Recombinant Technology and Probiotics

Icy D'Silva

Department of Food Science, Ontario Agricultural College, University of Guelph,

Guelph, Ontario N1G 2W1 Canada

idsilva@uoguelph.ca

Abstract - Recombinant technology has led the way to monumental advances in the development of useful molecules, including the development of safe probiotics. The development of novel approaches using recombinant technology and probiotics that allow accurate targeting of therapeutics to the mucosa is an interesting area of research. The creation and use of recombinant probiotics expressing recombinant ovalbumin, recombinant ovalbumin mutants and yet-to-be-designed recombinant hypo/non-allergenic molecules offer the opportunity to further investigate their effects for food, nutrition, environment and health. This review highlights advances in native probiotics and recombinant probiotics expressing native and recombinant molecules for food, nutrition, environment and health.

Keywords - Probiotics, Recombinant ovalbumin, Recombinant ovalbumin mutants, Recombinant technology

I. INTRODUCTION

Recombinant technology defined as transfer of the gene of interest from one organism to the plasmid of another organism, has led the way to monumental advances in the development of useful molecules, including the development of safe probiotics [1], [2], [3], [4]-[11]. Probiotics are live microorganisms having beneficial effects on human and animal health. Beneficial microbes have been used over the centuries for food, nutrition, environment and health, and are testimony of one of the ways to food safety. Probiotics, such as dietary lactic acid bacteria (LAB) present in yoghurt are non-invasive and non-pathogenic Gram-positive bacteria that are also present in the intestine. A minimum level of 10^6 viable probiotic bacteria per milliliter or gram of food is considered adequate although the amount of cells required to produce a beneficial health effect may vary according to the strain and the health benefit desired [12]. LAB are used for food processing and preservation, and are used as starter cultures, co-cultures and bioprotective cultures in the food industry [13]. In fermented foods, LAB have been shown to produce antimicrobial compounds, such as organic acids, bacteriocins and antifungal peptides. The United States Food and Drug Administration has categorized LAB as 'generally regarded as safe' (GRAS). Some of the functional effects of probiotics include strengthening of the gut mucosal barrier, antimutagenic/anticarcinogenic activities, stimulation of the immune system and lowering of blood cholesterol levels [14], [15], [16]. There is growing scientific evidence by mechanistic and clinical studies that probiotics have many health benefits, including modulation of host cell metabolism [17], reduction of lactose intolerance, maintenance of balanced gut microbiota [18], prevention of colonization by opportunistic pathogens [19] and treatment /prevention of allergies [20]. A correlation between intestinal microflora and allergic diseases has been epidemiologically demonstrated [21]. Epidemiological studies have shown that reduced consumption of fermented food is one of the factors associated with rise in allergic diseases. In animals, probiotics are used to improve health status and animal performance, while in ruminants probiotics are used to improve rumen fermentation, and may mitigate methane emissions known to contribute to global greenhouse effects [22].

While probiotics administered or consumed as a single species or as a mixture of species are being used as mucosal adjuvants, recombinant probiotics are increasingly being used to function as vehicles for the expression and targeted delivery of native or recombinant molecules to mucosal surfaces in nutrition and health [23]. The development of novel approaches using recombinant technology and probiotics that allow accurate targeting of therapeutics to the mucosa is an interesting area of research. Probiotic-mediated delivery systems obviate the need for large scale purification of molecules and enable delivery of molecules to the mucosa. The 'biodrug' concept is based on the oral administration of living recombinant microorganisms for the prevention or treatment of various diseases. This review highlights advances in native probiotics and recombinant probiotics expressing native and recombinant molecules for food, nutrition, environment and health.

II. SAFETY OF NATIVE AND RECOMBINANT PROBIOTICS

LAB are enterococci that are important in fermented foods for ripening and aroma development of certain cheeses and sausages, especially those produced in the Meditteranean, but can spoil processed meats and act as nosocomial pathogens causing bacteraemia, endocardities and other infections in humans. Some enterococci strains, such as *Enterococcus faecalis* and *Enterococcus faecium* harbor virulence traits, such as antibiotic resistance, adhesins, invasins and haemolysins [16]. Hence, it is imperative that each strain should be carefully evaluated for virulence determinants and sensitivity to clinically relevant antibiotics before being deemed suitable as a probiotic.

