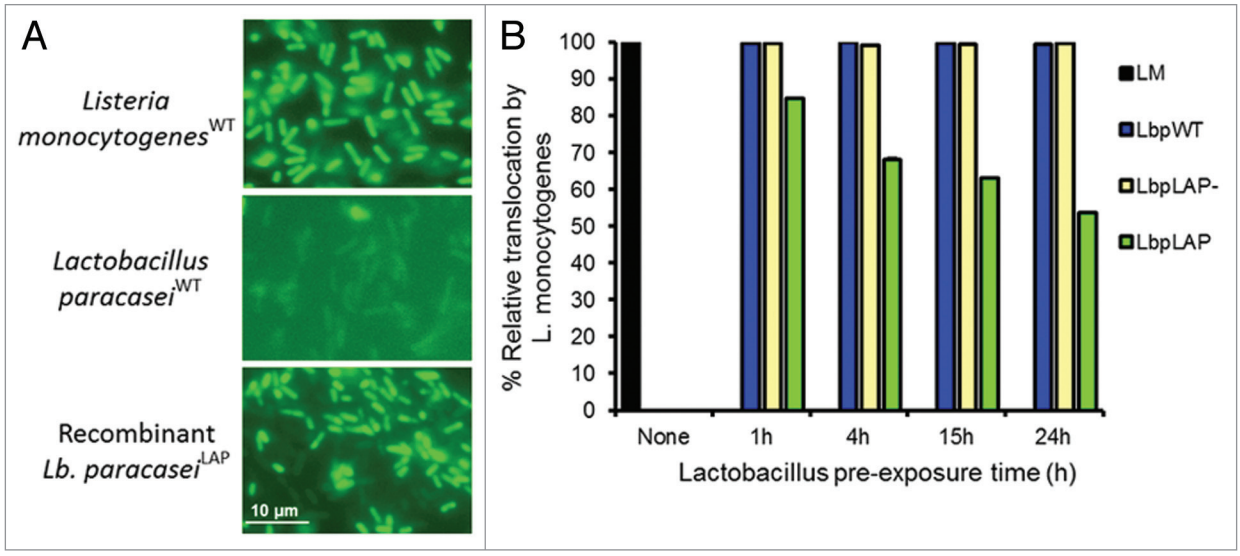

Figure 1. Inhibition of *Listeria monocytogenes* transepithelial translocation through epithelial barrier by bioengineered probiotic, *Lactobacillus paracasei* expressing Listeria adhesion protein (LAP). (**A**) Immunofluorescence staining of LAP expression by *Listeria monocytogenes*^{WT} and recombinant *Lb. paracasei*^{LAP}. (**B**) Recombinant *Lb. paracasei*^{LAP} (LbpLAP) showing about 46% reduction in *L. monocytogenes* translocation through epithelial barrier while, *Lb. paracasei*^{WT} (LbpWT) or *Lb. paracasei* containing empty vector (LbpLAP-) had no effect. Figures adapted from Koo et al.[64]

a similar approach, a recombinant *L. casei* strain expressing *S. Enteritidis* flagellar antigen FliC induced antigen specific protective immune response against *S. Enteritidis* in a mouse model.[74]

Similar strategies have also been adopted for viral pathogens. *L. jensenii* was engineered to secrete simian immunodeficiency virus (SIV) specific cyanovirin-N and the recombinant probiotic strain reduced the SIV infection by 62.9% in the Chinese macaque model.[75,76]

Regulation of virulence gene expression. Pathogenic bacteria have the ability to control the expression of virulence genes by sensing signals (termed quorum sensing) from their own species, other bacteria or their environment. Since the quorum sensing system senses population density, mediates colony-wide coordinated behavior, and controls virulence pathways,[77] interruption of quorum sensing pathway may serve as a viable option for disease prevention. *V. cholera* release cholera autoinducer-1 (CAI-1) and autoinducer-2 (AI-2) that accumulate when the population density increases at which point bacteria produce virulence factors. An AI-2 producing *E. coli* Nissle strain was engineered to co-express CAI-1, which suppressed virulence gene expression in *V. cholera* leading to its reduced lethality on infant mouse.[78]

