Chapter

# Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT

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## Abstract

Many diseases that affect the gastrointestinal tract (GIT) have great influence on the quality of life of the majority of patients. Many probiotic strains are being highly studied as a promising candidate due to their beneficial effect reported in the GIT. With the purpose of increasing the beneficial characteristics of some probiotics strains and, consequently, to improve further the reported results, many probiotic strains expressing or encoding different proteins, with anti-inflammatory activities, have been developed. These recombinant strains have been reported as good candidates for the treatment of different pathological conditions, especially colitis and mucositis disease since they have been shown to have positive results and good perspectives for GIT inflammation. Thus, this chapter will first address the aspects of the gastrointestinal tract in humans as well as its microbiota. In a second moment, it will discuss about chronic diseases, mainly the intestinal ones. Finally, it will discuss about probiotics, especially concerning on lactic acid bacteria (LAB), and its action in the prevention and treatment of these diseases. At the final part, we will point out aspects on the development of recombinant strains and the results found in the literature on disease models.

**Keywords:** *L. lactis*, *Lactobacillus*, DNA vaccine, heterologous protein

## 1. The human gastrointestinal tract

The human gastrointestinal tract is formed by a complex ecosystem which includes the gastrointestinal epithelium, immune cells, and resident microbiota [1] and comprehends one of the biggest existent interfaces between the host, environmental factors, and antigens in the human body.

The intestine encompasses a broad variety of microorganisms (bacteria, archaea, eukarya, and viruses) [2] from more than 3500 different species [3, 4] that coevolved with the host in a mutually beneficial relationship [5, 6]. The composition and density of bacterial populations in adult individuals differ considerably over the GIT. The area

of the GIT that has highest microorganism abundance is the colon ($10^{14}$) followed by dental plaque ($10^{12}$), ileum ($10^{11}$), saliva ($10^{11}$), and skin ($10^{11}$) [7]. However, low concentrations (up to $10^{2}$–$10^{7}$ cells/mL) and bacterial diversity are found in the upper GIT (stomach, duodenum, jejunum) [3, 4], since the presence of acid, bile salts, and pancreatic secretions hinders the bacterial colonization [8], so that there is no nutritional competition between the microbiota and the host [9]. Thus, both function and structure of microbial communities are significant and are closely related. However, function could be the more important measure of microbiome health, since bacterial ecology suggests that analogous ecosystems have similar function although they have moderately diverse composition [10, 11].

## 2. Gut microbiota

The importance and the specific functions that gut microbiota has in human nutrition and health are well settled. The attributed functions can be classified in three classes: metabolic, protective, and trophic [12]. The gene diversity of the microbial community provides a variety of enzymes and biochemical pathways, specific to the host, able to contribute to short-chain fatty acid (SCFA) production by carbohydrate fermentation and production of some vitamins such as K, B12, biotin, folic acid, and pantothenate. These factors added to synthesis of amino acids from ammonia or urea contributing to the metabolic function of the microbiota [13, 14].

The gut microbiota's protective function is related to barrier effect, once the resident bacteria generate a resistance line which avoid pathogens/opportunistic bacteria and maintain normal mucosal function. The activity of some bacteria to secrete antimicrobial substances, such as bacteriocins, is able to inhibit the growth of other bacteria and nutrient competition [15, 16].

Regarding trophic functions of gut microbiota, the interaction between resident microorganisms has influence in differentiation and proliferation of epithelial cells [17], as well as in the development and regulation of the immune system by numerous and varied interactions between microbes, epithelium, and gut lymphoid tissues [18].

It is important to highlight that the interactions between the gut microbiota and the host immune system are required to preserve the gut homeostasis [19–21]. When this relationship is affected, alterations in bacterial function and diversity lead to the imbalance in the composition of the resident microbiota, favoring either the growing of pathogenic bacteria or the decreasing in beneficial bacteria in a process known as dysbiosis [22], which appoint a great threat to gut integrity and is intrinsically related to the development and progression of several diseases, such as inflammatory bowel diseases.

## 3. Chronic inflammatory diseases

One of the most well-characterized chronic inflammatory diseases that mainly affect the digestive tract is inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD). The exact etiology of IBD is still unclear, but the strict relation between genetic and the environmental factors, such as enteric immune dysregulation and alterations in the intestinal microbiome [23, 24], is broadly known. Besides, these diseases generate substantial morbidity and have a high prevalence in developed countries (5 in 1000 individual are affected) they remain to increase in developing nations [25].

Both diseases, UC and CD, present different pathogenesis, symptomatology, inflammatory profiles, and gut microbiota composition. CD is characterized by the irregular transmural inflammation (extending deeply into the submucosal regions) which can

affect any portion of the GIT and often made difficult by strictures, abscesses, and fistulae. On the other hand, the inflammation presented in UC is restricted to the superficial layers of the intestinal mucosa characterized by mucosa erosion and/or ulcer, generally localized in the region of the gut most colonized by bacteria, the colon [26, 27]. In addition, regarding the immune response associated with these diseases, it is possible to relate CD with an increased IL-12, IL-23, IL-27, interferon γ (IFN-γ), and tumor necrosis factor-α (TNF-α) production, all associated with Th1 and Th17 immune responses, different from UC which is correlated with a Th2 immune response, with high levels of IL-5 and transforming growth factor-β (TGF-β) production [28].