Therapeutic safety of recombinant probiotic carrier organisms is crucial, especially when the strain has to be used under diseased conditions. Westendorf et al. [24] tested the potential of recombinant *Escherichia coli* Nissle 1917 (rEcN) to serve as a safe carrier for targeted delivery of recombinant proteins to the intestinal mucosa. rEcN was first approved by Dutch authorities as a therapeutic agent for experimental therapy of intestinal bowel disease in humans. In a well-defined and very sensitive immunological system, it was demonstrated that intestinal rEcN had no effect on migration, clonal expansion and activation status of specific $CD4^+$ T cells in healthy mice and in animals with acute colitis. Furthermore, rEcN had no effect on the induction or breakdown of peripheral T-cell tolerance in an autoimmune environment.

Risk exposure determination, risk assessment and safety assessment are essential to ensure protection for the population against any unintended consequences of the use of probiotics [25], as well as against general farm-to-fork challenges. Food safety is Canada's top priority, and the safety of probiotics, both native probiotics and recombinant probiotics expressing native and recombinant molecules is paramount in food safety. Health Canada, the Canadian Food Inspection Agency, Fisheries and Oceans Canada, and Environment Canada have joint responsibility to determine the safety of products. Regulations defining the conditions for activities and materials associated with the testing, manufacture, preparation, preservation, packaging, administration, storage and sale of products are set out in the Canadian *Food and Drugs Act*, *Food and Drug Regulations* and *Medical Devices Regulations*. Countries, such as India are fast emerging as potential markets for probiotics in food, and guidelines have been drafted for evaluation of probiotics in food [26].

III. TECHNOLOGICAL PERFORMANCE OF RECOMBINANT PROBIOTICS

Probiotics being of intestinal origin are sensitive to many environmental stresses, such as acidity, oxygen and heat; and hence it is technologically challenging to produce them [12]. It is important that biotechnologically produced probiotics fulfill several criteria, including scientifically validated health properties; demonstrated safety; good technological properties to be produced on a large scale; viability, functionality and lack of unpleasant flavors or textures on incorporating into food products; high survival rates in downstream processes and in food products during storage; high survival through the upper gastrointestinal tract; and high viability at the site of action in the intestine. Viability of *Lactobacillus delbrueckii* ssp *bulgaricus* has been shown to be increased 10^4 times at pH 2 and in the presence of bile salts when encapsulated in artificial oil bodies [27].

Recombinant LAB strains with improved efficacy and potency expressing specific genes encoding probiotic functions, such as adhesion factors to mucus, resistance to acid, and with specific cell wall components have been constructed and characterized, and could help unravel mechanisms underlying the cross-talk between probiotic bacteria and their host. A recombinant derivative of *Lactobacillus paracasei* NFBC338 overproducing heat shock protein chaperones GroES and GroEL was shown to exhibit improved thermal tolerance and acquired solvent tolerance [28]. *L. paracasei* NFBC 338 heterologously expressing the *Pediococcus parvulus* 2.6 glycosyltransferase (*gtf*) gene responsible for catalyzing the conversion of sugar nucleotides into β-glucan when grown in the presence of glucose displayed a "ropy" phenotype with strands of polysaccharide linking neighboring cells, and the biosynthesis of β-glucan [29]. This strain survived both technological and gastrointestinal stresses suggesting that production of a prebiotic β-glucan exopolysaccharide by strains destined for use as probiotics allow greater performance/protection during cultivation, processing, and ingestion. β-glucan has been reported to be associated with many health-promoting properties. Functional display of a fibrolytic enzyme β-glucanase from *Fibrobacter succinogenes* fused to the C-terminus of *L. reuteri* collagen-binding protein Cnb on the cell surface of *L. reuteri* Pg4, a probiotic strain isolated from the gastrointestinal tract of a healthy broiler was used to study the adhesion capability of the enzyme [30]. Persisting LAB, such as *Streptococcus gordonii*, *Lactobacillus plantarum*, and *Lactobacillus casei*; and nonpersisting LAB, such as *Lactococcus lactis* have been investigated as strain-specific immunoadjuvants for the protective antigen C subunit of the tetanus toxin [31]. *Lactococcus lactis* has been extensively consumed by humans without any