Production of antimicrobial factors. Some probiotics produce several antimicrobial compounds and peptides as a defense mechanism against pathogens. Engineering of probiotics to detect pathogen signals for timed production of antimicrobials would be a novel approach. Saeidi et al.[79] engineered a commensal *E. coli* to detect signals from the pathogen for the production of bacteriocin.[80] *Pseudomonas aeruginosa* quorum sensing system (LasI/LasR) controls virulence gene expression. LasI produces homoserine lactone that activates LasR and leads to virulence gene expression. A bacteriocin producing probiotic *E. coli* strain was engineered to express LasR (to detect homoserine lactone) under the control of the luxR promoter and E7 lysis protein to aid in release of the bacteriocin. Co-culture of *P. aeruginosa* and recombinant *E. coli* led to a decrease in *P. aeruginosa* growth and biofilm formation by 99% and 90%, respectively.

L. casei engineered to express human lactoferrin exhibited antimicrobial activity in the gastrointestinal tract against enteric pathogens both in vitro and in vivo.[81] This recombinant strain also protected from pathogen-induced tissue injury. Bioengineered probiotics were also able to control pathogen transmission by insects. Insect synbiotics residing in the midgut of insect (reduviid bug) was engineered to express antimicrobial peptide, cercopin A and reduced the carriage of *Trypanosoma cruzi*, a causative agent for Chagas disease.[82]

Immunomodulation and cytoprotection. The most effective strategies to prevent enteric pathogen colonization in a host are to develop strains that can provide protection on the mucosal surface. It would also be easier to combat an enteric infection by blocking the infection rather than trying to eliminate the organism after the infection has already been established. Live mucosal vaccines are viable options in the prophylaxis of enteric infections since they target mucosal surfaces and elicit strong localized immune response. Recombinant probiotic bacteria would serve as ideal vectors because of their inherent ability to bind to mucosal surfaces thereby promoting effective contact between the antigen and the immune system. Additionally, colonization of the gut by live probiotic cells would enable continued production of the immunogenic molecule to stimulate humoral and cellular immune responses.[55]

Several studies have reported the use of attenuated pathogens as vaccines; however, a risk of virulence reversion in the attenuated strains especially in immunocompromised individuals exists. This can be overcome by the use of recombinant probiotics strains that can efficiently deliver the immunogenic molecule to the target mucosal surface.[83] Such recombinant probiotics have been engineered as vaccine delivery vehicles against *Yersinia*

pseudotuberculosis,[84] *S. typhimurium*,[39,85] and *Streptococcus pneumoniae* infection.[86] Similar recombinant vaccine was developed using *L. acidophilus* engineered to express protective antigen (PA) of *Bacillus anthracis* to activate dendritic cell to protect host against anthrax.[87] Likewise, expression of PA in *L. gasseri* also provided 100% protection against anthrax in a mouse model.[88]

Probiotics were also engineered to deliver vaccines to the mucosal surfaces. The first recombinant probiotic oral vaccine was developed by expressing the tetanus toxin fragment C in *Lactococcus lactis*.[89] Recombinant *Lc. lactis* strain expressing Internalin A protein of *L. monocytogenes* enabled this non-invasive probiotic to invade the small intestine and to deliver the immunostimulatory molecule inside the epithelial cells.[90]

To control rotavirus infection, several live attenuated vaccines using the human and/or bovine rotavirus strain have been developed; however, these were ineffective due to lack of robust mucosal immune response. To help elicit strong mucosal immune response, recombinant *L. paracasei* expressing the variable domain of llama heavy-chain antibody was developed against rotavirus. This antibody expressing probiotic was able to markedly reduce disease length, severity, and viral load in a mouse model.[91] In another study, recombinant *Lc. lactis* expressing rotavirus spike-protein VP8 induced mucosal IgA and anti-VP8 antibodies at both intestinal and systemic levels in a mouse model[92] and provided 100% protection against rotavirus challenge.