## 4. IBD complications and microbiota manipulation

It is important to highlight that the principal cause of death in IBD patients is colorectal cancer (CRC) [29]. Frequent episodes of inflammatory process in the intestinal mucosa are related to development of this disease, which is the second most frequently identified cancer in females and the third in males.

There are increased evidences that environmental factors such as lifestyle and diet alterations have effect in CRC incidence [30]. This effect has been documented because there is evidence showing an essential relationship between dietary antigens and antigens of commensal bacteria with the regulatory T cells (Tregs), which maintain the immune tolerance and, consequently, reduce the risk of tumorigenesis associated with inflammation [31].

In this context, it was reported that the higher consumption of diet rich in grains and vegetables decreases the incidence of CRC. This effect involves different mechanisms such as the diminution in the fecal transit time due to the increase in the stool bulk, and consequently, it reduces the contact of carcinogen with colon cells and the fermentation of these fibers of colonic components [14, 32]. In addition, significant reduction in concentration of acetate, propionate, and butyrate with increase in fecal pH [33] and the decrease in the number of obligate anaerobe microorganisms have been reported in individuals with colon cancer [34] when compared with healthy people. Thus, intestinal environmental alterations are the keys to evolution toward adenoma and afterward to CRC progression [35].

It has been also reported that up to 30% of patients with UC need surgical management such as the restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) [36]. This procedure removes the entire colon and rectum while preserving the anal sphincter and, hence, normal bowel function and fecal continence, therefore acting as an internal pelvic place for intestinal contents [37]. Around 50–60% of UC patients with following IPAA develop inflammation in the ileal pouch, generating the condition called "pouchitis." The reported incidence of pouchitis is variable, generally because of the diagnostic criteria that have been used to define this syndrome [38, 39]. In addition, although its pathogenesis is uncertain, the main hypothesis for the mechanism by which the disease occurs is the break in the mucosal barrier generated by dysbiotic microbiome in susceptible patients, generating an unusual mucosal immune activation [40]; still the disease typically responds to antibiotics.

Corresponding to the increased attention given to the role of the intestinal microbiota in a variety of diseases, there has been an intense exploration of potential means to manipulate the intestinal microbiome either by probiotic administration or fecal microbiota transplant (FMT) for therapeutic effect [41].

In this context, a randomized clinical trial based on a 1-week treatment with anaerobically prepared donor FMT, compared with autologous FMT, resulted in a higher probability of remission in 8 weeks for patients with UC, revealing that stool administration from healthy donors to UC or CD patients is an intervention that seeks to restore a healthier balance of gut microbes and control IBD [42]. Data on FMT for

Crohn's disease is rather more limited than for UC, but it has been shown that single standardized FMT resulted in a clinical remission sustained for more than 9 months in CD patients [43]. However, the authors suggest that further studies are needed to enhance the knowledge about the use of stool transplantation for IBD treatment.

Alteration in the gut microbiome composition with increase in some groups of microorganisms, such as *Clostridium* and *Fusobacterium*, was also reported in patients with pouchitis [44, 45]. In this context, literature evidences indicate that the probiotic administration such as VSL#3 is effective in the chronic pouchitis prevention [46]. On the other hand, FMT to pouchitis treatment did not report the same beneficial results. Only three reports with this approach [47–49] exposed that neither clinical remission nor any adequate response was observed in the evaluated patients suggesting that the efficacy of FMT for pouchitis after proctocolectomy is limited [49]. The importance of standardization of this procedure needs to be highlighted to improve its efficacy, since frequency, route of administration (e.g., endoscopy, nasogastric tube, colonoscopy), and the criteria of choice of healthy donor are very important parameters to be considered.

## 5. Intestinal mucositis

Different chemotherapy regimens such as FOLFOX (5-fluorouracil and oxaliplatin), FOLFIRI (5-fluorouracil and irinotecan), and triple FOLFOXIRI regimen (5-fluorouracil, oxaliplatin, and irinotecan) [50, 51] are adopted for different types of cancer but with a broad range of collateral effects.

Mucositis is the most common side effect in patients undergoing chemotherapy/radiotherapy treatments, which consist in an inflammation and/or ulcers in the gastrointestinal tract [52] with consequent loss of cells from the epithelial barrier of the GIT. Many symptoms are related to gastrointestinal mucositis, such as diarrhea, severe abdominal pain, bleeding, fatigue, malnutrition, dehydration, electrolyte imbalance, and infections, with potential fatal complications which can conduce to reduction or interruption of antitumor treatment [53] and consequently leads to longer hospitalization.

This pathology occurs due to cytotoxic effects of anticancer drugs/radiotherapy that cause damage at the DNA of stem cell (epithelial cell progenitors) with intense oxidative stress and consequent cell death. This apoptotic process is exacerbated affecting the absorption by shortening the villi structure of enterocytes and causing the loss of epithelial barrier with an invasion of inflammatory cells (neutrophils, eosinophils, and macrophages) leading to an increased production of inflammatory mediators at the mucosal area with consequent epithelial erosion and ulceration. The progressive destruction of mucosal integrity causes the rupture of the *tight junctions* proteins, leading to an increase in the intestinal permeability with subsequent penetration of commensal microbiota to the submucosal layer generating bacteria translocation which exacerbates the inflammatory process and intensifies the symptoms [53–57]. Besides, the intestinal microbiota composition is also modified by the chemotherapeutic drugs and radiotherapy action [54, 58, 59] resulting in dysbiosis. After the end of treatment, recovery and restoration of the GIT structure occur [60].

