

Prospective Uses of Genetically Engineered Lactic Acid Bacteria for the Prevention of Inflammatory Bowel Diseases

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1. Introduction

Inflammatory bowel disease (IBD) is a term used to describe a group of intestinal disorders in which inflammation is a major feature. Although rare forms of IBD exist, these diseases normally pertain to ulcerative colitis (UC) (Head & Jurenka, 2003) and Crohn's disease (CD) (Baumgart & Sandborn, 2007). There is evidence that these do not represent distinct conditions but rather are the same disease with shared etiological factors (Price, 1992); however, clinical manifestations (such as the exact location of the pathology or the affected individual's immunological and constitutional endowment) are distinctive between both.

Despite many years of study, the exact etiology and pathogenesis of these disorders remain unclear but great advances have been made using experimental animal models and have provided insights into the complex, multi-factorial processes and mechanisms that can result in chronic intestinal inflammation (Elson & Weaver, 2003).

The aim of this chapter is to present an overview of the current expanding knowledge of the mechanisms by which lactic acid bacteria and other probiotic microorganisms participate in the prevention and treatment of IBD and how genetic engineering techniques can be used to improve their effectiveness or create novel therapeutic strains. In the following sections, the mechanisms by which these beneficial microorganisms exert their therapeutic effects, which include changes in the gut microbiota, stimulation of the host immune responses, enhancement of intestinal barrier function and reduction of the oxidative stress due to their antioxidant properties will be discussed.

2. Lactic acid bacteria and inflammatory bowel diseases

Lactic acid bacteria (LAB) constitute a phylogenetically heterogeneous group of ubiquitous microorganisms that are naturally present in high nutrient containing organic products such

as foods and occupy a wide range of ecological niches ranging from the surface of plants to the gastro-urogenital tract of animals. Currently, the LAB group includes a large number of cocci and bacilli, such as species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*, that normally contain a G+C content inferior to 55% in their chromosomal DNA. Although quite diverse, the members of this group have various characteristics in common, that include being: (i) Gram-positive; (ii) facultative anaerobes; (iii) non-sporulating; (iv) non-motile and (v) possess the capacity to convert sugars into lactic acid (Nouaille et al., 2003). LAB are one of the most important industrial groups of bacteria that are widely used in food production, health improvement and production of macromolecules, enzymes and metabolites.

From a historical point of view, LAB have been used since ancient times in food fermentation processes and preservation. Since the 1980's, many efforts have been made to better understand the molecular basis of LAB's technological properties in order to control the industrial processes involving these important microorganisms. Due to their lack of pathogenicity, most LAB species have received the GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration. In addition to their important technological properties in food production (production of lactic acid, decrease of lactose, improvement of organoleptic and physical characteristics), various species of LAB, such as *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus reuteri*, have been shown to possess therapeutic properties since they are able to prevent the development of some diseases as shown mostly using animal models and have the capacity to promote beneficial effects in human and animal health (LeBlanc et al., 2008). Because of all of their documented beneficial effects, certain strains of LAB have been designated as being *probiotic* that have been defined by the FAO/WHO as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001). Some of the health benefits which have been claimed for probiotics include: improvement of the normal microbiota, prevention of infectious diseases and food allergies, reduction of serum cholesterol, anticarcinogenic activity, stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms, and improvement of the digestion of lactose in intolerant hosts (Galdeano et al., 2007). The most commonly used strains as probiotics are members of lactobacilli, enterococci and bifidobacteria groups (Ouwehand et al., 2002). Currently, many products containing probiotics are available on retail shelves throughout the world because of the increase consumer demand for healthier natural foods that can increase their overall well-being (Galdeano et al., 2007). The specific health effects of selected probiotic strains have been confirmed by well documented double blind controlled human clinical trials and are becoming increasingly accepted. However, many proposed beneficial effects of probiotics still need further research and more information about their mechanisms of action is needed in order to confirm that they can be useful in the prevention and treatment of other specific diseases (Ouwehand et al., 2002).

