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Review Article

Future Clinical Applications of the Potential use of Lactic Acid Bacteria as Vehicles to Deliver DNA Vaccines

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Abstract

The use of Lactic Acid Bacteria (LAB) as DNA delivery vehicles represents an interesting strategy as they are regarded as safe. Within the group of LAB, the *Lactococcus lactis* is deemed as a model microorganism, which is being extensively used for antigen and cytokines production and delivery to the mucosal level. Recently studies about these bacteria have focused on their usage as vehicles for the delivery of genic vaccines. Wild type or recombinant invasive *L. lactis* are able to trigger DNA expression by epithelial cells, both *in vitro* and *in vivo*, important for effectiveness of the vaccine. For this, invasive strains of *L. lactis* have been developed in order to increase the delivery efficiency of these vaccines to host cells. DNA vaccines are plasmid structures with genes that encode antigenic/therapeutic proteins or peptides capable of triggering an immune response against a wide range of diseases. This review summarizes the potential use of Lactic Acid Bacteria as vehicles to deliver DNA vaccines.

Keywords: DNA vaccine; Delivery Vectors; Lactic Acid Bacteria; *Lactococcus Lactis*

Introduction

The use of DNA as a strategy for vaccination has progressed very quickly since the first publication, in 1992 [1]. DNA vaccines are the third generation vaccine that contains the best-required elements of standard vaccines to be used in humans. This vaccination strategy has the ability to induce potent cellular immune responses, in addition to antibodies and the elasticity to express multiple antigens or epitopes using a single DNA vector [2]. Genetic immunization involves the transfer of a gene encoding an antigenic protein cloned in expression vectors to a eukaryotic cell from the host, leading to the induction of an immune response against the expressed antigen [3]. Therefore theses transfected mammalian cells are able to express *in situ* the antigen (for vaccines) or the therapeutic protein (for gene therapy applications) [4]. Furthermore, they do not have the inconvenient of classical vaccines: they are safe, inexpensive, easy to produce, heat stable and amenable to genetic manipulation [3]. The DNA vaccine is composed of a plasmid backbone that contains a bacterial origin of replication needed for the vector's maintenance and propagation inside the bacteria, as well as a resistance marker, necessary to permit a selective growth of the bacteria that carries the plasmid; immunostimulatory sequences (ISS), for example, the "CpG motifs" (cytosine-phosphate-guanineunmethylated). They are responsible for increasing the magnitude of the immune response as they can enhance T lymphocyte recruitment or expansion [5–8]. Moreover, these ISS sequences can interact with Toll-like receptors (TLR), such as TLR9, and add adjuvant activity [9]. Another component of DNA vaccines is the transcriptional unit, necessary for eukaryotic expression, which harbors a promoter/enhancer region, introns with functional splicing donor and acceptor sites, as well as the ORF (open reading frame) 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 [3,10]. The polyA sequence usually is derived from the bovine growth hormone, SV40, or rabbit β-globin gene [8]. The ORF encoding the protein of interest contains a Kozak translation initiation sequence (ACCATGG) harboring an initiation codon (ATG) for appropriate translation [11–13]. The insert also contains a termination codon (TAA, TGA, or TAG) that signals a termination of translation. Major structures of DNA vaccines are illustrated in (Figure 1).

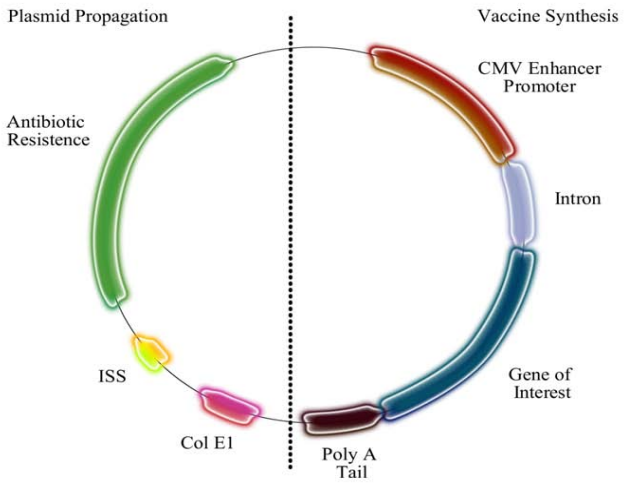

Figure 1: Genetic element of a plasmid DNA vaccine. The vaccine synthesis (eukaryotic expression region) harbors the enhancer/promoter, introns, transgene of interest and a transcriptional terminators (poly **A**), which together lead the protein synthesis. The Plasmid propagation in the microbial host comprises a prokaryotic origin of replication (ColE**1**) and a selectable marker and the immunostimulatory sequences (**ISS**).