associated form of pathology. Its main use lies in the manufacture of fermented milk, vegetable, and meat production. Recombinant *L. lactis* strains have been produced for synthesis and delivery of immunomodulatory proteins at the intestinal mucosa, and an adequate biologic containment system has been established [32], [33], [34]. A biologically contained *L. lactis* strain secreting human IL-10 was used in a phase I, open label clinical trial on Crohn's disease patients. This trial demonstrated that treatment of humans with viable *L lactis* secreting IL-10 is clinically and biologically safe and gave indications of its clinical efficacy [35]. Recombinant probiotic *Salmonella* strains were successfully used in DSS-treated animals [32], gene therapy of experimental colitis in mice [36], and as factories for production of recombinant therapeutic proteins [37], [38]. Recombinant strains producing sufficient antigen concentrations induced high serum IgG concentrations and high secretory IgA [39] while strains that co-delivered cytokines further enhanced host immune responses to prevent or treat inflammation in two murine colitis models [40], [41], [42]. Production and mucosal delivery of bioactive molecules, such as hypo/non-allergenic molecules, single-chain Fv antibodies, digestive enzymes and trefoil factors have been achieved in LAB [33], [43] for diseases, including vaginal candidiosis, dental caries, allergies, autoimmune diseases, HPV-induced tumors, and pancreatic insufficiency.

Although evidence of yeast strain variation with respect to immunological effects in the mammalian gut is becoming available [44], the probiotic yeast *Saccharomyces boulardii* taxonomically indistinguishable from strains of *S. cerevisiae* is the only strain demonstrated to suppress intestinal inflammation caused by a broad range of enteric pathogens [45]. It is particularly useful in the treatment of antibiotic induced diarrhoea and *Clostridium difficile* infections, and appears to act by modulating host signalling pathways through inhibition of interleukin-8 production. Improved characterization will drive further research into characterization of yeast-derived bioactive compounds in the gut, with clear opportunities for further biotechnological innovation [46]. A *S. cerevisiae* strain expressing plant cytochrome P450 73A1 (cinnamate-4-hydroxylase [CA4H] activity) used to study its survival and ability to bioconvert *trans*-cinnamic acid (CIN) into *p*-coumaric acid (COU) in the digestive tract of rats was found to be resistant to gastric and small intestinal secretions but was more sensitive to the conditions found in the large intestine [47]. Oral co-administration of the yeast and CIN resulted in increased CA4H activity, with COU being found throughout the rat's digestive tract and in its urine within the first 5 min. It is worth mentioning here that *Pichia pastoris* has become the favoured yeast vehicle for the production of proteins, both due to its higher protein secretion capacity and due to the very high cell densities that it achieves in industrial fermenters [46].

IV. RECOMBINANT PROBIOTICS AGAINST ALLERGY

Allergens are a serious food, nutrition, environment and health concern. There is a need for prevention and treatment of allergies, including diarrhea and asthma caused by food allergens, such as native egg ovalbumin. Clinical trials have shown a reduced incidence of allergic symptoms after ingestion of particular LAB strains [48]. The protective capacity of recombinant LAB strains against a food allergen β-lactoglobulin has been demonstrated [49]. A major advantage of mucosal application of recombinant LAB is the potential to induce local immune responses at the antigen-exposed mucosal site [43].

