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Novel Biotechnological and Therapeutic Applications for Wild Type and Genetically Engineered Lactic Acid Bacteria

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Abstract

The human gastrointestinal (GI) tract is colonized by a complex and dense microbial community that can be divided into three major phyla—Bacteroidetes, Firmicutes and Actinobacteria, which, under normal conditions, live in a symbiotic relationship with the host. However, it has been shown that a dysfunctional interaction between the microbiota and the host can lead to several intestinal disorders, thus being considered a field of growing interest by the scientific community. In this context, some studies have been carried out to elucidate functions of true resident bacteria, while other research has attempted to assess transient bacteria. In addition, some studies have focused on the group of lactic acid bacteria (LAB) that are widely used as starter cultures in food fermentation of a large variety of fermented foods. It has being reported that allochthonous LAB bacteria may have positive effects on the host when administrated in adequate amounts, thereby allowing them to be classified as probiotics microorganisms. Our research group recently investigated the mechanisms underlying the protective effects of dairy *Lactobacillus delbrueckii* Lb CNRZ327 *in vitro* and *in vivo* assays and have deposited the complete genome of *Lactococcus lactis* NCDO 2118, which will enable a greater understanding of its intrinsic characteristics. Beyond the classical employment of LAB, our group gathered research works using genetically engineered LABs, more specifically lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA

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vaccines. In this context, several studies have been conducted to develop new strains and efficient expression systems to use LAB as "cell factories" for the production of anti-inflammatory proteins, where we provide the recombinant *Lactococcus lactis* strains efficiency in the prevention of the intestinal damage associated with inflammatory bowel disease in murine models. Moreover, the use of LAB as cell carriers for the production and presentation of antigens has contributed significantly to the development of new vaccines. A growing number of publications on biotechnological or therapeutic employment of LAB has emerged showing their effectiveness against disease but also its safety and immune efficiency, the fact that there are varied ongoing studies with tests at different stages of clinical phase, strengthens our belief that their use will soon benefit the population against most diseases whose treatment and cure is difficult or non-existent.

Introduction

The human gastrointestinal (GI) tract is colonized by up to 10^{14} bacteria, ten times higher than the number of cells in the human body (Artis 2008; Ley et al. 2006). Most of these microorganisms are bacteria and fungi appear to be rare (Gill et al. 2006). The composition and density of bacterial populations vary along the GI of healthy adults (Wang et al. 2005; Zoetendal et al. 2006). Low numbers (10^3) of bacteria, mainly belonging to the streptococci and lactobacilli group, are present in the upper GI tract, while in contrast, much higher numbers reside in the lower compartments, where bacterial populations reach 10^{11}–10^{12} (Whitman et al. 1998). Bacterial species from both upper and lower GI are classified into three phyla—Bacteroidetes, Firmicutes and Actinobacteria (Eckburg et al. 2005).

Under normal conditions, the intestinal microbiota lives in a symbiotic relationship with the host and this interaction has become a field of growing interest for the scientific community, which is beginning to understand the diversity and function of this microbiota that plays an important role in human health. Metagenomic sequencing studies are currently being developed with the intent to (i) identify the different species that lives in the GI tract and (ii) understand their specific function that are thought to be essential for the proper functioning of the gut ecosystem. This includes functions known to be important for the host, such as degradation of complex polysaccharides, metabolism of mineral, carbohydrates, and lipids, synthesis of short chain fatty acids (SCFA), amino acids and vitamins, activation of bioactive food components, maturation and modulation of the immune system, as well as protection against potentially pathogenic species (Arumugam et al. 2011; Qin et al. 2010). It has been shown that a dysfunctional interaction between the microbiota of the gut and the mucosal immune system of the host can lead to inflammatory intestinal diseases known as inflammatory bowel diseases (IBDs) in genetically disposed individuals (Sartor 2006).

Some microbial members can be classified as true residents, indigenous or autochthonous species, which have a long-term association with the intestinal habitat forming a stable community. On the other hand, there are microbial species that under normal conditions do not colonize the intestine but they do occur in the GI, at least temporarily, as they are present in the food intake and disappear a few days after (Berg 1996). These transiting or allochthonous bacterial species are usually present in fermented food products like yogurt and cheeses. Studies aimed to assess

differences among true resident and transient bacteria are still incipient, and recent reports focus on the group of lactic acid bacteria (LAB) that are widely used as starter cultures in food fermentation of a large variety of fermented foods (Reuter 2001). Examples that illustrate this class of bacteria are *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum* and the thermophilic dairy lactobacilli *Lactobacillus delbrueckii* and *Lactobacillus helveticus* (Marteau and Shanahan 2003).

Probiotic Lactic Acid Bacteria

It has being reported that LAB allochthonous bacteria may have positive effect on their host. Actually, several health beneficial effects have been attributed to this group of bacteria, and the hypothesis of Metchnikoff that claimed that certain bacteria present in fermented food products might have positive effects on the consumers, improving their life expectancy proved to be correct (Metchnikoff 1907). Thus, the World Health Organization (WHO) in 2001 defined this group of microorganisms as probiotics–live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). Most of the probiotics used and exploited today are lactobacilli, especially *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *Lactobacillus johsonii*, most of which have been isolated from the human GI tract. Other representatives include *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri, Lactobacillus fermentum, Lactobacillus salivarius, Lactobacillus paracasei and Lactobacillus rhamnosus* (Borchers et al. 2009; FAO/WHO 2002; Ventura et al. 2009; Walter 2008). A number of other bacteria like *Escherichia coli* strain Nissle, *Enterococcus faecium*, *Enterococcus faecalis* and some species from the *Bifidobacterium* genus are considered as probiotics as well (Borchers et al. 2009; Ventura et al. 2009).

Many research projects have shown that probiotics can induce changes in the gut microbial species composition and diversity, suggesting that an increase in bacterial diversity may have a therapeutic role to attenuate intestinal inflammation. Probiotics can also inhibit growth of pathogens by producing antimicrobial compounds reducing their population at mucosal surfaces through competitive exclusion (Ljungh and Wadström 2006). Moreover, they are able to increase mucosal barrier function turning gut mucosa resistant to pathogens that in other conditions would be capable to translocate the epithelium and cause disease. Another characteristics of probiotics are that they can modulate inflammatory signaling pathways (for example nuclear factor kappa B, NF-κB"; in macrophages, dendritic cells (DCs) and intestinal epithelial cells (IECs) decreasing the secretion of pro-inflammatory cytokines, such as IL-8, TNF-α and INF-y (Fitzpatrick et al. 2008; Haller et al. 2002). Several studies have indicated that probiotics can induce the proliferation of regulatory DCs and T lymphocytes (Treg) establishing an anti-inflammatory environment with the predominance of TGF-β and IL-10 cytokines (Foligne et al. 2007; Di Giacinto et al. 2005; Pronio et al. 2008). Another very interesting feature is that certain probiotics were shown to be able to regulate apoptosis in IECs (Yan and Polk 2002).