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The promoter is the element used to drive the expression of the transgene/antigen in eukaryotic cells. The most broadly used promoter in traditional DNA formulations is the human cytomegalovirus (hCMV), which induces strong and constitutive expression of a protein in a variety of cell types [14,15]. An alternative to these viral promoters is the use of both human polyubiquitin C (UbC) and the elongation factor 1 α (EF1 α) promoters, which has been shown to persistent gene expression in mouse lung cells, leading to a four-fold increased protein expression level of an antigenic protein when compared to viral promoters [16]. Another element found in DNA vaccines localized after the promoter sequence and before the ORF from the gene of interests are the introns. These genetic elements were reported to increase promoter activity [6] and to avoid antigen expression by the prokaryotic machinery from bacteria, turning possible the Heterologous expression of the protein only by the eukaryotic system [7].

Plasmid backbones commercially available approved for gene therapy and vaccination include pVax1, pVAC, pDNAVACultra and pcDNA and others. They contain an *Escherichia coli* origin of replication such as pUC or pBR322, which allow plasmids to replicate in bacterial cell generating many copies of the plasmid in a short period of time [17]. The plasmids used in gene therapy and vaccination preferentially have TN903 gene, coding for amino glycoside enzyme that confers resistance to kanamycin, an antibiotic that is not widely used in humans preventing the risk of allergic responses when compared to others antibiotics [18]. DNA vaccines have a broad range of features that offer them many advantages over other vaccination platforms. The principal advantage concerning the DNA vaccine refers to the fact that they are easy to handle and rapid to construct. This is a fascinating attribute when considering an emerging pandemic threat [4]. In addition to this property DNA vaccines are (i) safe as plasmids do not replicate in human cells do not have the potential to integrate into the human cellular DNA (ii) No adverse effects have been reported neither tolerance to the antigen nor autoimmunity [19]; (iii) They have been shown to stimulate immunity through MHC I-mediated antigen presentation triggering both proliferation and activation of T and B cells antigen-specific; (iv) Vaccine manufacturing is simple and low cost as it requires only cloning techniques in order to clone the protein of interest; (v) they are stable at room temperature, easy to store and transport, presents thermal stability and long life time [20,21]. To sum up, DNA vaccines represents an attractive 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 [22].

Immunological Features of DNA Vaccine

Routes of administration

The therapeutic delivery of nucleic acid has recently been recognized as a promising tool for the treatment of several infectious and genetic diseases. In order to exert their protective effects, DNA vaccines needs a suitable delivery technology to produce a desired immune response.

Intramuscular injection is the most broadly used method to administrate DNA vaccine. Nevertheless, it has been shown that this method is inefficient to induce immune response in large animals as well as in humans for some reasons: the plasmid DNA administered by this manner is inefficiently expressed, poorly distributed, and rapidly degraded [23]. The low immunogenicity of DNA vaccines observed in these studies with humans and primates have indicated scientist to focus on other methods of administration where antigen-presenting cells (APCs) would be transfected in a higher efficiency and stimulating stronger immune response [24,25]. Considering the disadvantage of intramuscular injection, other possible routes of administration have been studied, such as intradermal, subcutaneous, intraperitoneal, sublingual, intrarectal, ocular, and application of the DNA vaccines to mucosal surfaces (vaginal, nasal and oral) [26]. The pathway of administration has been shown to influence the nature and the power of immune responses. Mucosal immune responses are most efficiently induced by administration of vaccines to the mucosa, where as systemic immunization strategies rarely induce long lasting or optimal mucosal immunity and are therefore less effective against infection at the mucosal surfaces.

Course of plasmid inside the cell and development of immunogenicity

Once the DNA vaccine is administrated, the DNA plasmid will transfect different types of cells, for instance myocytes, keratinocytes, resident Antigen Presenting Cells (APCs), such as dendritic cells (DCs) and macrophages [4,27]. Inside the cells, the plasmids will reach the nucleus surviving to the attack of endonuclease, using a microtubule net [28]. Inside the nucleus, the plasmid DNA has contact to the transcription machinery, which allows the transcription of the gene of interest [4,26]. The host cell offers the necessary post-translational modifications mimicking a real infection. The Bacterial mediated transfer for plasmid DNA is illustrated in Figure 2.