## 6. Metabolic syndrome

Besides IBD and mucositis, it has been reported that intestinal microbiota has an intrinsic effect on metabolism, potentially contributing to several features of the pathophysiology of metabolic syndrome [61, 62]. The metabolic syndrome is an accumulation of various risk factors (glucose intolerance, hyperinsulinemia,

hypertension, as well as dyslipidemia) which can often be associated with insulin resistance, hypertension with abdominal fat accumulation, and obesity [63–65].

The etiology of metabolic syndrome is not well-defined; however there are evident characteristics and life habits that could contribute to its development such as unbalanced diet, smoking, lack of physical activity, and the genetic predisposition [66]. These factors directly increase the risk of cardiovascular disease and chronic diseases as type 2 diabetes mellitus and obesity, and the interaction between components of both the clinical and biological phenotypes of the syndrome contributes to the development of a pro-inflammatory state [67].

The inflammatory process observed in MS is directly associated with increased oxidative stress. The reactive oxygen species (ROS) are capable of mediating symptoms of diabetes mellitus, such as insulin resistance and decrease in insulin secretion, and attend as precursors for the formation of LDLox (oxidized low-density lipoproteins), responsible for a large part of the development of atherosclerotic lesions, and the increase in circulating cholesterol fractions and glucose [68, 69]. In addition, chronic diseases are directly related to changes in the intestinal microbiome [70, 71], and they are also associated with elevated circulating levels of pro-inflammatory cytokines such as TNF and IL-6 [72].

The probiotic use in attenuating symptoms of different inflammatory diseases is widely reported in the literature. Among the commercial probiotics studied for treatment of these diseases, only a few products have been extensively tested in clinical trials in patients with MS, in order to demonstrate an effective result on weight loss, lipid metabolism, and reduction of inflammatory markers.

Studies performed with *Lactobacillus* strains have shown the ability of these probiotics in reducing the lipid accumulation in adipose tissues, as well as in inducing the subexpression of lipogenic genes [73, 74]. Animals that received diets with high concentrations of lipids and then treated with *L. gasseri* SBT2050 had shown lower intestinal permeability and bacterial translocation, as well as reduction of inflammatory parameters, suggesting that this strain improves the intestinal barrier function [75–78]. In addition, *L gasseri* BRN17 was studied to treat animals with MS caused by the carbohydrate-rich diet consumption. This strain reduced the accumulation of adipose tissue in mice, and it has a beneficial effect on weight loss [79–81]. Another important approach with associated probiotics (*Bifidobacterium*, *Lactobacillus*, and *S. thermophilus*) for treatment of overweight patients has shown an improvement in lipid profile, as well as insulin sensitivity [82]. Besides, recently Hsieh e collaborators [83] demonstrated that administration of live *Lactobacillus reuteri* ADR-1 and killed *Lactobacillus reuteri* ADR-3 strain ameliorated type 2 diabetes mellitus in a clinical trial. The results indicated that the consumption of ADR-1 displayed a reduction effect on serum glycated hemoglobin (HbA1c), triglyceride, and cholesterol levels. On the other hand, the intake of ADR-3 showed a beneficial effect on blood pressure reduction. Besides, a reduction in the levels of pro-inflammatory cytokines (IL-1β), increase in antioxidant enzyme (superoxide dismutase), and the changes in intestinal microflora composition (increase in intestinal level of *Lactobacillus* spp. and *Bifidobacterium* spp. and decrease in *Bacteroidetes*) were observed. Thus, these strategies highlight the beneficial and potential effect of interventions targeting gut microbiota modulation by the use of probiotic strains to treat components or complications of metabolic syndrome.

## 7. Functional foods

The human being for more than 4000 years has been consuming fermented products, by the fermentation process. At the beginning this practice was done

to preserve foods from either physical, chemical, or microbial alterations. The microorganisms participating in this process are the lactic acid bacteria, extensively widespread in nature and also belong to the GIT communities, able to convert the sugar in lactic acid as well as produce other metabolites which contribute to food modifications, either sensorial or nutritional value. Thus, the terminology "functional food" was attributed to food with health benefits to the consumer including nutritional and physiological function [84–86].

During the fermentation, these bacteria can contribute to improving the digestion of nutrients (lactose, proteins, small peptides, and polysaccharides); providing essential micronutrients (vitamins) as well as bioactive compounds (metabolites) with potential health benefits to the host, such as prevention against enteric inflammation [87, 88]; providing antimicrobial, antihypertensive, hypocholesterolemic, immunomodulatory, antioxidant, and anticancer effects [46, 85, 89–92]; showing ability to regulate the immunity; and, consequently, improving host quality of life [93].

Therefore, the gut communities and the microbial-derived molecules present in the gut lumen have been strongly influenced, either qualitatively or quantitatively, by consumption of dairy products [94] such as yogurts, cheeses, and fermented milk, among other fermented products using probiotic bacteria. Thus, the microbiota manipulation by functional food, probiotics, and prebiotics are evaluated as a beneficial option for treatment of GIT diseases [95].