Bacterial factors implied in the probiotic effect from different bacterial strains of LAB still remain to be identified. These molecules might be factors secreted by

the bacterium, as the induction of probiotic effects usually does not require direct cell contact. For instance, Lipoteichoic acid (LTA), a secreted factor from selected probiotics, has been shown to have a potent anti-inflammatory effect *in vitro* (Kim et al. 2008). In 2008, Mazmanian et al. (2008) demonstrated that the anti-inflammatory effect of *Bacteroides fragilis* was due to a single microbial molecule, polysaccharide A, PSA. Another example is the soluble peptides from the probiotic mixture VSL#3 that were shown to block NF-κB pathway decreasing the secretion of pro-inflammatory cytokines by the host (Petrof et al. 2004).

As strains of probiotics are capable of reverting an inflammatory to an anti-inflammatory environment, some are being tested as a therapeutic tool to fight against inflammatory intestinal diseases, such as IBD (Jurjus et al. 2004). With this purpose, several models of experimental colitis have been described in order to understand the pathogenesis and exploit probiotics as treatment for IBD (Jurjus et al. 2004; Prantera et al. 2002).

Lactobacillus delbrueckii

Our research group recently screened a collection of dairy *Lactobacillus delbrueckii* and tested its immune modulation effect *in vitro* through the quantification of (NF-κB) activation in a human intestinal epithelial cell line. All strains showed anti-inflammatory effects that varied from strong to light and this effect was due to bacterial surface exposed proteins. One strain (Lb CNZ327) that exhibited an extraordinarily anti-inflammatory function in the *in vitro* assays and was able to significantly reduce macroscopic and microscopic symptoms of dextran sulfate sodium (DSS) induced colitis in mice (Santos Rocha et al. 2012). In order to investigate the mechanisms underlying the protective effects of Lb CNRZ327 *in vivo*, mice were administrated with DSS and many immunological parameters were measured. It was observed that Lb CNRZ327 strain modulated the production of TGF-β, IL-6, and IL-12 in the colonic tissue and of TGF-β and IL-6 in the spleen causing the expansion of CD4+Foxp3+ regulatory T cells in the cecal lymph nodes, modulating not only mucosal but also systemic immune responses (Santos Rocha et al. 2014). Despite positive results from some pre-clinical or clinical trials using probiotics as treatment for intestinal inflammatory diseases, like IBD, our knowledge on the use of this group of bacteria is still preliminary. Due to the variability activity of different probiotic strains, well-designed studies and research projects are required to employ probiotics in medical practice.

Lactococcus lactis

Most beneficial effects of probiotics comprising the group of LAB have often been attributed to bacterial strains included into *Lactocbacillus* and *Bifidobacteria* genus. However, little is known about the effects of bacteria that are constantly present in our diet, such as *Lactococcus lactis*. This species is a facultative heterofermentative and mesophilic bacteria (optimum growth temperature around 30 °C) whose participation in the dairy industry is very relevant, especially for cheese production. There are two *Lactococcus lactis* subspecies reported to date: *lactis* and *cremoris*, both are found naturally in plants, especially grass. But they are also artificially found in the

fermented foods as yoghurt, bread and in some types of wines, once they are used as starter cultures (Carr et al. 2002).

In order to understand the beneficial effect of these bacteria and their mechanism of action some strains of *Lactococcus lactis* were selected for assessment of their immunomodulatory potential *in vitro.* For this purpose three strains were chosen: (i) *Lactococcus lactis* subsp. *lactis* IL1403, the first LAB sequenced and extensively used for the production of various metabolic products such as vitamin B, diacetyl and alanine, as well for the production of recombinant proteins (Bolotin et al. 2001; Kleerebezem et al. 2002); (ii) the *Lactococcus lactis* subsp. *cremoris* MG1363 strain, is most widely used in genetic and physiological research throughout the world and is employed in several biotechnological applications, such as oral vaccines or delivery of bioactive peptides to mucosal GI (Hanniffy et al. 2007); and (iii) the *Lactococcus lactis* subsp. *lactis* NCDO 2118 (LLNCDO2118) strain, isolated from frozen pea, it has been routinely used in our laboratory for cloning and expressing proteins. Recently, this later strain was described as a gamma-aminobutyric acid (GABA) producer (Mazzoli et al. 2010). GABA, the most widely distributed neurotransmitter in the central nervous system of vertebrates, is the product of L-glutamate decarboxylation mediated by the enzyme glutamate decarboxylase (GAD, EC 4.1.1.15) and is known to have positive effects on human health. This neurotransmitter is able to lower blood pressure in mildly hypertensive patients (Inoue et al. 2003), induce tranquilizer and diuretic effect (Jakobs et al. 1993; Wong et al. 2003), prevent diabetes (Hagiwara et al. 2004) and reduce the levels of inflammatory response in rheumatoid arthritis murine model (Tian et al. 2011).

The initial evaluation of *Lactococcus lactis* properties was performed in *in vitro* inflammation model, using intestinal epithelial Caco-2 cell line which, in culture, exhibit enterocytes characteristics (Pinto 1983). When these cells are stimulated with the pro-inflammatory cytokine IL-1β, transcriptional factor NF-κB is activated and consequently, the production of inflammatory mediators, including IL-8, TNF-α, IL-6, Cox2, iNOS. Our results demonstrated that LLNCDO2118 does not induce pro-inflammatory events, and the culture supernatant decreased the secretion of IL-8 levels by 45%, a cytokine which is overproduced in mucosal cells of IBD patients. The ability to inhibit IL-8 secretion or its pathway suggests an immunomodulatory effect of LLNCDO2118, and shows its potential use for IBD treatment, since its inhibition can result in improvement of symptoms of these intestinal diseases (Neurath et al. 1996) .

Later, LLNCDO2118 was evaluated *in vivo* for their potential in the prevention of ulcerative colitis (UC) chemically induced by DSS in mice. As UC is a chronic inflammation characterized by remission and recurrence periods, a protocol that mimics this behavior was employed (Travis et al. 2011). Thus, the animals were subjected to an initial 7-day cycle DSS ingestion followed by 7 days of rest (without DSS ingestion), allowing for a regression of symptoms as well as in the period of remission in UC, in which the treatment (*ad libitum*) with LLNCDO2118 strain was carried out. A second cycle of DSS was used to simulate the disease recurrence. This new DSS cycle started on 14th day and finished on the 21st, which were the days chosen to evaluate the effectiveness of the strain. On the 14th, the group treated with LLNCDO2118 showed improvement in clinical signs of colitis, particularly diarrhea, suggesting that this strain has a local effect, contributing to epithelial cells protection. The analysis carried out on day 21 showed that animals treated with LLNCDO2118