This feature is one of the biggest advantages of the genetic immunization [4,27]. The produced proteins are then presented to the surface of the cells becoming a target to the immune system. Antigenic proteins can be secreted as well generating both humoral and cellular immune responses. The direct transfection of the DNA plasmid to the APCs cells has a critical role in DNA vaccine immunogenicity. DCs are probably the most important APCs associated with the capture and processing of antigens via receptor-

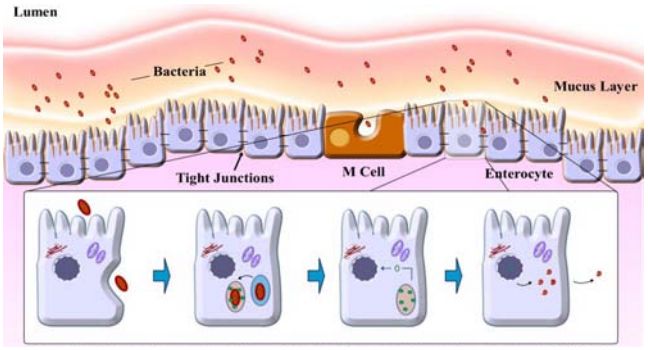

Figure 2: Schematic representation of bacterial mediated transfer of plasmid DNA into mammalian cells. First, the entrance of bacteria in mammalian cells. Second, the phagolysosome engulfs the bacteria to cleaves it. Third, the DNA plasmid escapes from the vesicle and reaches the nucleus of the mammalian cells. Fourth, in the nucleus the transgene is transcribed and the protein synthesis is realized by host cell. Finally, the protein is exposed the immune system.

mediated endocytosis as it presents antigens trough MHC class I or II directly to T *naïve* cells, leading to TCD4+ and TCD8+ lymphocytes activation. The stimulation of this immunological repertoire raises both humoral and cellular protection against the antigen inside the host [29,30].

Clinical advance in DNA vaccine platform

FDA still did not approve the use of DNA vaccines in humans. Phase I clinical studies have been reported for the prevention and/or treatment of HIV, malaria, hepatitis B, SARS and many other infectious agents [31]. In order to strongly trigger the innate immune mechanisms and to go guarantee an optimal expression of the antigen, it is essential to efficiently deliver the DNA into the cells ensuring their transfer to the nuclei [32]. Actually, the inefficient uptake/transfer of the plasmids into mammalian cells is one of the principal hurdles in DNA vaccinology [21]. The lack of reproducibility of many results obtained in mice after the applications in larger animals, as well as the failure of DNA vaccines to induce potent immune responses in humans have not yet been elucidated [33]. For this reason, novel strategies to improve transfection efficiency are emerging. Among them, can be highlighted the delivery methods employed to introduce the DNA vaccine in the organism [33]. These methods include physical and chemical approaches or the biological strategies, such as the use of viral and bacterial vectors. The physical methods of delivering include: gene gun, tattooing, ultrasound (US), electroporation (EP), laser and dermal patches. The chemical vectors most studied are lipid and polymer complexes. The principal advantages of these vectors are that they can tightly compact and protect the DNA and be recognized by specific cell-surface receptors expressed in DCs or macrophages. Moreover, they can disrupt the endosomal membrane to deliver DNA plasmids allowing their transfer to the nucleus when the pH of the medium is reduced to below to six [33]. Even though physical and chemical vectors have shown to be interesting tools to delivery DNA, other methods are being explored as well, such as viruses and bacteria. Regarding the use of viruses as delivery vehicles, a severe adverse effect has occurred during in a gene therapy trial raising serious safety concerns. Attenuated viruses have been studied for gene therapy/antigen delivery, however it was shown that their genome could integrate the host cellular chromatin (oncoretrovirus and lentivirus), which may cause genetic diseases or favor the development of cancerous cells [34].

Regarding the use of biological vehicles, bacterial vectors have been shown to be an excellent choice, as they can transfer the genetic material into mammalian cells such as viruses and does not present the same problems associated with their use. Both attenuated bacterial pathogens or live recombinant bacteria are considered good models for DNA delivery due to their ability to protect the DNA vaccine from enzymatic digestion, to stimulate the immune system as they can target inductive sites of the body generating effective adaptive immunity [35].