## 8. Lactic acid bacteria: the largest group of probiotic bacteria

There is a constant interaction between the host and the bowel commensal bacterial community in order to maintain the homeostasis [3, 96–98]. However, when this mutualist relationship is compromised, the intestinal microbiota may cause and/or contribute to either the establishment or the progression of inflammatory diseases [96–99]. In this context, the search for therapeutic strategies that minimize the development and progression of pathologies caused directly and indirectly by the unbalance of the commensal microbiota has grown. The consumption of probiotic bacteria is one of these strategies, as they present several effects, such as ability to improve the intestinal barrier, stimulate the systemic and mucosal immune system, regulate the composition of the intestinal microbiota, and provide essential micronutrients (such as vitamins and SCFAs) and other bioactive compounds (metabolites) with potential health benefits for the host [100–103].

Probiotics are defined as "live microorganisms that offer host health benefits when administered in adequate amounts" [104, 105]. The majority of the studied probiotics belongs to the group of lactic acid bacteria. However, other microorganisms with probiotic properties also deserve attention, such as yeasts (*Saccharomyces* spp.) and bacteria of the genus *Bifidobacterium* and *Faecalibacterium*, among others [106–108].

LAB, which include, mainly, species from the genus *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Streptococcus*, constitute a group of Gram-positive, anaerobic or aerotolerant, nonspore-forming, nonmobile, and highly low pH-tolerant microorganisms. However, the main characteristic of this group is its ability to produce lactic acid as the final product of the fermentation of carbohydrates [109–111].

## 9. Probiotic effects in gastrointestinal inflammation

LAB are often present in the human gut but also can be introduced by the ingestion of fermented foods, such as yogurt and other fermented milk products and fermented cured meat by-products [103], having the generally recognized as

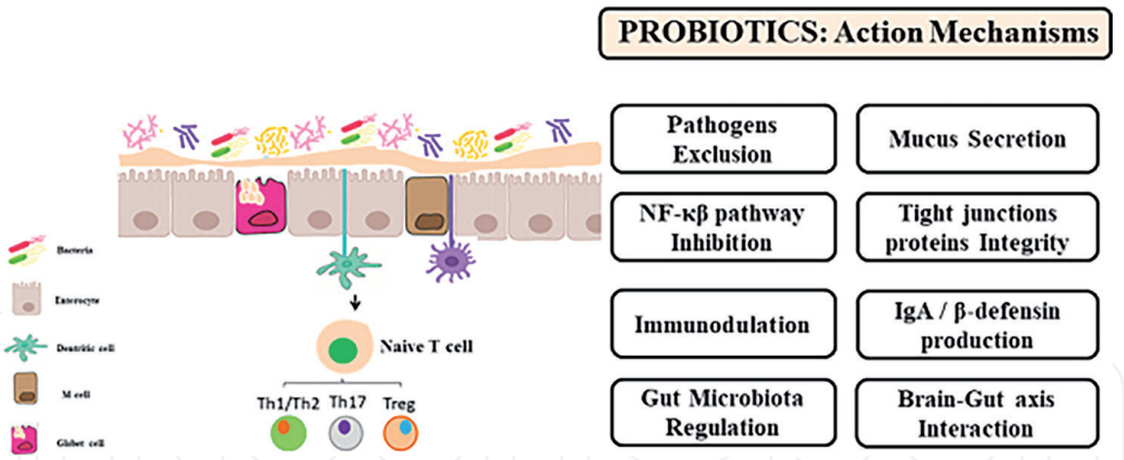


**Figure 1.**
*A schematic diagram about potential action mechanisms of probiotic bacteria.*

safe (GRAS) status by the Food and Drug Administration (FDA). *Lactobacillus* spp., *Streptococcus* spp., and *Lactococcus* spp. are the major LAB species with probiotic effects, and they have been used in therapeutic applications for treatment and prevention of various intestinal disorders [112, 113].

Scientific evidence reveals that the mechanisms by which probiotic bacteria ameliorate inflammatory bowel damage are heterogeneous, strain specific, and dependent on the number of available bacteria. Thus, administration of probiotic bacteria, specially LAB, improves intestinal inflammatory responses by (i) modulation and normalization of perturbed intestinal microbial communities; (ii) competitive exclusion of pathogens such as *Staphylococcus aureus* and *Salmonella typhimurium*, among others; (iii) bacteriocin and SCFA production; (iv) enzymatic activities related to metabolization of a number of carcinogens and other toxic substances; (v) adhesion to mucosal cells, cell antagonism, and mucin production; (vi) intestinal permeability reduction by tight junctions protein modulation (e.g., zonulin, claudin, occludin, junctional adhesion molecule); (vii) modulation of the immune system by stimulating Tregs cells, IgA production by B cells, and NF-kβ signaling pathway inhibition; and (viii) interaction with the brain-gut axis via the generation of bacterial metabolites (**Figure 1**) [103, 114–118].