had a reestablishment of the colon size, as well as microscopic scores improvement. This beneficial effect was not due to the production of Secretory Immunoglobulin A (sIgA) levels, an important factor to prevent bacterial translocation (Malin et al. 1996; O'Sullivan 2001), since they were unchanged. However, the cytokine profile of the LLNCDO2118 treated group was able to maintain intermediate levels of anti-inflammatory cytokine IL-10 in colon tissue, while animals that did not receive the strain showed reduced IL-10 levels. Furthermore, LLNCDO2118 administration was associated with an early increase in IL-6 production in the same tissue. IL-6 is a cytokine which can present both a pro-inflammatory and anti-inflammatory effect. In this case we suggest that IL-6 is related to increased mucosal repair by epithelial restitution (Chalaris et al. 2010; Dann et al. 2008; Grivennikov et al. 2009; Podolsky 1999; Scheller et al. 2011). As the DSS induced colitis is caused by the loss of immunological tolerance against the commensal microbiota antigens, being tolerance maintained primarily by Treg cells (and its relative ratio to activated T cells), the regulatory cells levels could then be associated with *Lactococcus lactis* anti-inflammatory mechanism. Thus, after colitis induction, the activated T cells ($CD69^+$) were quantified, which only animals fed with LLNCDO2118 showed higher levels in the spleen, suggesting that some *Lactococcus lactis* product could be able to activate T cells. The $CD4^+CD25^+CD45RB^{low}$ and $CD4^+CD25^+LAP^+$ cells was also analyzed in the mesenteric lymph node and spleen of animals treated with LLNCDO2118, since these specialized T cell response counterbalance pro-inflammatory ones (Bouma and Strober 2003; Strober et al. 2007). Although the anti-inflammatory activity of LLNCDO2118 did not increase $CD4^+CD25^+CD45RB^{low}$ Treg, there was, however, induction of T cells characterized by the surface expression of peptides associated with latency (LAP) both in the mesenteric lymph nodes and spleen of animals treated with this strain. Similar results were observed following treatment with VSL # 3 probiotic which was also administered during the remission period of colitis induced by TNBS (trinitrobenzenesulfonic acid), which has been shown to increase $CD4^+LAP^+$ cells, which is essential for the VSL # 3 probiotic effect (Di Giacinto et al. 2005).

As the differentiation of effector T cells (activated) and regulatory are modulated by DCs (Chen 2006), the profile of DCs expressing $CD103^+$ surface marker was investigated. These tolerogenic DCs are related to the differentiation of naïve $CD4^+$ T cells into Tregs (Coombes and Powrie 2008). In the inflamed group, which received only DSS, there was an increase in the population of $CD11c^+CD103^+$ cells compared to the control group (non-inflamed). LLNCDO2118 administration was able to further increase the amount of these cells, suggesting the expansion of Tregs, such as $CD4^+LAP^+$. So it was proposed that a second effect of LLNCDO2118 was observed on 21st day of experiment after the second DSS cycle, which no longer had the presence of the strain in the intestines of animals. In this second stage it was observed that the immunomodulatory capacity of LLNCDO2118 clearly depended on the expansion / recruitment of regulatory cells and their products, resulting in a milder form of UC. Similarly, Nishitani et al (2009). observed that the *Lactococcus lactis* subsp. *cremoris* FC strain when co-cultured with Caco-2 stimulated cells, was able to significantly reduce expression of IL-8 mRNA, and also inhibit nuclear translocation of NF-κB using RAW264.7 cells *in vitro* model. Now, LLNCDO2118 immunomodulation effect on T cells is considered the best characterized mechanisms of action. Recently our research group has deposited the complete genome of this probiotic strain, which will also enable a greater understanding of their intrinsic characteristics (Oliveira et al. 2014).

Heterologous Protein Production

Genetic engineering strategies in LAB have been employed to improve carbohydrate fermentation, metabolite production, enzymatic activities, or conferring them the capacity to produce beneficial compounds such as bacteriocins, and exopolysaccharides, vitamins, antioxidant enzymes and anti-inflammatory molecules (LeBlanc et al. 2013). In this context, several studies has been conducted to develop new strains and efficient expression systems to use LAB as "cell factories" for the production of proteins (de Moreno et al. 2011).

Lactococcus lactis is the best characterized LAB group member, being regarded as a model organism for the production of heterologous proteins for (i) being an easy to handle microorganism, and (ii) being safe for human use. It was the first LAB to have its genome fully sequenced (Bolotin et al. 1999) thus, it has currently a large number of genetic tools for cloning and expressing (Guimarães et al. 2009; de Vos 1999).

Gene expression regulation in Lactococcus lactis

Transcription

Bacteria gene transcription starts when the sigma subunit (σ) of RNA polymerase recognizes a specific region located on the DNA. This region, called the "promoter" is located on an upstream sequence of gene or operon characterized by the presence of 2 consensus motives, –35 (TTGACA) and –10 (TATAAT) base pairs from the transcription start site. After recognition of these hexanucleotides, the transcription process is carried out (Bolotin et al. 1999). In *Lactococcus lactis* a number of promoters have been described through comparative and functional analysis of already identified genes (Kuipers et al. 1993). They feature sequences –35 and –10 similar to those found in *Escherichia coli* and *Bacillus subtilis* and also a "TG" (thymine-guanine) motif located on the first upstream base pair of the –10 sequence. The primary sigma factor in *Lactococcus lactis* is encoded by rpoD gene (Araya-Kojima et al. 1995; Bolotin et al. 2001) and shows homology to the σ^{70} and σ^{A} factors genes of *Escherichia coli* and *Bacillus subtilis,* respectively. The transcription stops in the 3' portion of genes and operons, where a palindromic sequence of nucleotides rich in guanine, cytosine and thymine, called "transcriptional terminators", signals the end of the process. Most genes and operons in *Lactococcus lactis* have such sequences.

Translation

Once transcription has occurred, the translation process initiates. In *Lactococcus lactis,* the translation start signals are also similar to those described in *Escherichia coli* and *Bacillus subtilis*. The ribosome attachment site or "RBS" is located in the 5' portion of mRNA to be translated, and is complementary to the sequence 3' to the 16S rRNA (3'CUUUCCUCC 5') of *Lactococcus lactis* (Chiaruttini and Milet 1993). Although most of the initiation codons are AUG, other codons, such as GUG were also observed (van de Guchte et al. 1992).

Genetic tools for the production of heterologous proteins in *Lactococcus lactis*

Heterologous proteins expression systems in *Lactococcus lactis* were obtained by the progress of genetic knowledge, the development of molecular biology techniques and studies of regulatory elements of gene expression, such as constitutive or inductive promoters (Miyoshi et al. 2004). This combination has allowed a variety of proteins from different sources to be cloned and highly expressed in *Lactococcus lactis* through several plasmidial vectors (Bermúdez-Humarán et al. 2011; Langella and Le Loir 1999; Mercenier et al. 2000).

Heterologous proteins expression systems and cell targeting

An early gene expression system for use in *Lactococcus lactis* was based on the promoter Plac and the regulatory gene *lacR* from the bacterial lactose operon. This operon is activated when the Plac promoter is induced in the presence of lactose and the transcription repressor gene (*lacR*) is suppressed in the same condition, allowing the target gene to be expressed (van Rooijen et al. 1992).

Subsequently another system was developed, consisting of three vectors that matched the lac operon elements and 2 more elements from the bacteriophage T7 of *Escherichia coli*, allowing a higher level of induction of the protein of interest (Wells et al. 1993). In this system, the gene coding RNA polymerase from phage T7 (T7 RNA pol) was placed under the control of Plac promoter in a first vector while in a second vector, the target protein is under control of the T7 promoter. Thus, this system works in a way that when lactose was added to the culture medium, the Plac induces the expression of T7 RNA pol, which activates expression of the gene of interest controlled by the T7 promoter. However, in order to the cell to be capable of metabolizing soluble lactose in the medium, a third vector containing the lac operon was necessary. Although this system allowed fine control of gene expression and higher levels of production, it became infeasible because it required three antibiotic resistance markers making it unsuitable for food and pharmaceutical industry employment (Wells et al. 1993).