2.1 Mechanisms of action of LAB against IBD

It has been shown that LAB and other probiotic microorganisms can counteract inflammatory processes in the gut by stabilizing the microbial environment and the permeability of the intestinal barrier, and by enhancing the degradation of enteral antigens

and altering their immunogenicity (Isolauri et al., 2004). Some of these disease preventing effects have been recently reviewed (del Carmen et al., 2011); however, a few examples of each of the mechanisms by which LAB exert anti-IBD effects will be given to demonstrate their potential uses.

2.1.1 Modulation of the gut microbiota

The first mechanism that LAB use to prevent IBD is the modulation of the gastrointestinal microbiota of animals. It was reported that *Lactobacillus (L.) reuteri* could be used to prevent colitis in IL-10 knock-out (KO) mice by increasing the number of LAB in the gastrointestinal tract (Madsen et al., 1999). Neonatal mice presented a decreased concentration of colonic *Lactobacillus* species and an increased concentration of mucosal adherent bacteria. Oral administration of the prebiotic lactulose increased the levels of *Lactobacillus* species and rectal swabbing with *L. reuteri* restored *Lactobacillus* levels to normal and reduced the number of adherent bacteria within the colon. These effects were associated with the attenuation of UC (Madsen et al., 1999). In a placebo-controlled trial, orally administered *L. salivarius* UCC118 reduced prevalence of colon cancer and mucosal inflammatory activity in IL-10 KO mice by modifying the intestinal microbiota in these animals: *Clostridium (C.) perfringens*, coliforms, and enterococcus levels were significantly reduced in the probiotic fed group (O'Mahony et al., 2001).

Gut microbiota can antagonize pathogenic bacteria by conferring a physiologically restrictive environment inhibiting bacterial adherence and translocation. Probiotic bacteria also decrease luminal pH, as has been demonstrated in patients with UC following ingestion of the probiotic preparation VSL#3, a mixture of 4 lactobacilli strains (*L. plantarum*, *L. casei*, *L. acidophilus*, and *L. delbrueckii ssp. bulgaricus*), 3 bifidobacteria strains (*Bifidobacterium (B.) infantis*, *B. breve*, and *B. longum*), and 1 strain of *Streptococcus (S.) salivarius ssp. thermophilus* (Venturi et al., 1999).

Another mechanism by which probiotics can exert a positive effect by inhibiting pathogenic microorganisms is by producing antimicrobial substances such as bacteriocins. Several bacteriocins produced by different *Lactobacillus* species have been described (Klaenhammer, 1988). The inhibitory activity of these bacteriocins varies; for example, the probiotic *L. salivarius* UCC118 produces a peptide that inhibits a broad range of pathogens such as *Bacillus*, *Staphylococcus*, *Enterococcus*, *Listeria*, and *Salmonella* species (Flynn et al., 2002). Lacticin 3147, a broad-spectrum bacteriocin produced by a *Lactococcus (Lc.) lactis* strain, inhibits a range of genetically distinct *C. difficile* isolated from healthy subjects, patients with IBD and from different origins (Rea et al., 2007).

The Symbiotic Instant Mixture (SIM) containing a prebiotic compound inulin, and a combination of probiotic microorganisms (*L. acidophilus* La-5 and *B. lactis* Bb-12) significantly reduced inflammation in transgenic rats that produce human HLA-B27- β 2-microglobulin. The effect was enhanced by combination with metronidazole, suggesting a synergistic effect of the combination of antibiotics and probiotics in the treatment of experimental colitis (Schultz et al., 2004). In a double-blind randomized study, the efficacy of VSL#3 combined with antibiotic treatment on the post-operative recurrence of CD was compared with treatment with mesalazine alone. Combination of antibiotic and probiotic treatment was more efficient in prophylaxis of post-operative recurrence of Crohn's disease (Campieri et al., 2000).

2.1.2 Modulation of the host immune responses and enhancement of intestinal barrier function

The intestinal mucosa is the body's first line of defense against pathogenic and toxic invasions from food. After ingestion, orally administered antigens encounter the GALT (Gut Associated Lymphoid Tissue), which is a well-organized immune network that protects the host from pathogens and prevents ingested proteins from hyperstimulating the immune response through a mechanism called oral tolerance (Weiner et al., 1997).