## 10. Recombinant LAB probiotics

In order to potentialize the beneficial effects of probiotic strains, research has been conducted over the last decades, based on genetic engineering techniques, especially those related to DNA manipulation. Thus, modern methods of genetic engineering open the new opportunities to design and create genetically modified probiotic strains with the desired characteristics or to exclusively target a specific pathogen or toxin to be used either as a vaccine or for drug delivery [119, 120]. Since most of the probiotic strains are part of the LAB group, most of the genetic manipulation studies are carried out with species that belong to this group, such as *Lactococcus* and *Lactobacillus* genera. Consequently, recombinant probiotics have been created for mucosal delivery of therapeutic and/or prophylactic molecules comprising DNA, peptides, single-chain variable fragments, cytokines, enzymes, and allergens [121, 122], leading to the concept of "biodrug" for the prevention and treatment of various diseases [123]. Thus, researches have emphasized the use of species of these genera in two different approaches: the first as producers of heterologous protein and the second as vehicle for delivery of DNA vaccines [124].

## 10.1 LAB as producers of heterologous protein

Many studies are carried out with *Lactococcus lactis* due to its economic importance in the production of cheese and its easy growth and manipulation. In addition, it was the first species of LAB to have its genome completely sequenced, which allowed a greater understanding of its genetic and physiological mechanisms, aiding in the development of technological packages for its genetic manipulation in a laboratory environment [124–128].

There are several ways to make LAB produce heterologous proteins, and the most used form is through the insertion of a plasmid into its cytoplasm. Plasmids are elements of extrachromosomal DNA that are naturally found in prokaryotes. With the advent of the recombinant DNA technique, these elements have been manipulated to act as molecular vehicles that allow the production of proteins of interest by the bacterium [129].

The first heterologous protein production system based on plasmid insertion in LAB was developed for *L. lactis.* These systems included both inducible and constitutive promoters, which ensure efficient expression of the antigen of interest under different conditions [130, 131]. Although it is possible to choose the type of promoter to be used in the vector, the vast majority of expression vectors present inducible promoters that allow controlled expression of the protein of interest by protecting against aggregation and protein degradation in the bacterial cytoplasm. On the other hand, these vectors present safety issues that need to be analyzed since it is necessary to introduce chemical compounds into the culture medium to induce protein expression prior to animal administration [132–134].

With the improvement of cloning and expression techniques, several production systems were developed, specifically for LAB, allowing the production of different molecules of interest, including pathogen antigens, by a large number of LAB species [135–139]. The most commonly used regulation systems in LAB are the following:

### *10.1.1 Nisin-controlled gene expression (NICE)*

Among the heterologous production systems, the most widely studied is the nisin-controlled gene expression system. This system is based on the expression of three genes (nisA, nisF, and nisR) that are involved in the production and regulation of the antimicrobial peptide nisin, which is naturally secreted by different strains of *L. lactis*. In this system the membrane-located histidine kinase NisK senses the signal inducer nisin and autophosphorylates and then transfers the phosphorous group to the intracellular response regulator protein NisR which acts as a transcription activator of nisA/nisF and induces gene expression under pNis promoter. Depending on the presence or absence of the corresponding targeting signals, the protein is either expressed into the cytoplasm or the cell envelope or secreted into the external medium [140]. Thus, it has already been successfully used for the expression of different proteins of medical and biotechnological interest [141, 142].

### *10.1.2 Xylose-inducible expression system (XIES)*

In 2004, Miyoshi and colleagues [143] developed the xylose-inducible expression system whose promoter is the xylose permease gene (pxylT) found in *L. lactis* NCDO2118. This system produces either cytoplasmic or secreted proteins being activated in the presence of xylose and strongly repressed in the presence of glucose, fructose, or mannose [143].

### *10.1.3 Stress-inducible controlled expression system (SICE)*

More recently, the stress-inducible controlled expression system was developed using the *L. lactis* groESL promoter [134]. This system induces expression of proteins of interest via stress stimuli such as those found in the GIT (e.g., bile salt, acid pH, antimicrobial peptide, and heat shock proteins) [134, 144]. This system does not require the induction of bacterial culture or the presence of regulatory genes, being a good alternative in the delivery and production of therapeutic proteins at mucosal surfaces.

## 10.2 LAB as a live vehicle to deliver DNA vaccine plasmids to eukaryotic cells

Among the available approaches to stimulate efficient mucosal responses, the use of bacterial system for DNA delivery and its expression using the eukaryotic cell machinery have been extensively explored. Unlike the production of heterologous protein, in which the bacterium is responsible for the synthesis of the protein of interest, in the DNA vaccine platform, the bacteria only act as a delivery vehicle for prophylactic and therapeutic purposes [109, 145].

New vectors had been developed to approach the DNA vaccine using LAB as live delivery vehicles [146, 147, 148–150]. These vectors present a series of common characteristics such as the presence of a eukaryotic promoter, which allows protein expression by eukaryotic cells; a prokaryotic region, which has a selection marker (usually antibiotic resistance); a multiple cloning site, where the open reading frame (ORF) of interest will be inserted; and a prokaryotic origin of replication, which ensures that the plasmid replicates only in prokaryotic cells [151]. Some molecules (IL-10, IL-4, and HSP65) have been cloned in these vectors to evaluate their effect, especially as a treatment approach in diseases related to the bowel [152, 153], as well as reporters (GFP and Cherry) which allowed the understanding of this platform in the mammalian body [148, 154]. Although further studies need to be conducted in order to elucidate whether the cloning of ORFs of interest in these vectors is really effective pointing to disease prevention and treatment, this approach is undoubtedly an important tool for the development of new techniques with potential in the medical clinic.