Gene expression regulation studies on the common phages from the *Lactococcus* sp. were the basis for developing more simple expression systems like the "operator-repressor system" based on r1t, a *Lactococcus lactis* phage (Nauta et al. 1996). In the same vector, the gene coding of the protein of interest is placed under the control of P_{ORF5} phage promoter, which is repressed by the phage protein Rro. When added to the medium, the mutagen mitomycin C causes the proteolytic breakdown of the repressor protein Rro, and the consequent release of the PORF5 promoter. Free of repression, the promoter induces the expression from of the gene under its control. This system was tested using the *lac* Z reporter gene rom *Escherichia coli* and subsequently using the *acmA* gene (autolysin) from *Lactococcus* sp. However, the use of mitomycin C as inductor prevents the use of this system for protein production in fermenters as well in food products.

In another system, the genetic elements from phage φ31 were used to develop an expression system that matched the P_{15A10} promoter and the replication origin, ori31 (O'Sullivan 2001). Here, as in other systems, the gene of interest cloned under the control of P_{15A10}, and ori31, are in the same vector. After the start of φ31 phage

infection, ori31 becomes target of the phage replication machinery and the amount of vector copies within the cell is increased. Due to this increase and due to the strength of P_{15A10} promoter, the gene of interest expression level is also increased. After cell lysis caused by phage replication, the protein molecules in question are released into the environment. The major disadvantage of this system is the need to obtain cell infection induction; which leads to the destruction of the cell culture, thus impeding their industrial use, in fermenters.

In this context, many studies have been conducted in order to develop safer and more suitable vectors for food industry. One of the most powerful expression systems already developed for use in food industry are based on genes involved in biosynthesis and regulation of the antimicrobial nisin, a peptide naturally secreted by several strains of *Lactococcus lactis.* Because of its antimicrobial properties, it is widely used as a natural food preservative. The Nisin Controlled Gene Expression–NICE system was developed in *Lactococcus lactis* where the genes *nis*R and *nis*K were inserted into the chromosome in the MG1363 strain, and the P_{nisA} promoter in the expression vector followed by multiple cloning sites (MCS) for insertion of genes of interest (Kuipers et al. 1993; Mierau and Kleerebezem 2005). In this system, induction of expression of heterologous proteins can be achieved by adding nisin in the extracellular medium, in which the *nis*K gene functions as a membrane sensor that recognizes the extracellular presence of nisin, while the signal is transferred to NisR by a phosphorylation process, turning NisR capable of binding to P_{nisA} promoter and consequently activate the gene of interest transcription.

This system is also versatile, making the heterologous protein able to accomplish their desired biological activity by addressing them properly to its final cell destination: (i) cytoplasm (ii) membrane or (iii) the extracellular medium. In bacteria, the protein secretion is accomplished by the addressing of specific sequences which encode a hydrophobic negatively charged signal peptide (SP) located at their amino-terminal portion (N-terminal). This SP is recognized and cleaved by the secretion machinery allowing translocation of the protein across the cell membrane, and thereby released in the extracellular medium. Another signal sequence is Cell Wall Anchor (CWA) that encondes a peptide composed of 30 amino acids which is located in the carboxy-terminal portion (C-terminus) of the protein. The CWA has a conserved motif (LPXTG) which is recognized by anchoring machinery. Thereby, the protein containing this motif is covalently attached to peptidoglycan present in the cell membrane (Le Loir et al. 1994; Mierau and Kleerebezem 2005; Piard et al. 1997).

Xylose-Inducible Expression System

In 2004, Miyoshi *and collaborators.* developed a new gene expression system for *Lactococcus lactis*. The system, called Xylose-Inducible Expression System (XIES) based on the xylose permease gene promoter (PxylT), from *Lactococcus lactis* NCDO2118, Described by Jamet and Renult (2001). In the presence of some sugars, as glucose, fructose and/or mannose, PxylT was shown to be repressed; otherwise, PxylT is transcriptionally activated by xylose in *Lactococcus lactis* (Miyoshi et al. 2004). Thereby, this promoter could be successively turned on by adding xylose and off by washing the cells and growing them on glucose (Jamet and Renault 2001). Myoshi and collaborators (2004) developed a new lactococcal XIES that also incorporates

the ability to target heterologous proteins to cytoplasm or extracellular medium. This system contains two plasmids that are derived from two broad-host-range expression vectors, pCYT:Nuc and pSEC:Nuc that would send the protein to the cytoplasm or to the extracellular medium, respectively (Bermúdez-Humarán et al. 2003). The system combines the PxylT (Jamet 2001), the ribosome-binding site (RBS) and the signal peptide (SP) of the lactococcal secreted protein, Usp45 (van Asseldonk et al. 1990) and the *Staphylococcus aureus* nuclease gene (*nuc*) as the reporter (Le Loir et al. 1994; Shortle 1983) and was successfully applied to high-level Nuc production and correct protein targeting and was tested in the *Lactococcus lactis* subsp. *lactis* strain NCDO2118. These systems have great advantages once they are considered less expensive and safer for laboratory use as compared to the other available expressions methods (Azevedo et al. 2012).

De Azevedo et al. (2012) constructed the recombinant *Lactococcus lactis* strains that were able to produce and properly send the *Mycobacterium leprae* 65-kDa HSP (Hsp65) to the cytoplasm or to the extracellular medium, using XIES. Heat shock proteins (HSPs) expression in host is induced by a wide variety of stresses (including high temperature, anoxia, and ethanol) (Lindquist and Craig 1988). Hsp65 are also known to play a major role in immune modulation, controlling autoimmune responses. Some authors showed that oral administration of a recombinant *Lactococcus lactis* strain that produces and releases LPS-free Hsp65 prevented the development of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, reduced the incidence of type I diabetes in non-obese diabetic mice and attenuated atherosclerosis in low-density lipoprotein receptor-deficient mice (Jing et al. 2011; Ma et al. 2014; Rezende et al. 2013). Some *Lactococcus lactis* produced recombinant Hsp65 that could be used for biotechnological and therapeutic applications. The rHsp65 protein was efficiently produced in both the cytoplasm and secreted forms to the extracellular medium, confirming the ability of the XIES to produce and correctly address recombinant proteins (Azevedo et al. 2012).

del Carmen et al. (2011) have used the XIES to express anti-inflammatory molecules as an alternative therapy against IBDs, like use of a fermented dairy product containing IL-10-producing *Lactococcus lactis* for the prevention and/or treatment of IBD using a rodent model of Crohn`s disease (CD). IL-10 has a central role in down-regulating inflammatory cascades, which makes it a good candidate for use in the therapeutic intervention in inflammatory processes (Marinho et al. 2010). The XIES was effective in dairy matrix, as observed by significant increases of the cytokine in fermented milks with IL-10-producing strains (Cyt and Sec). Once, XIES is a LAB expression system that can be added into the food matrix that is tightly regulated by xylose, which is rarely found in conventional foods, it can act perfectly as an inductor. In this way IL-10 expression can be up- or down regulated, which is especially useful for expression studies of the cytokines in media or milk. The use of an inducible expression system is very interesting from a genetics point of view, because genes in this expression system are only expressed when required by adding the inducer (in this case xylose). A constitutive expression system would continuously produce the IL-10 (or other genes under its control) when it might not be required, especially in the cases of bacterial strains that could persist in the gastrointestinal tract (del Carmen et al. 2011). Induction with xylose increased the cytokine levels production by IL-10 *Lactococcus lactis* producers (>500 pg/ml for the Cyt strain and >1,000 pg/ml for the Sec strain) (Marinho et al. 2010). There is some controversial data in the literature about the more