Bacterial as delivery vectors for DNA vaccines

The innate tropism of some pathogenic bacterial strains have for specific tissues of the host directed the attention of researchers to study them as a vehicle to deliver DNA vaccine, as this characteristic is indispensable for the elicitation of immune response. Several advantages can be highlighted: they can keep the plasmid in a high copy number, they are easy to manufacture, they are less laborious and has low cost as there is no need to amplify and purify the plasmid before [7,36], large-size plasmid are able to be housed inside the bacteria, permitting the insertion of multiple genes of interest, and the bacterial cell can protect the DNA against endonuclease degradation [37,38].

Additionally to these features of bacterial vectors is the possibility to use them for oral administration, important feature to stimulate both mucosal and systemic immune responses [39]. Considering the increased numbers of vaccines administered all over the word, the fact that they can be inoculated without the use of a needle turns them a cheap and safe method [40].

DNA delivery from bacterial to mucosal surfaces: immunological features

Bacteria carrying a DNA vaccine are able to cross the intestinal barrier, mainly via M cell (specialized epithelial cells named Micro fold cells) overlying Peyer's patches (PPs). The PPs are isolated lymphoid follicles in draining gut mesenteric lymph nodes, considered more accessible to antigens and bacteria present in the luminal compartment. Another manner by which bacteria may have access to the body is through immature dendritic cells (DC) that reside in PPs. They are capable to open tight junctions between epithelial cells, extend their dendrites outside the epithelium and directly sample bacteria, thereby monitoring the contents of the intestinal lumen [41]. Moreover, bacterial vectors are able to enter inside the host body by invading intestinal epithelial cells (IECs) lining mucosal surfaces through the expression of some proteins named invasins. This characteristic refers to the capacity of attenuated pathogenic vectors to deliver DNA vaccines as they naturally produce invasins.

Regarding the vectors based on attenuated pathogenic species, once inside the cells they 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 [36,37]. The plasmids can then reach the nucleus through the microtubules net; once 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 [36,42]. The antigenic proteins may be secreted outside the cell or be presented on the surface of epithelial cell or DCs. The Major Histocompatibility Complex (MHC) class-II, from APCs, presents the exogenous proteins, turning naïve T cells activated into T helper cells (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 cytotoxic T-cells (CD8+ T-cells), stimulating them [43].

Other important components of immunity are the pattern recognition receptors (Toll-like and Nod-like receptors) expressed by IECs, B-lymphocytes and DCs that are located in the sub epithelial lamina propria. These receptors are able to recognize some bacterial components known as microbe-associated molecular patterns (MAMPs), triggering intracellular signaling pathways that lead to cytokine secretion and immune cell activation [44,45]. The production of a merged immune response encompassing the induction of humoral and cell mediated immunity (CMI) effectors like CD8+ and CD4+ T cells after DNA vaccination using bacterial

vectors is a well established event known as cross-priming [46,47]. The bacterial recognition by the immune system modulates the innate immune response, therefore, supporting a vigorous and lasting adaptive response [37].

Another important characteristic is the communication between activated immune cells localized in the mucosal surface with the systemic blood where they can travel around the body via the lymph [48]. The production and secretion of antigen-specific secretory immunoglobulin A (SIgA) responses by plasmocytes is another important advantage to be considered when using bacteria as mucosal delivery vehicles for DNA vaccines.

Commensal bacteria can interact with IECs and deliver tolerogenic signals that are transmitted to the underlying cells of the immune system [49]; consequently, they are not ignored by the intestinal immune system. The gastrointestinal mucosa offers immunological tolerance against nonpathogenic bacteria and antigens; this phenomenon avoids reactions against proteins and commensal bacteria. Thus, mucosal tolerance protects the mucosa from detrimental inflammatory immune responses [50]. Actually, allergic disease development and cancer, especially colon cancer, has been associated with alterations in the intestinal micro biota [51].

Bacteria as a delivery vector for gene transfer

Salmonella typhi, Listeria monocytogenes, Shigella Flexner, Yesinia enterocolitica and *Escherichia coli* are the principal enteropathogenic species most widely used as bacterial delivery systems into mammalian cells [36] due to their natural tropism for DCs and macrophages in the lymphoid tissue of the intestinal mucosal surface [7]. There are several reports showing the use of these strains as bacterial vectors. For example: *Salmonella typhimurium* is probably the most broadly used bacteria for antigen delivery applications, such as encoding duck enteritis virus UL24 [52], encoding HIV gp140 [53], among others; *Yesinia enterocolitica* encoding *Brucella abortus* antigens [54]; *Listeria monocytogenes* as a gene delivery vector for targeting cancer cells [55] as it could effectively target tumors expressing surface bound antigens (Her2/neu), intracellular antigens (HPV-16 E7) or secreted antigens (PSA) [56].