rEcN has excellent colonization properties rendering it as an ideal carrier for gut-focused *in situ* synthesis of therapeutic molecules [24]. Adam et al. [50] reported that EcN represents an efficient adjuvant to prevent allergic responses. EcN activated dendritic cells through TLR4 signaling pathway dependent up-regulation of CD40, CD80 and CD86 and stimulation of NF-κB and MAPK. Intra-nasal co-administration of EcN with recombinant dust mite allergen ProDer p 1 in a murine model of mite allergy prevented subsequent allergic response following Der p 1 sensitization and airway challenge with aerosolized mite extracts through the induction of an allergen-specific IgG2a response, the prevention of specific IgE production and a strong reduction of IL-5 secretion by allergen-restimulated splenocytes. EcN alone or in combination with ProDer p 1 inhibited the development of airway eosinophilia and neutrophilia. This *in vivo* protective effect of EcN was, in part, mediated by TLR4 signaling.

Subcutaneous immunization, as well as intranasal pretreatment of mice with recombinant *Lactobacillus plantarum* and *Lactococcus lactis* strains producing the major birch pollen allergen Bet v I lead to a shift towards non-allergic Th1 immune responses along with enhanced allergen-specific mucosal IgA levels thus offering a promising approach to prevent systemic and local allergic immune responses [51]. Mucosal delivery of OVA by genetically modified *L. lactis* induces suppression of local and systemic OVA-specific T cell responses in OVA T cell receptor transgenic mice, this effect being dependent on both OVA and the bacterium. Oral administration resulted in induction of systemic tolerance mediated by antigen specific CD4+CD25- regulatory T cells that seem to function through a TGF-β-dependent mechanism. The intestinal delivery system

through the oral route by *L. lactis* is superior than oral administration of low or high doses of soluble OVA antigen as the latter were not able to diminish delayed hypersensitive response [52].

Recombinant hypo/non-allergenic molecules exhibit Th1 promoting capacity and offer less invasive treatments when administered as mucosal vaccines. Several studies show that oral administration of *Bifidobacterium* or *Lactobacillus* alleviate food allergy [53]. Oral administration of *B. bifidum* BGN4 inhibited serum IgE and IgG1 production in a food allergy mouse model [54], while *L. casei* Shirota suppressed antigen-specific IgE by stimulating secretion of IL-12 [55]. Spleen cells of the mice administrated with *L. casei* Shirota produced high levels of Th1-associated cytokines, such as IFN-γ and IL-2, but low levels of Th2-associated cytokines, such as IL-4, IL-5, IL-6 [56]. Promotion of anti-allergenic processes through maintenance of balance on Th1 and Th2 cytokines [57], [58] and enhancement of regulatory lymphocytes [59], [60] by specific probiotic species have been reported. Torii et al. [61] supported this hypothesis by showing that oral administration of *L. acidophilus* L-92 suppressed ovalbumin (OVA)-specific IgE production through regulation of Th1 and Th2 cytokines and induction of Treg associated TGF-β production. Additionally, the specific IgE suppressive effect of *B. bifidum* G9-1 was suggested to be mediated by Treg cells independent of IFN-γ production [20]. Supplementation with *L. acidophilus* resulted in an increased allergic sensitization rate – strain specificity [62]. Some commercial probiotics strains and different *L. acidophilus* strains have the potential to induce differential cytokine profiles [53], [63].

Native egg ovalbumin is a phosphoglycoprotein constituting 58% of the protein fraction of the egg white and is a common ingredient in processed foods. It is used to add nutritional value, texture and taste to food, in addition to its use as an emulsifying agent, but it is also an allergen. Recombinant ovalbumin (rOVA) and eight recombinant ovalbumin mutants (rOVAm) were designed and developed [4], [5]. The recombinant molecules were tested for their protective efficiency as orally administered immunospecific agents against anaphylaxis / egg allergy in a mouse model, and their mechanisms of action were studied [4], [5], [8]. The creation and use of recombinant probiotics expressing rOVA, rOVAm and yet-to-be-designed recombinant hypo/non-allergenic molecules offer the opportunity to further investigate their effects for food, nutrition, environment and health.

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