effective IL-10 producer *Lactococcus lactis* strain. It was demonstrated by Marinho et al. (2010) that *Lactococcus lactis*- producing IL-10 in the cytoplasm showed a higher immunomodulatory potential in a murine lung inflammation model, hypothesizing that the recombinant IL-10 produced in the cytoplasmic form stored IL-10 for a longer period of time and is slowly released in the tissue when the bacterial host lysis occurs. However, Del Carmen et al. (2011) found that the secreting IL-10 strain showed a higher anti-inflammatory effect compared to the cytoplasmatic producing IL-10 strain. This could be due to the fact that this cytokine and the *Lactococcus lactis* Sec strain are probably both protected by the food matrix (milk), resulting in a more efficient delivery of IL-10 in the gut. The IL-10 produced by these lactococci strains was able to induce an anti-inflammatory effect in our TNBS model and this effect was attributed to IL-10 producers while the wild-type (wt) strains did not exert any effect. Prevention of intestinal damages (macroscopic and microscopic) was also observed in mice that received the milks fermented by both of these strains (Cyt and Sec producers) thereby proving the anti-inflammatory effect of these products. The promising results obtained in these studies showed that the employment of fermented milks as a new form of administration of IL-10-producing *Lactococcus lactis* could represent an alternative treatment for IBDs (del Carmen et al. 2011; Marinho et al. 2010).

In another study, researchers evaluated the production and the delivery of 15-lipoxygenase-1 (15-LOX-1) by *Lactococcus lactis* NCDO2118 containing XIES in a TNBS- induced colitis model in mice (Saraiva et al. 2015). 15-LOX-1 enzyme is found in endothelial/epithelial cells that plays a key role in the oxidative metabolism of arachidonic acid responsible for lipoxins production, lipid mediators with potent anti-inflammatory actions (Lee et al. 2011; Serhan 2005). They concluded that 15-LOX-1 producing *Lactococcus lactis* was effective in the prevention of the intestinal damage associated to inflammatory bowel disease in a murine model, proving the effectiveness and efficiency of XIES (Saraiva et al. 2015).

Production of several staphylococcal proteins in *Lactococcus lactis* has been reported. However, these studies were not dedicated to the development of an antigen for oral vaccination, most of them were dedicated to expression-secretion systems development, such as staphylococcal nuclease to be used as a reporter protein, used latter as a reporter protein in XIES (Le Loir et al. 1994), for the characterization of staphylococcal virulence factors as ClfA and FnbA (Que et al. 2001), ClfB (Clarke et al. 2009), IsdA (Innocentin et al. 2009), or to increase adhesion properties of recombinant *Lactococcus lactis* strains (Harro et al. 2012). Asensi et al. (2013) were the first to evaluate the production of a staphylococcal antigen in a recombinant LAB strain to be used for oral vaccination where two recombinant *Lactococcus lactis* strains allowed the production of Staphylococcal enterotoxin type B (rSEB), a potent superantigenic exotoxin, either cytoplasmatic or secreted in the intestinal mucosa of mice, using XIES. Oral immunization with the recombinant strains induced a protective immune response against a lethal challenge with *Staphylococcus aureus* ATCC 14458, an SEB producer strain, in murine model.

DNA Vaccines

Vaccination is one of the main tools to combat and eradicate diverse pathogenic and/or infectious agents widespread around the world.

DNA vaccines are the third generation vaccine, which utilizes genetically engineered DNA to produce an immunologic response. The first study about this platform started in the early 1990s when Wolff et al. (1992) observed that injection of a "naked" plasmid DNA encoding foreign antigens in mice made their muscle cells capable of expressing these same antigens since this first publication, the use of DNA as a strategy for vaccination has progressed very quickly. The Norwegian Biotechnology Advisory Board defines this platform as "the intentional transfer of genetic material (DNA or RNA) to somatic cells for the purpose of influencing the immune system" (The Norwegian Biotechnology Advisory Board 2003).

This platform has the propriety to induce humoral and cellular immune response against different kind of microorganisms such as parasites, bacteria and disease-producing viruses (Ulmer et al. 1993; Wolff et al. 1992). Moreover, it was also utilized on several tumor models (Cheng et al. 2005). The components of DNA vaccine are: (i) the plasmid backbone which contains a bacterial origin of replication needed for the vector's maintenance and propagation inside the bacteria; the cytosine-phosphate-guanine unmethylated (CpG) motifs, called immunostimulatory sequences (ISS), these motifs could be a contribution to DNA immunogenicity. In the mammalian genome CpG have a low frequency and are mainly methylated, but bacterial DNA contains many unmethylated CpG motifs allowing this motif to be recognized by mammals as a pathogen associated molecular pattern (PAMP). In this way, CpG motifs are the ones in charge of increasing the magnitude of the immune response because they can interact with Toll-like receptors (TLR), such as TLR9, adding adjuvant activity (Tudor et al. 2005), a resistance marker, required to permit selective growth of the bacteria that carries the plasmid; (ii) the transcriptional unit, essential for eukaryotic expression, which harbors a promoter/enhancer region, introns with functional splicing donor and acceptor sites, as well as the open reading frame (ORF) encoding the antigenic protein of interest, and the polyadenylation sequence (poly A), signal required for efficient and correct transcription termination of the ORF and transfer of the stable mRNA from the nucleus to the cytoplasm (Azevedo et al. 1999; Kowalczyk and Ertl 1999).

After an intramuscular or intradermal injection, the naked plasmid DNA will transfect somatic cells, such as myocytes and keratinocytes, and/or resident Antigen Presenting Cells (APCs) like DCs and macrophages located in the lamina propria (Kutzler and Weiner 2008; Liu 2011). Due to the fact that antigens are expressed intracellularly, both humoral and cell-mediated immunity can be activated to generate a broad immune protection. After the transcription and translation of the transgene the host-synthesized antigens become the target of immune surveillance in the context of both major histocompatibility complexes (MHC) class I and class II molecules of APCs. The APC cells have the propriety to move to the draining lymph nodes where they present the antigenic peptide-MHC complexes to stimulate naïve T cells. In the other hand, B cells are activated, beginning the antibody production cascades. Although plasmid DNA vaccines vectors can induce antibody and CD4+ T cell helper responses, they are particularly suited to induce CD8+ T cell responses (Anderson and Schneider 2007). The CD8+ T cells, the cytotoxic T lymphocytes, which are important in controlling infections (Leifert and Whitton 2000) induced by DNA vaccine, can occur in two main pathways: (i) the direct DNA transfection

of the APCs like as dendritic cells (DCs) and (ii) cross-presentation approach, when somatic cells such as myocytes are transfected with DNA and the expressed antigens are taken up by the APCs, or when the transfected apoptotic cells are phagocytosed by the APCs (Leifert and whitton 2000, Xu et al. 2014).