The use of human enteric bacterial strains, as a bacterial carrier, is being considered an advantage because of their capacity to infect human colonic mucosa after oral administration. However, for this proposal enteropathogenic species needs to be attenuated or inactivated. Nonetheless, attenuated strains has a restrict use as they present the risk to revert to the virulent phenotype compromising its safety, as reported for the oral polio vaccine (e.g., Sabin 3) [57]. Therefore, World and Health Organization (WHO) does not recommend their use in children and immunocompromised individuals. Thus, to counteract this severe problem, it has been investigated the use of non-pathogenic bacteria, such as LAB as vectors for genetic immunization [58].

Lactic acid bacteria

Lactic acid bacteria (LAB) are distributed in diverse ecological niches; for example in plants, fermented foods, as well as in the gastrointestinal tract of many animals, including humans, where some species can live as commensal microorganisms [59]. LAB comprises mainly species of *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus and Streptococcus* [60,61]. They are widely used in the industry for the production and the preservation of foods due to their ability to acidify the medium (pH 3.5 to 4.5). Therefore, LAB are ingested diary by humans in different types of foods such as cheese, wine, yogurt, fermented milks, pickles, kefir, butter, among others. It have been reported the importance of enteric lactic bacteria during their life into the human gastrointestinal tract, as they have an important impact on host metabolism, participating in microbial-mammalian co-metabolism [62]. Therefore, FDA has granted some strains of LAB group as "Generally Recognized as Safe" (GRAS) for human consumption [63]. Another important characteristics of LAB are their capacity to restore the normal intestinal flora, eliminate intestinal pathogens, reinforce the intestinal barrier capacity to foreign antigens, stimulate nonspecific immunity such as phagocytosis, stimulation of humoral immunity and production of anti-inflammatory products [64,65]. Due to all these positive effects, some strains are considered as probiotics because when consumed at adequate levels they can positively influence the human health. Thus, scientific is extensively exploring these probiotics as an alternative treatment for some diseases, and to serve as a tool for genetic immunization [66].

L. lactis is the best-characterized member within the LAB group being considered the model organism. They are facultative anaerobic, catalase negative, and do not form endospores. *Lactococcus lactis ssp. lactis* was originally found as a milk-souring isolate, but it is also associated with plants. *Lactococcus lactis ssp. cremoris* is used as a starter culture for the manufacture of Cheddar cheese, in which it contributes with highly prized flavor [60]. *L. lactis* does not colonize the digestive tract of men and animals. Besides to this economic significance of *L. lactis*, other important features have been described: (i) it has a completely sequenced genome [67]; (ii) they are "GRAS", (iii) it is genetically easy to manipulate; and (iv) many genetic tools have already been developed for this species [68], and (v) does not contain Lip polysaccharide (LPS) avoiding endotoxin shock after being administered to humans [69].

Lactococcus lactis: heterologous protein delivery

Besides its traditional and safe use in the food industry, *L. lactis* is presenting to be a very an interesting tool to be used as a "cell factory" for the high-level protein production. This new role assigned for *L. lactis* is due to the fact that this bacterium does not produce endotoxins or other toxic metabolic products [70]; few proteins are secreted in *L. lactis*; Usp45 (Unknown Secreted Protein of 45 kDa) is the only one on e secreted in quantities large enough to be detected in Coomassie-stained protein gels [71]. This feature is very important, as it simplifies purification step after bacterial growth in fomenters [72].

Transformation protocols, cloning or screening-vectors, mutagenesis systems, protein expression and targeting-systems are examples of the availability of genetic tools that have been developed for *L. lactis*. These tools have been used to engineer *L. lactis* to produce intra- or extra-cellular recombinant proteins of viral, bacterial or eukaryotic origins [72]. These tools also allow the expression of these proteins in a controlled manner. To this end, various vectors containing constitutive or inducible promoters PlacA [73]; PnisA [74]; PT7 [75]; P170 [76]; P59 [77]; PxylT [78]; Pzn [79] have been developed and represent the basis of all expression systems in *L. lactis* and other LAB [80].