Besides the use of heterologous antigens to stimulate immune responses, expression of cytokines can also help in immunostimulation. Several probiotic strains have been engineered to express cytokines and other anti-inflammatory molecules to help suppress intestinal inflammation and provide cytoprotection. Murine IL-10, an immunosuppressive and anti-inflammatory cytokine was cloned and expressed in *Lc. lactis* strain, and the recombinant strain reduced inflammation and colitis in 40% of the mice.[93] Oral administration of IL-10 secreting probiotic in a colitis murine model resulted in a reduction in inflammatory symptoms. Human interferon-β (huIFN-β) is immunomodulatory and increases IL-10 expression. *Lc. lactis* secreting huIFN-β was shown to significantly reduce microbial colitis and inflamation.[94] In addition to huIFN-β, heme oxygenase-I (HO-1) has also been shown to modulate the anti-inflammatory effect of IL-10. *Lc. lactis* secreting HO-1, when administered in rats, prevented mucosal injury by LPS, reduced LPS-induced endotoxemia, and significantly increased survival rate in rats.[95] Oral immunization of mice with *L. casei* expressing IL-1β and heat-killed *S. Enteritidis* (SE) enhanced anti-SE antibodies demonstrating adjuvant properties of recombinant probiotics.[96] Recombinant *L. plantarum* surface displaying invasin protein of *Y. pseudotuberculosis* served as a potent activator of NF-κB and was demonstrated to be a promising mucosal delivery vehicle for vaccine antigen.[97] *L. acidophilus* was engineered to express hemagglutinin of the avian influenza virus H5N1 and induced strong mucosal and serum antibody response to H5N1.[98]

Safety of Probiotic Therapy and Biocontainment

The ultimate goal of developing a recombinant probiotic is its use in humans and animals. Prior to the approval of a recombinant probiotic for human use, it is essential that the bacteria be screened for potential pathogenicity and virulence traits.[10,21,99] Providing evidence for the absence of virulence properties is relatively straightforward in elucidating the pathogenic potential. Besides phenotypic characterization, it is also essential to genetically screen potential candidates for use as probiotics. Another critical consideration is the scope for antimicrobial resistance. In addition to being sensitive to antibiotics, it is also essential that the probiotic bacteria do not carry any transferrable antibiotic resistance genes, which can serve as genetic reservoirs for other potentially pathogenic bacteria. Besides acquisition of antibiotic resistant genes, there is also the risk for uptake of virulence genes from pathogens that co-inhabit the intestinal tract at the same time. However, there is no evidence in the literature for such event taking place in the gut. This could partly be due to the transient colonization of the gut by probiotics. Considering all the factors that are essential in assessment of safety of probiotic therapy, it is paramount that the general conclusion "probiotics are safe" cannot be broadly made. Prior to the use of a probiotic or probiotic cocktail in foods or dietary supplement, they need to be determined to be safe for the general population. Therefore, when intended for use as drugs, the safety assessment must balance risk with benefit.[99]

Another important consideration for genetically modified probiotic is preventing its accumulation in the environment and preventing lateral dissemination of the genetic material to other bacteria. The best approach to address this concern is to use a biological system that is propagated along with the probiotic termed as biological containment systems.[100] Biocontainment systems can be active or passive. Active containment involves the conditional production of a bacterial toxin through tightly regulated gene expression that is controlled by an environmental cue. Passive containment results in growth dependence on the complementation of an auxotrophy or gene defect, by supplementing another gene or essential metabolite.[101] Hillman[102] used the passive approach to contain recombinant *Streptococcus mutans*. They deleted the *alr* gene necessary for d-alanine synthesis that is essential for biosynthesis of cell wall. Similarly, Fu and Xu[103] developed a containment system for recombinant *L. acidophilus* using the thymidilmate synthase gene (*thyA*) from *L. casei* as a marker for plasmid maintenance.

Conclusions and Future Perspectives

Although probiotics have been used in food to enhance flavor or to provide health benefits, currently there is an increasing trend for their use in medicine. They provide a viable alternative especially in the treatment and prevention of enteric diseases. Over the years, several probiotics have been demonstrated to be effective against enteropathogens and their mode of action has been elucidated. A better understanding of the host pathogen interaction has also enabled the development of bioengineered probiotics that can be used for the targeted elimination of pathogens. The use of engineered probiotics helps overcome the short-half

life and stability of other therapeutic alternatives and also provides access to a cost-effective alternative. Recombinant probiotics can be used in a variety of applications. However, there is a need to contain the modified organism to prevent its uninhibited spread. Also, it is essential to consider their biosafety and their ability to cause allergy due to prolonged consumption. Although there are several hurdles in the development of safe and effective bioengineered probiotics, advancements in technologies and further refinements in techniques will continue to provide novel bio-therapeutics for the treatment and prevention of enteric infections both in rich and economically challenged countries.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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