DNA vaccines have an extensive range of features that give them many advantages over other vaccination platforms like traditional vaccines developed against pathogens, including either killed or attenuated pathogenic agents. DNA vaccines are relatively cheap and easy to produce, which is an important feature when considering an emerging pandemic threat (Liu 2011). An essential concern about vaccine products is safety. DNA vaccine are considered safe because they lack the risk of reversion to a disease causing state or secondary infection. Also the risk of integration of the plasmid into the host cell causing insertional mutagenesis, which may lead to the inactivation of tumor suppressor genes or activation of oncogenes, is found to be significantly lower than the spontaneous mutation rate (Nichols et al. 1995; Wang et al. 2004). No adverse effects have been reported either tolerance to the antigen or autoimmunity (Liu and Ulmer 2005), animal studies showed that there is no increase in anti-nuclear or anti-DNA antibodies after DNA vaccination. There has been no evidence that autoimmunity is associated with DNA vaccines (Le et al. 2000; Tavel et al. 2007).

Vaccine manufacturing is a simple and low cost method as it requires only the use of cloning techniques in order to clone the protein of interest. They are stable at room temperature, easy to store and transport, presents thermal stability and have a long shelf life (Grunwald and Ulbert 2015; Pereira et al. 2014). A systemic inflammation, which might conduce the increase of cardiovascular risk, is a rising concern about vaccination in general (Gherardi and Authier 2012; Ramakrishnan et al. 2012), although DNA vaccines are still contemplated as a relatively new approach to vaccination, and its potential to induce systemic inflammation must not be overlooked (Xu et al. 2014). Many studies have shown that DNA vaccines are generally satisfactory with an acceptably good safety profile, and no systemic inflammation has been reported (Goepfert et al. 2011; Jaoko et al. 2008; Kalams et al. 2012; Ledgerwood et al. 2011) .

Therefore, DNA vaccines portray as a smart tool due to its property to induce all three points of adaptive immunity: antibodies, helper T cells (TH) and cytotoxic T lymphocytes (CTLs), as well as being capable of stimulating innate immune responses (Li et al. 2012) with safety. However, among the disadvantages, the poor immunogenicity of naked-DNA platform when is administrated in large animals (Kim et al. 2010) can be highlighted along with the necessity of using adjuvants besides the gene encoding of the protein. These platform are limited to protein immunogens and are not useful for non-protein based antigens such as bacterial polysaccharides (Kuby et al. 2007).

To circumvent this problem a delivery vehicle is needed to protect the DNA vaccine against endonucleases degradation. Thus, pathogenic bacteria attenuated strains appear as an interesting delivery method as they have innate tropism for specific tissues of host, which makes them attractive to use as a vehicle delivery to DNA vaccine. The use of bacteria as a delivery vector has numerous benefits: they can maintain the plasmid in a high copy number, they are easy to manufacture, they are

less laborious and the cost is low as there is no need to amplify and purify the plasmid before handling (Becker et al. 2008; Schoen et al. 2004), and large-size plasmid can be housed inside the bacteria, permitting the insertion of multiple genes of interest (Hoebe et al. 2004; Seow and Wood 2009). Another important features is the possibility of these vectors being used for mucosal administration, without the use of a needle, thus having the ability to stimulate both mucosal and systemic immune responses (Srivastava and Liu 2003).

In the intestinal mucosa, the bacteria carrying a DNA vaccine are able to cross the intestinal barrier, mainly via specialized epithelial cells called Microfold cells (M cells). M cells overlying Peyer's patches (PPs) whose lymphoid follicles are isolated while draining gut mesenteric lymph nodes are considered more accessible to antigens and bacteria present in the luminal compartment. DCs located in the PPs, are another pathway that bacteria have to access the body. Immature DC are able to open the tight junctions between epithelial cells, extend their dendrites outside the epithelium and directly sample bacteria, thereby monitoring the contents of the intestinal lumen (Rescigno et al. 2001). The IECs lining mucosal surfaces, can be invaded by bacteria through bacterial proteins called invasins. This characteristic refers to the capacity of attenuated pathogenic vectors to deliver DNA vaccines as they are able to naturally produce invasins.

Once inside the cells, bacteria vector have the ability to escape from the phagolysosome vesicles by the secretion of a variety of phospholipases and pore-forming cytolysins and enter the cytoplasm of the host cells (Hoebe et al. 2004; Schoen et al. 2004). The microtubules net are used by the plasmid to reach the nucleus. In the nucleus, using the host cell's transcription machinery, the protein of interest carried by the plasmid can be encoded, translated, and secreted afterwards (Grillot-Courvalin et al. 1999; Schoen et al. 2004) by the cell or be presented on the surface of epithelial cell or DCs. The MHC class-II, from APCs, presents the exogenous proteins, turning naïve T cells activated into CD4+ T cells. Furthermore, the exogenous protein may also be processed into small peptides, which are then presented on the surface of MHC class-I molecules to CD8+ T cells, and stimulate them (Saha et al. 2011).

The pattern recognition receptors (Toll-like and Nod-like receptors) expressed by IECs, B-lymphocytes and DCs located in the sub epithelial lamina propria are the other components of immunity used by bacteria. The bacterial components known as microbe-associated molecular patterns (MAMPs) are recognized by pattern recognition receptors and trigger intracellular signaling pathways that lead to cytokine secretion and immune cell activation (Barbosa and Rescigno 2010; Steinhagen et al. 2011). The bacterial recognition by the immune system modulates the innate immune response, thereby supporting a vigorous and lasting adaptive response (Hoebe et al. 2004).

Enteropathogenic bacteria like *Salmonella* typhi, *Listeria monocytogenes, Shigella flexneri, Yersinia enterocolitica* and *Escherichia coli* are the species that are most widely used as bacterial delivery systems into mammalian cells (Schoen et al. 2004) because of their natural tropism for macrophages as well as DCs in the lymphoid tissue of the intestinal mucosal surface (Becker et al. 2008).

The method that use senteropathogenic species as a bacterial carrier is being considered an advantage because of their capacity to infect human colonic mucosa after

oral administration. However, they need to be attenuated or inactivated as they present the risk to revert to the virulent phenotype, there by compromising its safety. Therefore, World and Health Organization (WHO) does not recommend their use in children and immunocompromised individuals. Thus, to counteract this severe problem, the use of non-pathogenic bacteria, such as LAB as vectors for genetic immunization has been investigated (Wells and Mercenier 2008).

Lactic acid bacteria vehicles for DNA vaccine delivery

Regarding *Lactococcus lactis* as a vehicle to deliver DNA vaccines, many interesting features can be highlighted: (i) it was proved in different laboratories all over the world that they can carry recombinant plasmids and express antigens and therapeutic molecules at different cellular localizations (Le Loir et al. 2001; Wells et al. 1993); (ii) it was successfully demonstrated that *Lactococcus lactis* can deliver DNA into eukaryotic cells and *in vivo* to mice IECs (Chatel et al. 2008a; Guimarães et al. 2005a; Innocentin et al. 2009); (iii) they can induce both systemic and mucosal immunity when administrated at mucosa surfaces (Chang et al. 2003; Robinson et al. 1997); (iv) they can resist the acid environment of the stomach, are able to survive into the gastrointestinal tract, ensuring recombinant protein or plasmid delivery (Pereira et al. 2014). Regarding its extraordinary safety profile (Salminen et al. 1998), because *Lactococcus lactis* is not very immunogenic, it can be orally administrated several times (Guimarães et al. 2006). All these characteristics makes it a good option for being used in immunization programs (Macauley-Patrick et al. 2005).