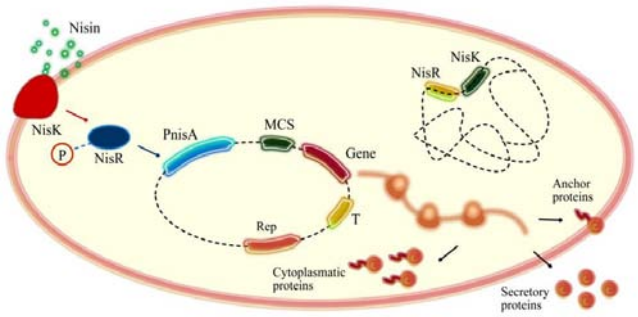

Figure 3: Schematic representation of the NICE system development in *L. lactis*. The expression of genes in response to nisin stimulus involves a membrane-located sensor protein (NisK) and a cytoplasmic response regulator (NisR) that controls transcriptional activation from the misA promoter (PnisA). The auto-induction mechanism of nisin is used as a controlled heterologous gene expression system in *L. Lactic.*

Nisin Controlled Gene Expression (NICE) is the most used amongst all expression systems developed for use in LAB. It offers significant potential for regulated gene expression. NisR and NisK genes encode a two-component system (NisRK), which controls the expression of the nisA gene via signal transduction. NisK functions as a membrane sensor that detects extracellular nisin. The signal is transferred to NisR through a phosphorylation process turning this gene capable of activating gene transcription, which is controlled by the PnisA promoter [74].

Therefore, in the presence of Nisin the promoter can induce the transcription of the molecule of interest [81] (Figure 3). NisK and NisR genes were isolated from the nisin gene cluster and inserted into the chromosome of *L. lactis* subsp. *cremoris* MG1363 (nisin-negative), creating NZ9000 strain (nisin positive) [82,83]. Depending on the presence or absence of the corresponding targeting signals, the protein is expressed into the cytoplasm, anchored to the cell wall or secreted to the extracellular medium. Many exogenous proteins have been expressed in *L. lactis* through this system [74,84,85]. NICE system has been tested in other LAB, such as *Leuconostoc lactis, Lactobacillus Helveticas, Streptococcus sp., Bacillus sp., Enterococcus sp.* [86] *and Lactobacillus Plant arum* [87], proving its versatility.

Another expression and targeting system that allows different cellular locations of the gene of interest for use in *L. lactis* is the xylose-inducible expression system (XIES), which is based on the use of a xylose inducible lactococcal promoter, PxylT from *L. lactis* NCDO2118 strain. In the presence of sugars (glucose, fructose and/or mannose) PxylT is tightly repressed. However, in the presence of xylose, PxylT is transcription ally activated [88,89]. *Staphylococcus aurous* nuclease genes (nuc) fused or not to the lactococci Usp45 signal peptide was adopted to test the capacity of this system to express the cytoplasm or secreted form of nuc protein. Xylose-inducible nuc expression was found to be tightly controlled resulting in high-level, long-term protein production, and correct targeting either to the cytoplasm or to the extracellular medium. This expression system is versatile and can be easily switched on or off by adding either xylose or glucose, respectively [78]. XIES system has been employed in the biotechnology field for production of different heterologous protein, for example: 65-kDa heat shock proteins (HSPs) of *Mycobacterium leprae* [90]; S-layer protein (SlpA) of *Lactobacillus brevis* [91], among others.

Lactococcus lactis as live mucosal delivery vectors for vaccine

Regarding *L. lactis* as a vehicle to deliver DNA vaccines, many interesting features can be highlighted: (1) 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 [73,92]; (2) it was successfully demonstrated that *L. lactis* can deliver DNA into eukaryotic cells and *in vivo* to mice IECs [93–95]; (3) they can induce both systemic and mucosal immunity when administrated at mucosa surfaces [96,97]; (4) they can resist to the acid environment of the stomach, being able to survive into the gastrointestinal tract, ensuring recombinant protein or plasmid delivery [20]. Because *L. lactis* is not very immunogenic, it can be orally administrated multiple times [98], regarding its extraordinary safety profile [99]. Furthermore, *L. lactis* has the ability to stimulate the phagocytic system of the host [100]. All this characteristics turns it a good option for being used in immunization programs [101]. In accordance with the benefits of *L. lactis*, several researchers have developed different recombinant strains to be used for genetic immunization. Table 1 summarizes some studies that were performed in different laboratories around the world corroborating the ability of *L. lactis* in inducing long-lasting immune responses.