The main mechanism of protection given by the GALT is humoral immune response mediated by secretory IgA (s-IgA) which prevents the entry of potentially harmful antigens, while also interacting with mucosal pathogens without potentiating damage. The stimulation of this immune response could thus be used to prevent certain infectious diseases that enter the host through the oral route. An increasing number of probiotic strains have shown to highly increase s-IgA, therefore the stimulation of IgA-producing cells is often considered a must in probiotic screening trials (O'Sullivan, 2001).

Numerous studies have shown that certain strains of lactobacilli and bifidobacteria can modulate the production of cytokines (mediators produced by immune cells) that are involved in the regulation, activation, growth, and differentiation of immune cells and have been recently reviewed (de Moreno de LeBlanc et al., 2011). These probiotic microorganisms are able to prevent and treat certain inflammatory diseases in the gastrointestinal tract through the repression of pro-inflammatory cytokines. In this sense, the anti-inflammatory effect of yoghurt administration to mice was studied using a TNBS induced acute intestinal inflammation model. The animals that received yoghurt continuously (before and after TNBS) had lower intestinal damages and improved anti-inflammatory response in the gut (de Moreno de LeBlanc et al., 2009). This improvement of the immune response was related with beneficial changes in the intestinal microbiota and differences in the cytokine production and secretion with increases of IL-10 and decreases of IL-17 in the mice given yoghurt. The same yoghurt was analyzed in a mouse model of recurrent inflammation where the administration of the fermented product after the acute episode, when the animals were recovered, prevented the recurrence of the inflammation (Chaves et al., 2011).

One of the central transcription factors mediating inflammatory responses is the nuclear factor κ B (NF- κ B). NF- κ B is required for the transcriptional activation of a number of inflammatory effectors, including IL-8, TNF- α , IL-6, Cox2, iNOS and many others and, its deregulation has been detected in many inflammatory conditions. It was shown that a number of LABs can suppress inflammatory signals mediated by NF- κ B. These include strains of the phylogenetically closely related species *L. acidophilus* and *L. johnsonii*, which have been isolated from the human GI tract and form part of the acidophilus complex.

One of the ways by which probiotics can exert immunomodulatory activities is by increasing IL-10 production that can in turn help in preventing certain IBD that are caused by abnormal inflammatory responses (de Moreno de LeBlanc et al., 2011). However, not all probiotic strains act in the same manner. Anti-inflammatory effects, such as stimulation of IL-10 producing cells, are strain dependent traits, and their effectiveness also depends on the concentrations used and the method of administration. By increasing IL-10 levels and in consequence decreasing inflammatory cytokines such as TNF- α , IFN- γ , and IL-17 some LAB can prevent the appearance of local inflammatory diseases and could be used as an adjunct therapy with conventional treatments (de Moreno de LeBlanc et al., 2011).

Enhancement of intestinal barrier function is another mechanism by which probiotic bacteria may benefit the host. The exact mechanisms by which probiotic bacteria enhance

gut mucosal barrier function are unclear, but may relate to alterations in mucus or chloride secretion or changes in mucosal cell-cell interactions and cellular stability through modulation of cytoskeletal and tight junction protein phosphorylation (Hilsden et al., 1996, Madsen et al., 2001, Meddings, 2008, Ng et al., 2009, Schmitz et al., 1999).

Oral treatment with VSL#3 normalized colonic physiologic function and barrier integrity in IL-10 KO mice as assessed by short circuit currents, transepithelial potential differences, and mannitol fluxes in excised tissues from mice (Madsen et al., 2001).

L. plantarum and *L. reuteri* enhanced barrier function on a methotrexate-induced enterocolitis rat model (Mao et al., 1996); and colonization of healthy mice intestinal loops with *L. brevis* reduced intestinal permeability (Garcia-Lafuente et al., 2001).