## 11. Next-generation recombinants: using CRISPR-Cas system

Among the different techniques used to construct recombinant LAB strains, the most recent is associated with the use of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system, based on the use of a system present in several bacterial strains that works as part of the adaptive immune system of bacteria and archaea against the presence of external DNA, such as plasmids and bacteriophages [155–159].

Although this system has been studied for more than 30 years [160], it was only in 2013 that the first experiments were carried out emphasizing its use as a tool for genome editing [161, 162]. Evaluating the CRISPR databases, it is possible to observe that about 46% of all bacterial genomes presents the CRISPR-Cas system, and this percentage reaches approximately 63% of the sequenced *Lactobacillus* genomes [163]. The natural presence of this system in most of the LAB strains expands the possibilities of genetic manipulation of microorganisms of this group, including probiotic ones [164].

The first gene editing experiment in LAB based on the CRISPR-Cas system was conducted by Oh and van Pijkeren [165] where they were able to edit three different

regions of the genome, with efficiency up to 100% in the selected clones. After this pioneering work, few others were published focusing on LAB gene editing [166–168].

Therefore, the use of this technology is presented as a widely viable strategy to be applied in LAB, enabling the development of food-grade recombinant strains in order to allow their future use in the clinic [169].

## 12. Use of recombinant LAB to treat GIT-related disorders

The use of recombinant *L. lactis* strains, as well as others recombinant LAB strains, using different systems has shown promising results in many studies as an alternative therapy to treat, especially, GIT inflammation and other diseases (**Table 1**).

To arrive at mucosa in sufficient quantities to exert their therapeutic effects, many LAB strains must survive, during their passage through the GIT, stressor factors such as pH, temperature, bile salt concentration, and the presence of antimicrobial peptides [170–172]. In this context, an interest approach was recently developed by Coelho-Rocha and colleagues [154] using an encapsulated recombinant strain (*L. lactis* pExu:*mcherry*) and tested it through the GIT at different times post-administration. They have shown that the microencapsulation process is an effective method to improve DNA delivery, guaranteeing a greater number of viable bacteria able to reach different sections of the bowel [154].

The use of recombinant probiotics to improve therapeutic approaches has been widely studied using different systems with different molecules. As IBDs are a serious clinical topic, many strategies have been tested trying to improve previous results found with wild type strains.

*L. lactis* MG1363 strain carrying the pTREX1 vector expressing the mouse IL-27 protected mice against the inflammatory effects of dextran sulfate sodium (DSS)-induced colitis. This recombinant strain was able to reduce disease activity scores and pathology features of the large and small bowels and also led to reduced levels of inflammatory cytokines IL-1β, TNF-α, and IFN-γ in colonic tissue. In addition, reduction in the number of $CD4^+$ and $IL\text{-}17^+$ T cells in gut-associated lymphoid tissue and increase in IL-10 production were observed [173].

Besides, it was also demonstrated in a DSS-induced colitis mouse model that the oral administration of *L. lactis* NZ900 strain harboring the NICE system expressing either the anti-inflammatory cytokine IL-10, TGF-β1, secretory leukocyte protease inhibitor (SLPI), or elafin was able to ameliorate some clinical parameters in inflamed mice. Even though it was possible to observe the reduction of weight loss and diarrhea, microscopic colonic damage scores, colon thickness, and myeloperoxidase (MPO) activity, the authors reported that treatments with recombinant *L. lactis* strain delivering either SLPI or elafin were more efficient to reduce signs of colitis than treatments with anti-inflammatory cytokines. Altogether these recombinant strains display anti-inflammatory effects in inflamed mice [174].

Approaches using the invasive *L. lactis* MG1363 $FnBPA^+$, by expressing the FnBpA protein at their surface and carrying the pValac eukaryotic expression vector coding either the IL-10 cytokine [r*L. lactis* $FnPBA^+$ (pValac:*il-10*)] or the IL-4 cytokine [r*L. lactis* $FnPBA^+$ (pValac:*il-4*)] in DSS or trinitrobenzenesulfonic acid (TNBS)-induced acute model of colitis, respectively, were also investigated. The administration of *L. lactis* $FnPBA^+$ (pValac:*il-10*) recombinant strain was capable to reduce the intestinal inflammation by increasing IL-10 levels and sIgA production, accompanied by decreasing IL-6, as well as the restoration of intestinal architecture of mice colon [153]. Besides, the engineered *L. lactis* $FnPBA^+$ (pValac:*il-4*) was able to slump the level of pro-inflammatory cytokine (IL-12, IL-6) and myeloperoxidase activity and increase levels of IL-4 and IL-10, consequently decreasing the colitis harshness [153].