Research that used wild-type (wt) *Lactococcus lactis* as a vector for genetic immunizationhave demonstrated both *in vitro* (Guimarães et al. 2006) and *in vivo* (Chatel et al. 2008a) that the percentage of gene transferred observed was low, as well as a low and transitory Th1-type immune response after immunization trials (Chatel et al. 2008a). To solve this problem scientist developed recombinant *Lactococcus lactis* expressing different invasins to improve bacterial interaction with IECs (Azevedo et al. 2012; Guimarães et al. 2005; Innocentin et al. 2009).

Regarding non-invasive LABs, Guimarães et al. (2006) carried out *in vitro* studies using non-invasin strains of *Lactococcus lactis* as DNA delivering vehicles. The pLIG:BLG plasmid, containing an eukaryotic expression cassette with the cDNA of the bovine -lactoglobulin (BLG) under the control of the human cytomegalovirus eukaryotic promoter (Pcmv) was used to transform *Lactococcus lactis* MG1363. Caco-2 human cells were co-incubated with purified pLIG:BLG, MG1363 (pLIG:BLG), MG1363 and a mix of MG1363(pLIG) and pLIG:BLG. Only the cells that were co-incubated with MG1363 (pLIG:BLG) exhibited the presence of BLG cDNA and the subsequent expression of BLG. This result indicated that there was the delivery of the BLG cDNA to the mammalian epithelial cells. The authors suggested that after the co-culture, some bacteria are internalized and lysed by the host phagolysosome and, consequently, the BLG cDNA was released in the cytosol.

After these *in vitro* results Chatel et al. (2008) described for the first time *in vivo* the transfer of functional genetic material from non-invasive food-grade transiting from bacteria to host. The delivery of a eukaryotic expression plasmid coding of the BLG to the epithelial cells of the intestinal membrane of mice using *Lactococcus lactis* is possible. This demonstrates the capacity of using these bacteria in the delivery of DNA vaccines. In this study, mice were submitted to intragastrically gavage. The BLG cDNA was detected in the epithelial membrane of the small intestine in

40% of the mice. Moreover, the BLG was produced by 53% of them. In addition, the BLG production was responsible for inducing a protective immune response when the mice were sensitized with cow's milk proteins. In this case, the induction of a Th1 immune response counteracting a Th2 response was observed. The delivery of a functional plasmid by *Lactococcus lactis* to the mice intestinal wall provides us with the understanding of the host-bacterium interaction and the modulation of host immune response due to the delivered DNA.

Regarding the use of invasive LABs, Guimarães et al. (2005) constructed a *Lactococcus lactis* strain capable of invading epithelial cells by cloning and expressing the internalin A gene (*inlA*) of *Listeria monocytogenes* under the control of a native promoter. Western Blot and immunofluorescence experiments showed that the cell wall anchored form of InlA was efficiently exhibited by the recombinant lactococci, that favored the internalization of *Lactococcus lactis inlA+* in Caco-2 cells. Invasivity test showed that *Lactococcus lactisinlA+* was 100 times more invasive than for wt *Lactococcus lactis*. Moreover, *Lactococcus lactis inlA+* could deliver the eukaryotic expression plasmid coding the Green fluorescent protein (GFP) gene to Caco-2 cells, as it was possible to detect the GFP in 1% of the invaded cells. Finally, *in vivo* studies using *Lactococcus lactis inlA+* for oral inoculation of guinea pigs revealed that *Lactococcus lactis inlA+* was able to penetrate intestinal cells.

With these invasive lactococci, DNA delivery by this bacterium can be measured. In order to achieve this, a new vector has been developed resulting from the co-integration of two replicons: one from *Eschericha coli* and the other from *Lactococcus lactis,* named vaccination using lactic acid bacteria (pValac). The pValac is formed by the fusion of (i) cytomegalovirus promoter (CMV), that allows the expression of the antigen of interest in eukaryotic cells, (ii) polyadenylation sequences from the Bovine Growth Hormone (BGH), essential to stabilize the RNA transcript, (iii) origins of replication that allow its propagation in both *Escherichia coli* and *Lactococcus lactis* hosts, and (iv) a chloramphenicol resistance gene for selection of strains harboring the plasmid. The functionality of pValac was observed after transfecting plasmids harboring the *gfp* ORF into mammalian cells, PK15. The PK15 cells were able to express GFP.

Although the interesting results obtained by the utilization of *Lactococcus lactis inlA+*, *in vivo* experimental studies are limited to guinea pigs or mutated mice, InlA cannot bind the murine E-cadherin. Thus, Innocentin et al. (2009) performed comparative studies using both *Lactococcus lactis* expressing the Fibronectin-Binding Protein A of *Staphylococcus aureus* (LL-FnBPA+) as a InlA. In this study, it was verified that LL-FnBPA+ or the truncated form coding only C and D domains of FnBPA (LL-CD+) were internalized by the Caco-2 intestinal epithelial cells as efficiently as *Lactococcus lactis inlA+*. Also in this study, it was evidenced for the first time that lactococci can be internalized in high levels and they as heterogeneously distributed in the cell monolayer. Finally, studies were performed using *Lactococcus lactis* InlA, *Lactococcus lactis* FnBPA and *Lactococcus lactis* CD carrying GFP and all of them were able to trigger GFP expression in Caco-2 cells.

Pontes et al. (2012) in *in vitro* and *in vivo* studies used invasive *Lactococcus lactis* expressing FnBPA of *Staphylococcus aureus* (LL-FnBPA+) and demonstrated that the production of FnBPA increased the plasmid transfer to Caco-2 cells (Pontes et al. 2012). When the invasiveness of Caco-2 cells by LL-FnBPA+ carrying the pValacBLG plasmid (LL-FnBPA+BLG) or not (LL-FnBPA+) was compared with

the LL-wide type (LL-wt) and LL-BLG, it was observed that LL-FnBPA+BLG and LL-FnBPA+ were 10 times more invasive than LL-wt and LL-BLG. After the Caco-2 cells were co-incubated with LL-FnBPA+BLG and LL-BLG. It was found that the cells incubated with LL-FnBPA+BLG produced 30 times more BLG than the cells co-incubated with the non-invasive strain. Moreover, using BLG and GFP under the control of a eukaryotic promoter, the potential of LL-FnBPA+ as a DNA vaccine delivery vehicle was characterized *in vivo*. After the oral administration of LL-FnBPA+BLG and LL-BLG to mice, it was detected the plasmid transfer to enterocytes had no difference between both strains. The same result was observed when LL-FnBPA+GFP were used. Regarding the expression of BLG by mice, the oral administration of LL-FnBPA+BLG led to an increase in the number of mice able to produce BLG, but there was no difference in the levels of the BLG produced. In other words, *Lactococcus lactis* increased the plasmid transfer but not the quantity of plasmid transferred.

As mentioned before, InlA cannot bind the murine E-cadherin. Moreover, FnBPA requires an adequate amount of fibronectin to be used by the integrins. Therefore, to bypass these problems and to better understand the steps of DNA transfer to mammalian cells, De Azevedo et al. (2012), engineered *Lactococcus lactis* to express a mutated form of InlA (mInLA+) which allowed the affinity to murine E-cadherin and, consequently, *in vivo* experiments using conventional mice. The results of the tests with Caco-2 cells demonstrated that LL-mInlA+ were 1000 times more invasive than LL. To analyze the role of this strain of *Lactococcus lactis* as a DNA delivery vector, a plasmid carrying the BLG cDNA (pValacBLG) was used and the transfer to intestinal epithelial cells (IECs) was measured. *In vitro* results showed that LL-mInlA+BLG were 10 times more invasive than LL-BLG. *In vivo*, after oral administration of LL-mInlA+BLG and LL-BLG, the number of mice producing BLG in isolated enterocytes was slightly higher in mice administered with LL-mInlA+BLG than with LL-BLG.