Some probiotic bacteria modify MUC gene expression and mucus secretion. For example, *L. plantarum* 299v induced intestinal mucin gene (MUC2 and MUC3) expression in vitro (Mack et al., 1999). VSL#3 also induced expression of mucins in vitro and increased transepithelial resistance (TER), prevented pathogen-induced decrease in TER, and stabilized tight junctions. In this way, probiotics and protein(s) released by these organisms may functionally modulate the intestinal epithelium of the host by different mechanisms, including the competition of whole organisms for contact with the epithelial surface as well as stabilization of the cytoskeleton and barrier function and the induction of mucin expression (Otte & Podolsky, 2004).

2.1.3 Reduction of the oxidative stress

As a result of recurrent and abnormal inflammation, IBD appears to be associated with oxidative stress, which is characterized by an uncontrolled increase in reactive oxygen species (ROS) concentrations in the gastrointestinal tract. Several studies have established a correlation between the increase in ROS production and disease activity in inflamed biopsies of IBD patients. Therefore, a suggested mechanism by which LAB could prevent inflammation is through the expression of antioxidant enzymes that are able to decrease ROS levels or at least impair their formation.

ROS are normal byproducts of oxygen metabolism (such as superoxide ions, free radicals and peroxides). These small molecules can be generated in aerobiosis by flavoproteins and by phagocytes during inflammatory reactions. At low concentrations, ROS participate in cell signaling and regulatory pathways. However, when present in large amounts, they act to eliminate infectious agents by causing significant damages to cell structures and macromolecular constituents such as DNA, RNA, proteins and lipids. When ROS concentration exceeds the capacity of cell defense systems, toxicity is triggered. It is well known that oxidative damage occurs during the pathogenesis of cancer, cirrhosis, atherosclerosis and other chronic diseases. It has been shown for example that human tumor cells produce and excrete large amounts of H₂O₂ that might participate in tumor invasion and proliferation (Szatrowski & Nathan, 1991). Thus, oxidative stress plays an important role in pathologies of the gastrointestinal tract of humans such as IBD as well as in certain types of cancers.

In order to offset oxidative stress, aerobic cells like those of the normal intestinal mucosa are equipped with a complex antioxidant defense system which includes enzymatic and non-enzymatic components having synergistic and interdependent effects on each other. The normal intestinal mucosa is equipped with a network of antioxidant enzymes such as catalase (Cat), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione-s-transferase (GST), and superoxide dismutase (SOD) that are able to neutralize ROS. The

activities of these enzymes are usually balanced to maintain a low and continual steady-state level of ROS; however, the levels of these enzymes are frequently depleted in IBD patients (Kruidenier et al., 2003). Probiotic LAB strains expressing high levels of antioxidant enzymes could increase these enzymatic activities in specific locations of the gastrointestinal tract and could thus contribute to prevent oxidative epithelial damages, giving rise to potential applications for IBD treatment or post-cancer drug treatments. Since few microorganisms produce antioxidant enzymes at the required concentrations to exert biological effects, genetic engineering strategies have been employed to produce antioxidant producing LAB, these will be discussed in another section of this chapter.

Superoxide dismutase (SOD) is considered as the first line of defense against ROS and is a member of the family of metalloenzymes that catalyze the oxido-reduction of superoxide anion to H₂O₂. There are three different forms of this enzyme according to their metal center: manganese, copper-zinc, or iron. These enzymes are found across a broad range of organisms, which can use one, two, or all three enzymes to meet their antioxidant needs. In most *Streptococcus* and *Lactococcus* spp., elimination of ROS is accomplished through the action of Mn-SOD (Sanders et al., 1995). It has also been reported that two strains of *L. fermentum*, named E-3 and E-18, and a strain of *S. thermophilus* showed significant antioxidative activity due to production of Mn-SOD (Kullisaar et al., 2002). Furthermore, recent experimental data indicate that subcutaneous treatment with SOD significantly reduces peroxidation reactions in the inflamed colon and confers significant amelioration of colonic inflammatory changes in a rat model of TNBS colitis (Segui et al., 2004). In addition, treatment with SOD decreases oxidative stress and adhesion molecule upregulation in response to abdominal irradiation in mice.