| Microorganism | Gene | Expression System | Inflamation Condition | Anti-Inflamatory Properties | References |
|---|---|---|---|---|---|
| *L. lactis* MG1363 | Mouse IL-10 | SICE | Mouse model of DNBS-induced colitis | Restoration of intestinal architecture; IgA production and IL-6 reduction; Reduced tissue damage | [134] |
| *L. lactis* MG1363 | Mouse IL-10 and IL-4 | pValac vector | Mouse model of DSS/TNBS-induced colitis | Decreased IL-6, IL-12 and MPO activity Reduced tissue damage | [152-153] |
| *L. lactis* NZ9000 | Mouse TGF-β1; IL-10 and leukocyte protease inhibitor Human Elafin | NICE | Mouse model of DSS-induced colitis | Reduced tissue damage Decreased pro-inflammatory cytokines | [174] |
| *L. lactis* NCDO 2118 | Human 15-lipoxygenase-1 | XIES | Mouse model of DSS-induced colitis | Reduced tissue damage | [175] |
| *L. lactis* NCDO 2118 | *M. leprae* Hsp65 protein | XIES | Mouse model of DSS-induced colitis | Restoration of intestinal architecture CD4+Foxp3+ and CD4+LAP+ regulatory T cells production | [176] |
| *B. bifidum* BS42 | Mouse IL-10 | BEST | Mouse model of DNBS-induced colitis | Reduced tissue damage | [177] |
| *L. casei* BL23 | Superoxide dismutase A from *L.lactis* MG1363 Catalase from *L.plantarum* ATCC | pLEM415 vector | Mouse model of TNBS-induced Crohn´s disease | Reduced tissue damage Reduced microbial translocation Increase IL-10/ INF-γ reduction | [180] |
| *S. thermophilus* CLR807 | Superoxide dismutase A from *L.lactis* MG1363 Catalase from *L.plantarum* ATCC | pIL253 vector | Mouse model of TNBS-induced colitis | Reduced tissue damage Reduced microbial translocation IL-17 reduction | [181] |
| *L.lactis* AG013 | Human Trefoil Factor 1 (Htff-1) | ThyA native promoter of *L.lactis* | Hamster model of radiation-induced oral mucositis | Reduced clicnical scores of oral mucositis | [186] |
| *L. lactis* NZ9000 | Human pancreatitis associated protein (Reg3A) | NICE | Mouse model of 5-FU-induced intestinal mucositis | Microbiota Regulation Villus architecture preservation Increased Paneth cells activity | [185, 187] |

| Microorganism | Gene | Expression System | Inflamation Condition | Anti-Inflamatory Properties | References |
|---|---|---|---|---|---|
| *L.lactis* NCDO2118 | *M. leprae* Hsp65 protein | XIES | Mice model of experimental encephalomyelitis | Increased CD4+Foxp3+ regulatory T cells<br>Reduced encephalytogenic CD4+ T cells | [184] |
| *L.lactis* MG1363 | Mouse IL-17 | SICE | Mice model HPV-induced cancer | Reduced tumor size<br>Induced IL-6 and IL-17 secretion | [182] |
| *L.lactis* NZ9000 | *M. leprae* Hsp65 protein and peptide derived of human Hsp60 protein | NICE | Mice model of diabetes type 1 | Reduction of insulitis<br>Inhibition of T cell proliferation | [183] |

**Table 1.**
*Protein with anti-inflammatory properties produced in different strains of bacteria.*

The human 15-lipoxygenase-1-producing *L. lactis* NCDO2118 harboring the xylose-inducible expression system (pXylt:CYT:*15-LOX-1*) was also effective in attenuating the symptoms of DSS-induced colitis in a murine model [175]. Its oral administration improved the body weight, decreased pro-inflammatory cytokines (IFN-γ and IL-4) while increasing the anti-inflammatory cytokine IL-10, and, consequently, ameliorated the macroscopic damage scores associated with the inflammation.

The oral pretreatment with genetically modified *L. lactis* NCDO2118 able to secrete HSP65 protein from *Mycobacterium leprae*, using XIES system (pXylt:SEC:*hsp65*), prevented DSS-induced colitis in C57BL/6 mice [176]. This protection was associated with reduced pro-inflammatory cytokines, such as IFN-γ, IL-6, and TNF-α; it also increased IL-10 production in colonic tissue and expansion of $CD4^+FoxP3^+$ and $CD4^+$ latency-associated peptide ($LAP^+$) regulatory T cells in the spleen and mesenteric lymph nodes. Besides, the authors showed that this effect was dependent on IL-10 and toll-like receptor 2 (TLR-2) [176].

Although *L. lactis* represents an excellent candidate for a live mucosal vector delivery system, other bacteria have also been explored as promising live vehicles for molecule expression with therapeutic properties, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*. In this context, Mauras et al. [177] using the new *Bifidobacteria* Expression SysTem (BEST) allowing the production of IL-10 in *Bifidobacterium bifidum* BS42(pBESTExp4:*il-10* and pBESTBL1181:*il-10*) demonstrated that the use of these recombinant strains in a DNBS-induced colitis model showed its ability to decrease local inflammation and confirmed therefore its potential for delivery of therapeutic molecules in the colon.

It is well known that IBD is associated with oxidative stress by the increase in concentration of reactive oxygen species in the GIT and impaired antioxidant defenses [178, 179]. In this context, it has been shown that some probiotic LAB strains may play a protective role in IBD by expressing antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [180, 181].