Our research group was very interested to know whether uptake of *Lactococcus lactis* DNA vaccines by DCs could also lead to antigen expression, as observed in IECs, as they are unique in their ability to induce antigen-specific T cell responses. We demonstrated that both non-invasive and invasive lactococci could transfect bone-marrow DCs (BMDCs), inducing the secretion of the pro-inflammatory cytokine IL-12. This plasmid transfer to BMDCs was also measured through a polarized monolayer of IECs, mimicking the situation found in the GI tract. Co-incubation of strains in this co-culture model showed that DCs incubated with LL-mInlA+ containing pValac:BLG could express significant levels of BLG, suggesting that DCs could sample bacteria containing the DNA vaccine across the epithelial barrier and express the antigen (de Azevedo et al. 2015).

With reference to the IBDs, del Carmem et al. (2014) used LL-FnBPA+ carrying pValac:il-10. The Interleukin-10 (il-10) is an important anti-inflammatory cytokine involved in the intestinal immune system (del Carmen et al. 2014). Transfection and invasiveness assays using cell cultures showed the functionality of the plasmid and the invasive strain. Fluorescence microscopy using mice confirmed the *in vitro* results. After that, a trinitrobenzene sulfonic acid (TNBS) model for induction of intestinal inflammation in mouse was performed. Mice that received LL-FnBPA+ carrying pValac:il-10 plasmid exhibited lower damage scores by macroscopic and microscopic analysis of the large intestine, lower microbial translocation to liver and

the anti-inflammatory/pro-inflammatory cytokine ratios were increased more than the mice that received *Lactococcus lactis* FnBPA+ without the pValac:il-10 plasmid. These results suggest that this DNA delivery strategy was efficient in preventing inflammation in this colitis murine model.

Continuing in the IBDs research line, Zurita-Turk et al. (2014) used LL-FnBPA+pValac:Il-10, LL-FnBPA+, LL-pValac:Il-10 and LL-wt in a different colitis model, the dextran sodium sulphate (DSS) model for induction of intestinal inflammation (Zurita-Turk et al. 2014). The results showed that both LL-FnBPA+pValac:Il-10 and LL-pValac:Il-10 were able to diminish the intestinal inflammation. Therefore, both strains delivered the eukaryotic expression vector to host cells directly at the sites of inflammation and lead *in situ* IL-10 production and its anti-inflammatory properties.

Christophe et al. (2015), working with another LAB, *Lactobacillus plantarum*, and aiming to increase the DNA delivery by these bacteria, constructed a strain targeting DEC-205, a receptor located at the surface of dendritic cells (Christophe et al. 2015). The objective was to increase the bacterial uptake and, consequently, improve the delivery of the cDNA to immune cells. For that, anti-DEC-205 antibody (aDec) was displayed at the surface of *Lactobacillus plantarum* using a covalent anchoring of aDec to the cell membrane, a covalent anchoring to the cell wall and a non-covalent anchoring to the cell wall. The results show that aDec was successfully expressed in the three strains, but surface location of the antibody could only be demonstrated for the strains with a covalent than a non-covalent anchoring to the cell wall. To verify the plasmid transfer, a plasmid for GFP expression under the control of a eukaryotic promoter was used to transform the three strains. GFP expression in DC cells was increased when using the strains producing cell-wall anchored aDec. However, *in vivo* tests using the mouse model exhibited a higher expression of GFP when the strain with a covalent anchoring to the cell membrane was used. It seems to be that the more embedded location of aDec in this strain is beneficial when cells are exposed to the gastro-intestinal tract conditions.

For further reading on this topic, the following works can be of great value: Almeida et al. (2014) evaluated the invasiveness of recombinant strains of *Lactococcus lactis* expressing FnBPA under the control of its constitutive promoter or driven by the strong NICE system (Almeida et al. 2014; Pontes et al. 2014) compared immune responses elicited by DNA immunization using LL-FnBPA+BLG and LL-BLG and they verified that the immune response could be modified by production of invasins on the cell surface (Pontes et al. 2014). They showed that intranasal or oral DNA administration using invasive LL-FnBPA+BLG elicited a TH2 primary immune response whereas the LL-BLG elicited a classical TH1 immune response; Pontes et al. (2012) revised not only the expression of heterologous protein but also the delivery systems developed for *Lactococcus lactis*, and its use as an oral vaccine carrier (Pontes et al. 2012); Bermúdez-Humarán et al. (2011) gathered research works using LABs, more specifically lactococci and lactobacilli, as mucosal delivery vectors for therapeutic proteins and DNA vaccines (Bermúdez-Humarán et al. 2011).

Recently, our team has developed another vector called pExu, to be used in *Lactococcus lactis*. This vector will be also used in genetic immunization like the pValac.

DNA vaccines in clinical trial phase and already licensed for use

Since the early 1990s, when studies of DNA vaccines were started, and till date, more than 18,000 scientific papers have been published on this subject. Among these, almost 500 were published in the first half of 2015's (PUBMED 2015). However, while research has advanced, currently, only four DNA vaccines are licensed and commercially available in the world, all of them for veterinary use.

The first two prophylactic vaccines based on recombinant DNA technology have been approved for use and licensed in 2005. The first one against horses West Nile virus (West Nile- Innovation®) (Davidson et al. 2005; Davis et al. 2001) and the second one against salmonids infectious hematopoietic necrosis virus (IHNV) (Apex-IHN®) (Anderson et al. 1996; Garver et al. 2005).

In 2008, a gene therapy based on the same technology has been licensed for pigs' treatment in Australia. Administration of a single dose of LifeTide® (plasmid containing the GHRH gene–growth hormone releasing hormone), in reproductive age females was able to reduce perinatal morbidity and mortality, thereby increasing productivity (Khan et al. 2010a,b).

The latter permit a DNA vaccine occurred in 2010. Once pt™ was developed to be used as immunotherapy for melanoma in dogs and its effectiveness is related to antibodies production that prevent the development and aggravation of the disease (Bergman et al. 2003; Liao et al. 2006).

While the number of studies with DNA-based vaccines are high, and currently there are already licensed treatment for veterinary use and any type of vaccine or treatment based on this technology are available for use in humans.

According to clinical trials (www.clinicaltrials.gov), currently there are 33 studies involving DNA vaccines in clinical trial phase worldwide. The vast majority of studies, more than 57%, are related to cancers. The testing HIV vaccines are also significant, accounting for over 27% of all ongoing studies. The other 21% are related to other diseases such as Ebola virus, HPV, hepatitis, etc.

As the licensing process for the commercialization of DNA vaccines in humans is long and meticulous, taking into account not only effectiveness against disease but also their safety and immune efficiency, the fact that there are already licensed DNA vaccines for veterinary use and various ongoing studies with tests at different stages of clinical phase, makes us believe that vaccinology based on recombinant DNA technology is a tool that will soon benefit the population against most diseases whose treatment and cure is difficult or non-existent.

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