Catalase is another major antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen. Catalases are widespread in aerobic (facultative or not) bacteria such as *E. coli* and *Bacillus (B.) subtilis* (Rochat et al., 2005). There are two different classes of catalases according to their active-site composition: one is heme-dependent and the other, also named pseudocatalase is manganese-dependent. By definition, LAB are catalase negative microorganisms, thus genetic modifications are necessary in order for them to produce this important antioxidant enzyme.

3. Genetics of LAB and recombinant protein production strategies

Comparative genomics has facilitated our understanding of LAB evolution and has indicated that a combination of gene gain and loss occurred during the evolution of these bacteria in various environmental habitats (Makarova et al., 2006). Beginning with the genome sequencing of *Lc. lactis* IL-1403 in 1999 (Bolotin et al., 1999), by July 2011 there were 314 LAB genomes (98 complete and 216 in progress) publicly available at the National Center for Biotechnology Information (NCBI, 2011).

Lc. lactis is widely used in the dairy industry and is normally used as the model for other LAB, not only because of its economic importance, but also because of the following features: (1) it has a completely sequenced genome; (2) it is genetically easy to manipulate; and (3) many genetic tools have already been developed for this species.

Studies based on the identification and isolation of wild-type plasmids from *L. lactis* and other LAB have made it possible to develop various cloning vectors. Using molecular biology techniques, these plasmids have been manipulated so that they have become important tools for cloning and studying genes of interest, both those of prokaryotes and

eukaryotes. They basically consist of (1) origin of replication (*ori*), (2) selection marker (gene) for antibiotic resistance, and (3) multiple-cloning site.

The expression of heterologous proteins in *Lc. lactis* has been favored both by advances in genetic knowledge and by new developments in molecular biology techniques. Using this duet of tools to obtain increased levels of these proteins and control their production, various vectors containing constitutive or inductive promoters were developed and currently constitute the basis of all expression systems in *Lc. lactis* and other LAB.

3.1 LAB expression systems

LAB are potential candidates to be used as vehicles for the production and delivery of heterologous proteins of vaccinal, medical, or technological interest and various delivery systems are now available for these probiotic microorganisms (Miyoshi et al., 2010). Different genetic engineering strategies in LAB have been used to: improve their carbohydrate fermenting properties (lactose, galactose), increase specific metabolite production (diacetyl, acetoin), produce or increase enzymatic activities (proteolytic enzymes, α - and β -galactosidase, α -amylase), or conferring them the capacity to produce beneficial compounds such as bacteriocins, exopolysaccharide (EPS) and other sugars, vitamins, antioxidant enzymes and anti-inflammatory cytokines (Sybesma et al., 2006).

A series of studies to develop new strains and efficient expression systems has been conducted to use LAB as “cell factories” for the production of proteins (Djordjevic & Klaenhammer, 1998). However, in order for some of these proteins (enzymes and antigens) produced by bacteria to attain the desired biological activity levels, it is necessary that they correctly target specific cellular locations: (1) cytoplasm, (2) membrane, or (3) extracellular environment.

3.1.1 Nisin-Controlled Expression (NICE) system

A versatile and tightly controlled gene expression system was first described in 1995, based on the auto-regulation mechanism of the bacteriocin nisin, denominated the Nisin-Controlled Expression (NICE) system (Kuipers et al., 1995). This system has become one of the most successful and widely used tools for regulated gene expression in Gram-positive bacteria. An extensive overview of the different applications in lactococci and other Gram-positive bacteria has been published 10 years after the NICE system was first published (Mierau & Kleerebezem, 2005) showing its potential use in: (1) over-expression of homologous and heterologous genes for functional studies and to obtain large quantities of specific gene products, (2) metabolic engineering, (3) expression of prokaryotic and eukaryotic membrane proteins, (4) protein secretion and anchoring in the cell envelope, (5) expression of genes with toxic products and analysis of essential genes and (6) large scale applications.