L. lactis expressing invasins for DNA delivering

Studies demonstrated both *in vitro* [98] and *in vivo* [93] that wild-type (wt) *L. lactis* could be used as a vector for genetic immunization. However, the percentage of gene transferred observed was low, as well as a low and transitory Th1-type immune response after immunization trials [93]. For this reason, with the aim to increase the capacity of *lactococci* to deliver DNA, some strains of *L. lactis* expressing invasins have been developed. The first engineered *L. lactis* to express invasins was reported in 2005 by Guimarães and co-workers; InlA gene from *L. monocytogenes* was cloned and expressed under transcriptional control of the native promoter. This work showed that recombinant *lactococci* could efficiently express the cell wall anchored form of InlA, and the invasion rates of LL-InlA+ strain in Caco-2 cells was approximately 100-fold higher than the wt *lactococci.* Moreover, after oral inoculation in guinea pigs, this recombinant strain was capable to invade intestinal cells [94]. However, the use of LL-InlA+ strain showed to be inconvenient because InlA cannot bind to its receptor in mice, murine E-cadherin, thus limiting the *in vivo* studies as the effect of LL-InlA+ strain was only possible to be explored in guinea pigs or transgenic mice, which may be laborious and/or expensive [102]. To improve this strategy another invasive strain of *L. lactis* was constructed by cloning the gene encoding fibronectin-binding protein A (FnBPA), from *Staphylococcus aureus* (LL-FnBPA+) [103]. FnBPA production at the surface of *L. lactis* increases invasiveness of the cells 1000 fold and increased plasmid transfer 30-fold *in vitro* (Innocent in et al., 2009). *L. lactis InlA+* and *L. lactis FnBPA+*, showed comparable internalization rates in Caco-2 cells [93]. Therefore, another recombinant invasive *lactococci* was developed producing a mutated form of Internal in A (mInlA), appropriate to be used in a murine model [90].

In order to evaluate recombinant *L. lactis* expressing invasins a

Table 1: Antigens expressed by *L. lactis,* inducing long-lasting immune responses.

Antigens Expressed by *L. lactis*	Immune Response Observed	Application	Reference
EDIII antigen from dengue virus type 2	Neutralization of the virus *in vitro*	Dengue virus control strategy	Sim et al., 2008 (107)
Envelope protein of the human immunodeficiency virus 1 (HIV-1)	High levels of IgG and IgA antibodies against the antigen observed	HIV vaccine	Xin et al., 2003 (108)
Antigenic protein of *Proteus mirabilis* (HBPM)	Protection to the animals against challenge with *P.mirabilis* virulent strain	Control of urinary tract infections	Scavone et al., 2007 (109)
PspA antigen derived from *Streptococcus pneumoniae*	Better protected against challenge with the virulent strain	Vaccine against pneumonia	Hanniffy et al., 2007 (110)
Antigen lcrV from *Yersinia pseudotuberculosis*	Humoral and cellular immune responses, conferring protection against challenge	Vaccine against Far East scarlet-like fever	Daniel et al., 2009 (111)
EspB antigen from the type III secretion system (T3SS) of *E. coli* serotype O157:H7	Significant levels of specific serum Ig and faecal IgA	New strategie to fight against enterohemorrhagic *E. coli*	Ahmed et al., 2012 (112)
Leishmania antigen LACK and the proinflammatory cytokine IL-12	Protection against challenge	Vaccine to combat Leishmaniosis	Hugentobler et al., 2012 (113)
Mature murine IFNgamma	--	Adjuvant tool	Bermúdez-Humarán et al., 2008 (114)
Antigen envolved with LPS transport (wzm) from *Vibrio cholerae* O1 strain	Increase of systemic and mucosal immunity	Vaccine against cholera	Zamri et al., 2012 (115)
Catalase-producing *L. lactis*	Inhibition of chemically induced colon cancer	Cancer therapy	De Moreno de LeBlanc et al., 2008 (116)
IL-10	Anti-inflammatory effects controlling intestinal inflammation	Treatment of allergy and inflammatory bowel diseases (IBD)	Cortes-Perez et al., 2007 (117); Braat et al. 2006(118) ; Marinho et al., 2010 (119)

new vector have been developed resulted from the co-integration of two replicons: one from *E. coli* and the other from *L. lactis,* named pValac (Vaccination using Lactic acid bacteria). 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 *E. coli* and *L. 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 Pk15 cells. The vector demonstrated to be functional as PK15 cells were able to express GFP protein. Moreover, invasiveness assays of *L. lactis* inlA+ carrying pValac: gfp into Caco-2 cells showed that this strain could deliver the vector to epithelial cells, *in vitro* [104]. This assay demonstrated that *L. lactis* expressing invasins and harboring functional plasmids can serve as tools for genetic immunization [93].