LeBlanc et al. and Del Carmen et al. [180, 181] showed, respectively, that *L. casei* BL23 and *S. thermophilus* CRL807 transformed with two different plasmids (pLEM415:*mnkat*; pLEM415:*sodA*) (pIL253:*sodA* and pIL253:*mnkat*) harboring

the genes encoding catalase (CAT) or superoxide dismutase (SOD) antioxidant enzymes exhibited anti-inflammatory activities in a mouse model of Crohn's and colitis disease induced by trinitrobenzenesulfonic acid (TNBS). The authors observed a reduction in weight loss, fewer liver microbial translocation, lower macroscopic and microscopic damage scores, and modulation of the IFN-γ/IL-10 [180] and IL-10/IL-17 [181] cytokine production in the large intestines of mice treated with either CAT- or SOD-producing lactobacilli/streptococci.

The stress-inducible controlled expression (SICE) system represented by *L. lactis* MG1363 strain harboring the pLB333 plasmid was developed to avoid the external induction of culture before the host administration [134]. Several interesting molecules were cloned in this system such as IL-10 [134] and IL-17 [182], and the effect of *L. lactis* secreting them was evaluated in mice models. *L. lactis* (pSICE:*il-10*) was tested in a DNBS-induced colitis mice model, resulting in a significant reduction in colitis parameters with improvement in weight loss and a decrease in macroscopic scores [134]. The intranasal administration with *L. lactis* secreting IL-17A (pSICE:*il-17*), in a mice model of human papilloma virus (HPV)-induced cancer, was able to reduce tumor size and induce IL-6 and IL-17 secretion in reactivated splenocytes from mice challenged with the tumoral cell line [182]. Both works confirmed the potential use of *L. lactis* harboring the SICE system to deliver interesting molecules either to colitis or colon cancer patients [134, 182].

Although many studies have focused on the use of recombinant bacteria for the treatment of IBDs, as was previously discussed, the use of recombinant probiotic strains expressing/delivering therapeutic molecules has been explored for treatment or prevention of other diseases such as mucositis, cancer, obesity, multiple sclerosis, and diabetes [182–185].

An in vivo study reported by Caluwaerts et al. [186] showed that recombinant *L. lactis* AG013 secreting human trefoil factor 1(hTFF-1) was able to reduce the severity and course of radiation-induced oral mucositis. Carvalho et al. [187] also demonstrated that a recombinant strain of *L. lactis* NZ9000 using the inducible NICE system to express the human pancreatitis-associated protein (PAP) was able to prevent 5-FU-induced intestinal mucositis in a murine model. It was observed that this protein preserved villous architecture, increased Paneth cell activity [187], and suppressed the growth of *Enterobacteriaceae* during inflammation [185].

It also has been shown that oral administration of a recombinant *L. lactis* NCDO2118 strain (pXylT:SEC:*hsp65*) prevented the development of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice [184]. Mice fed daily with this recombinant strain increased the number of natural and inducible $CD4^+FoxP3^+$ and $CD4^+$ latency-associated peptide ($LAP^+$) regulatory T cells in the spleen, inguinal and mesenteric lymph nodes, as well as in the spinal cord. In addition, a reduction in the recruitment of encephalitogenic $CD4^+$ T cells to the spinal cord was observed, which decreased IgG response against HSP65 and induced an anti-inflammatory cytokine profile (IL-17 reduction and IL-10 increase) during EAE development.

The oral administration of recombinant *L. lactis* expressing HSP65 and tandemly repeated P277 (pCYT:*HSP65-6P277*) was also analyzed in a model of type 1 diabetes mellitus (DM1) [183]. The authors observed that oral administration of recombinant *L. Lactis* resulted in the prevention of hyperglycemia, improved glucose tolerance and reduced insulitis, and induced HSP65- and P277-specific T-cell immunotolerance, as well as antigen-specific proliferation of splenocytes, demonstrating to be an effective therapeutic approach in preventing DM1 [183].

Another study using the *E. coli* Nissle 1917 strain engineered to secrete N-acylphosphatidylethanolamines (NAPEs) (pDEST-At1g78690 expression

plasmid) demonstrated that this strain was able to reduce the obesity of mice fed with a high-fat diet when added to drinking water. N-acyl phosphatidylethanolamines are precursors to the N-acylethanolamine (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and reducing food intake and obesity. Mice that received modified bacteria had dramatically lower food intake, adiposity, insulin resistance, and hepatosteatosis than mice receiving standard water or control bacteria [188]. In addition, it was observed that changes on intestinal microbiota significantly decreased the abundance of *Firmicutes* and increased the abundance of *Proteobacteria*. Thus, these results provide evidence of the potential efficacy of this approach to inhibit the development of metabolic disorders and related diseases.

## 13. Conclusion

Currently the association between disease progression, especially chronic inflammatory diseases, and intestinal dysbiosis has been more frequently observed. As a clinical strategy, the use of probiotic bacteria, which naturally benefit the host, has been increasingly used on the treatment of diseases related to the GIT. In view of the good results obtained with this approach, researchers have sought through bacterial genetic modification to increase the beneficial potential of probiotics, either through their use for heterologous protein production or as a vehicle for vaccinal plasmid delivery, by developing recombinant bacterial strains and by testing their action in different disease models. And while there are still a number of questions that need to be answered about the use of genetically modified organisms for health care, especially in human, the use of these strains has proven to be a potentially effective therapeutic alternative, so much so that clinical trials using recombinant lineages have already been authorized and conducted in humans.

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