3.1.2 Xylose-Inducible Expression System (XIES)

Another controlled production system to target heterologous proteins to cytoplasm or extracellular medium was described for *Lc. lactis* NCDO2118 based on the use of a xylose-inducible lactococcal promoter, PxyIT (Miyoshi et al., 2004). The capacities of the Xylose-Inducible Expression System (XIES) to produce cytoplasmic and secreted proteins were tested using the *Staphylococcus aureus* nuclease gene (*nuc*) fused or not to the lactococcal Usp45 signal peptide. Xylose-inducible gene expression is tightly controlled and resulted in

high-level and long-term protein production, and correct targeting either to the cytoplasm or to the extracellular medium. Furthermore, this expression system is versatile and can be switched on or off easily by adding either xylose or glucose, respectively, and has potential as an alternative and useful tool for the production of proteins of interest in *Lc. lactis*.

3.1.3 pValac

A new plasmid vector for DNA delivery using lactococci, the pValac plasmid was constructed by the fusion of: i) an eukaryotic region, allowing the cloning of an antigen of interest under the control of the pCMV eukaryotic promoter to be expressed by a host cell and ii) a prokaryotic region allowing replication and selection of bacteria (Guimaraes et al., 2009). In order to evaluate pValac functionality, the *gfp* ORF was cloned into pValac (pValac:*gfp*) and was analyzed by transfection in PK15 cells. Invasiveness assays of *Lc. lactis* *inlA*+ strains harbouring pValac:*gfp* into Caco-2 cells demonstrated the potential of pValac to deliver DNA and trigger DNA expression by epithelial cells.

4. Prevention of IBD using genetically modified LAB

As described above, LAB are potential candidates to be used as vehicles for the production and delivery of heterologous proteins of vaccinal, medical, or technological interest and various delivery systems are now available for these probiotic microorganisms (Miyoshi et al., 2010). The use of LAB that produce anti-inflammatory compounds (such as IL-10 and antioxidant enzymes) in the treatment of colitis and IBD will be discussed in the following sections.

4.1 Antioxidant enzyme producing strains

As stated earlier, in IBD patients, oxidative stress occurs as a result of recurrent and abnormal inflammation with an associated increase in ROS concentrations.

Since few microorganisms produce antioxidant enzymes at concentrations required to exert biological effects, genetic engineering strategies have been employed to produce antioxidant producing LAB. Recent reviews have shown the potential uses of such strains in the treatment of IBD using a variety of animal models (Spyropoulos et al., 2010). LAB have been used to locally deliver antioxidant enzymes (such as SOD) directly to the intestines, an important breakthrough since oral administration of SOD is greatly limited by its short lifespan (5-10 min) in the hostile conditions of the gastrointestinal tract. It has been shown that genetically engineered *L. plantarum* and *Lc. lactis* capable of producing and releasing SOD exhibit anti-inflammatory effects in a TNBS colitis model (Han et al., 2006). Another experimental study demonstrated that *L. gasseri* producing manganese SOD had significant anti-inflammatory activity reducing the severity of colitis in IL-10-deficient mice (Carroll et al., 2007). Recent data has shown that SOD producing *L. casei* BL23 was able to significantly attenuate the TNBS-induced damages as shown by higher survival rates, decreased animal weight loss, lower bacterial translocation to the liver and the prevention of damage to the large intestines (LeBlanc et al., 2011). This is in agreement with previous results that have shown that the same SOD-expressing strain of *L. casei* was able to slightly attenuate the colonic histological damage score of a DSS-induced colitis model (Watterlot et al., 2010). Since *Lc. lactis* has no catalase (as is also the case for the majority of LAB), the *B. subtilis* heme catalase Kat E gene was introduced into this industrially important microorganism

giving rise to a strain capable of producing active catalase that can provide efficient antioxidant activity (Rochat et al., 2005). Recently, the heterologous expression of non-heme catalase in bacteria relevant to dairy industries (*L. casei*) has also been reported (Rochat et al., 2006). This latter strain offers the advantage that no exogenous heme has to be added to the culture medium in order to exert an efficient catalase activity. We have previously shown that the catalase-producing *Lc. lactis* strain was able to prevent tumor appearance in the colon (de Moreno de LeBlanc et al., 2008). In another study, we have shown that a catalase producing strain of *L. casei* BL23 significantly decreased the physiological damages caused by the TNBS administration (LeBlanc et al., 2011). This result is similar to those obtained previously where it was shown that both the native strain of *L. casei* BL23 and its catalase producing derivative presented a significant reduction of caecal and colonic inflammatory scores (Rochat et al., 2007).