Besides the effort to construct invasive *L. lactis* strains and plasmids to be used for genetic immunization, other works have attempted to test *L. lactis* in the vaccination field.

De Azevedo and co-workers used *L. lactis* expressing a mutated form of *L.* monocytogenes Internal in A (LL-mInlA) carrying pValac: BLG. BLG is the bovine β lacto globulin, a major cow's milk allergen. They were able to show the production of mInlA enhanced invasivity and allowed plasmid transfer in a higher efficiency in *in vitro* experiments. Besides than, were done *in vivo* experiments showing slightly increased plasmid transfer after oral administration [90].

An elegant work has been done with recombinants *L. lactis* FnBPA, *L. lactis* mInlA and wt strains (*L. lactis* non invasive), all of them carrying pValac: BLG. It was showed that the intranasal immunization of *L. lactis* non invasive strain carrying pValac: BLG elicited a TH1 immune response. However, when immune response elicited by *L. lactis* FnBPA and *L. lactis* mInlA were evaluated, both strains carrying pValac: BLG, it was observed the secretion of IL-4 and IL-5 in medium of BLG reactivated splenocytes, after both oral and intranasal administration. It was concluded that non invasive *lactococci* elicits a Th1 immune profile while the immunization with the recombinant invasive strains elicited a Th2 immune response [105].

Another work using *L. lactis* as DNA delivery vehicle involved the construction and evaluation of a DNA vaccine against Tuberculoses. In this work BALB/C mice were orally administered with *L. lactis* expressing FnBPA carrying pValac coding for the 6-kDa early secreted antigenic target (ESAT-6) gene of *Mycobacterium tuberculosis*. This report showed significant increase of interferon gamma (IFN-γ) production in spleen cells, as well as a significant increase of specific secretory immunoglobulin A (SIgA) production in colon tissue and fecal extracts [20].

The administration of *L. lactis* FnBPA, has been used for the administration of other therapeutic molecule, such as IL10, for the treatment of Inflammatory Bowel Disease (IBD), an inflammatory condition of the TGI being presented in two forms: Ulcerative colitis and Crohn's disease. To this end, the therapeutic effect of *L. lactis* expressing FnBPA carrying pValac coding for Interleukin-10 from *Mus musculus* was evaluated using a model of acute trinitrobenzenesulfonic acid (TNBS)-induced colitis in mouse. It was observed a decrease in the severity of the inflammation with lower macroscopic and microscopic inflammatory scores in the large intestine, decrease of microbial translocation to the liver and less body weight loss. Furthermore, they were able to shown a decrease in anti-inflammatory cytokine, IL-17. This work suggested that recombinant *L .lactis* expressing FnBPA invasins (pValac:il-10) is a suitable option to maintain an anti-inflammatory status in the GIT, especially for chronic Crohn's disease patients [106].

Conclusion

Food-grade bacteria, such as LAB, have recently been proposed as a vehicle to express recombinant antigens and therapeutic molecules, as well as to deliver DNA vaccines. One of the LAB models, *L. lactis*, has been shown to act as DNA delivery vehicles and to deliver many

different proteins with biomedical and biotechnological interest at the mucosal level. In spite of the necessity of other studies, the approach of using wild type or recombinant *L. lactis* as a tool to deliver therapeutic plasmids can be considered a promising future. An important point regarding the use of *L. lactis* as a DNA delivery vector is the strategy to use *L. lactis* expressing invasins. As recombinant invasive strains improved the delivery of DNA, especially *in vitro*, scientists explored new horizons testing them for many applications, for instance in the vaccination area. To date, there are no DNA vaccines available to be administered in humans. The only licensed DNA vaccine is for veterinary purposes. Being *L. lactis* safe for use in humans and capable to efficiently deliver DNA vaccines, it Fs use as immunization vehicles is an excellent choice for clinical applications in near future. Especially because this bacterium is easy to handle, GRAS and has a large number of genetic tools already developed.

Recombinant strains, as well as new vectors have attracted researcher's attention and a great number of studies are in progress with the aim to develop systems using LAB to deliver DNA vaccine.

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