4.2 IL-10 producing strains

Genetically modified *Lc. lactis* secreting IL-10 provides a novel therapeutic approach for IBD. The first description of *Lc. lactis* that can secrete biologically active IL-10 was published in 2000 (Schotte et al., 2000). In this pioneer study, murine-IL-10 was synthesized as a fusion protein, consisting of the mature part of the eukaryotic protein fused to the secretion signal of the lactococcal Usp45 protein. Intragastric administration of this recombinant *Lc. lactis* strain prevented the onset of colitis in IL-10 KO mice and caused a 50% reduction of the inflammation in DSS-induced chronic colitis (Steidler et al., 2000).

The application of IL-10 producing LAB is not only limited to the treatment of IBD. It was recently shown that treatment of asthma with a *Lc. lactis* expressing murine IL-10 was efficient since this LAB modulated experimental airway inflammation in the mouse model (Marinho et al., 2010). *Lc. lactis* producing recombinant IL-10 used in this study was efficient in suppressing lung inflammation, independently of Treg cells, since this cytokine plays a central role in the regulation of inflammatory cascades, allergen-induced airway inflammation and non-specific airway responsiveness (Tournoy et al., 2000). In another study, it was shown that oral administration of an IL-10-secreting *Lc. lactis* strain could prevent food-induced IgE sensitization in a mouse model of food allergy (Frossard et al., 2007). These studies confirm that IL-10 secreting LAB hold potential for the treatment of many inflammatory diseases where this cytokine acts as a modulating compound.

Although a clear positive effect of these recombinant strains has been demonstrated, the exact mechanism by which the beneficial effect of the IL-10-producing *Lc. lactis* on the mucosa is produced remains unclear. A recent study has demonstrated the uptake of IL-10-secreting *Lc. lactis* by the paracellular route in inflamed mucosal tissue in mouse models of chronic colitis, suggesting that IL-10 production by these LAB residing inside the mucosa in the vicinity of responsive cells can improve the local action of IL-10 in inflamed tissue and the efficiency of the treatment (Waeytens et al., 2008). In another study, it was shown that genetically engineered *Lc. lactis* secreting murine IL-10 could modulate the functions of bone marrow-derived DC in the presence of LPS (Loos et al., 2009). This data suggest that the beneficial effects of IL-10 secreting LAB during chronic colitis might involve inhibition of CD4⁺ Th17 cells and a reduced accumulation of these cells as well as other immune cells at the site of inflammation.

In another study, we have evaluate the anti-inflammatory effect of the administration of milks fermented by *Lc. lactis* strains that produce IL-10 under the control of the XIES, using a Trinitrobenzenesulfonic acid induced colitis murine model. Mice that received milks

fermented by *Lc. lactis* strains producing IL-10 in the cytoplasm (Cyt strain) or secreted to the product (Sec strain) showed lower damage scores in their large intestines, decreased IFN- γ levels in their intestinal fluids and lower microbial translocation to liver, compared to mice receiving milk fermented by the wild-type (Wt) strain or those not receiving any treatment (unpublished data). The results obtained in this study show that the employment of fermented milks as a new form of administration of IL-10 producing *L. lactis* is effective in the prevention of IBD in a murine model. This new approach could lead to the development of novel fermented products with therapeutical purposes; suitable for specific populations suffering from gastrointestinal disorders or prone to acquiring them. Similar strains could also be included in probiotic mixtures together with other strains that are able to prevent inflammation by other mechanisms such as immune stimulation or that possess antioxidant properties.

5. Prospective uses of GM-LAB

Although there is no scientific evidence that supports the notion that genetically modified (GM) foods or microorganisms are dangerous for human consumption, it is necessary to demonstrate that these are innocuous in order to alleviate the fears held by the general public associated with the use of genetically modified organisms, if we want to use designer probiotics to extend the range of applications covered by natural probiotics. Also, the proper design of GM-LAB is essential in order to eliminate the risks of dissipation in the environment and prevent the transfer of certain genes (such as antibiotic resistance genes) to other microorganisms.

The construction of a biological containment system for a genetically modified *Lc. lactis* for intestinal delivery of human IL-10 is an important step forward for the safe use of GM-LAB for human therapeutic purposes (Steidler et al., 2003). In this study, the thymidylate synthase gene of *Lc. lactis* was replaced by the human IL-10 gene, making this strain incapable of growing when deprived of thymidine or thymine. This strain does not contain any antibiotic resistance markers and because of its thymidine auxotrophy, it cannot disseminate in the environment making it one of the safest GM strains ever engineered. This containment system was recently evaluated in CD patients and it was shown that no adverse effects were produced after consuming this GM-LAB and that it could only be recovered in feces when thymidine was added (Braat et al., 2006). Although only preliminary results from this phase 1 trial were obtained, the use of genetically modified bacteria for mucosal delivery of proteins is a feasible strategy in human with chronic intestinal inflammation (Braat et al., 2006).

Intragastric administration of *Lc. lactis* genetically modified to secrete IL-10 *in situ* in the intestine was shown to be effective in healing and preventing chronic colitis in mice. However, its use in humans is hindered by the sensitivity of *Lc. lactis* to freeze-drying and its poor survival in the gastrointestinal tract, reasons for which novel means for more effective mucosal delivery of therapeutic LAB are currently being developed (Huyghebaert et al., 2005a, b, Termont et al., 2006).

6. Conclusion

This review has shown that probiotics have been extensively used in order to prevent and treat inflammatory bowel diseases. The mechanism of action of these beneficial

microorganisms, which includes changes in the gut microbiota, stimulation of the host immune responses, enhancement of intestinal barrier function and reduction of the oxidative stress due to their antioxidant properties and antioxidant enzyme production has been demonstrated principally using animal models and in specific human trials. It has recently been suggested that there is a lack of well-designed, large, randomized, placebo-controlled trials that can certify that probiotics are effective in the prevention and treatment of IBD (Mallon et al., 2007). Additional studies such as the double-blind, randomized, placebo-controlled clinical trial of the treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment (Tursi et al., 2010) are required to confirm animal data and are necessary to convince the medical and general community of the benefits and potential application of probiotics in the prevention and treatment of IBD.

Although probiotic effects are a strain dependent trait, using modern genetic engineering techniques it is theoretically possible to obtain strains that can exert a variety of beneficial properties. For example, the introduction of antioxidant enzyme genes or cytokine producing capabilities in current probiotic strains that have natural anti-inflammatory properties, such as the ability to modulate the immune dependant anti-inflammatory processes, could generate very useful strains that could be applied in the treatment of a variety of inflammatory diseases. These strains could also be included in treatment protocols since it has been shown that probiotics can enhance the effectiveness of traditional IBD treatments. However, before proposing the genetic modification of anti-inflammatory strains, the innate mechanisms of the potential host strains should be demonstrated in properly designed large scale human clinical trials. These trials are essential in future studies using the engineered strains to demonstrate the differences between the native and modified microorganisms.

The consumption of engineered strains by humans is still highly controversial due to the public perception that genetic manipulation is not "natural". Scientists must perform well-designed studies where the results are divulged to the general populations in order to inform consumers of the obvious beneficial effects these novel techniques can confer with the minimum of risk to their health and to the environment. Throughout the course of history, most novel treatments have met resistance from potential benefactors, it is thus important to show that the potential benefits are highly superior than the risks for novel treatments to be completely accepted by the population as a whole.

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8. References

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