Use of bacteria in DNA vaccine delivery

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DNA vaccines consist in the administration of vectors that contain a eukaryotic expression cassette, responsible for coding the antigen of interest in host cells. To turn possible its transcription, a number of barriers must be surpassed. Thus, the use of bacteria, like *Lactococcus lactis,* as a vehicle to the deliver vaccine plasmids by the oral route is a promising strategy. Bacteria can invade epithelial cells or be phagocytized, allowing plasmid escape from the cytoplasm to the nucleus, resulting in the expression of the ORF of interest for its subsequent presentation to the immune system. Native *L. lactis* were used for delivering DNA both *in vitro* and *in vivo*. Aiming to make the vector delivery more efficient, recombinant *L. lactis* strains expressing invasins were tested and they showed to be able to deliver a eukaryotic expression cassette more efficiently than the wild type strain *in vitro*. In this chapter, we present a general view about DNA vaccines and the use of bacteria, especially *L. lactis* to deliver them to eukaryotic cells, which may represent a new strategy for the control of many infectious diseases.

Keywords DNA vaccine; bacterial delivery; oral mucosa; *Lactococcus lactis*

1. Concepts in DNA Vaccines

Since the inception of DNA vaccines in the early 1950s [1], several years elapsed before it was demonstrated that administration of a recombinant DNA plasmid into an animal resulted in the expression of the desired protein [2-4]. Following these findings, in 1992, Tang and colleagues showed that injection of plasmids containing the genomic copy of the human growth hormone (hGH) gene under the transcriptional control of a specific promoter was capable of eliciting a specific humoral immune reaction against hGH, suggesting that DNA could be used to induce specific immune responses [5]. Soon after, in 1993, two research groups demonstrated that immunization with a DNA plasmid could protect mice against a lethal influenza challenge [6,7]. Since then, the use of DNA as a form of vaccination has progressed very rapidly. In the past decade, several DNA vaccines designed to strike against various pathogens and tumor antigens, besides therapeutic proteins, have been tested as they are a simple way to induce immune responses, affecting not only humoral but also cellular immunity [8,9].

Therefore, up to date, four DNA vaccines have been approved in the area of veterinary medicine, being two of them prophylactic vaccines against infectious diseases, one an immunotherapy for cancer and one a gene therapy [10]. In 2005, the West Nile Virus DNA vaccine to horses and the DNA vaccine against the Infectious Hematopoietic Necrosis Virus to Salmon were licensed in the United States and Canada, respectively. Then, in 2008, a therapeutic DNA vaccine capable to express the natural form of the growth hormone, which was released in pigs, was licensed in Australia. Also, in 2010, a DNA vaccine for cancer treatment was approved and licensed for dogs affected by oral melanoma in the United States.

Despite the first successes and licensing of DNA vaccines for veterinary use, most of the studies in clinical trials have demonstrated that DNA vaccines are able to generate immune responses [9]. This vaccine platform has entered into a variety of human clinical trials for prophylactic vaccines against viral, bacterial or parasitic infections and also as a potential therapy to treat infectious diseases, many types of cancers, Alzheimer disease, allergy and autoimmune disorders. It is clear that DNA vaccination technology has established itself in the field of experimental immunotherapy, and prudent design and experimentation may represent an important component of the next generation of prophylactic and therapeutic vaccines that are efficient and economically accessible to peoples worldwide.

DNA vaccination is a vaccine strategy that conceptually combines some of the most desirable attributes of standard vaccine approaches. This vaccination strategy offers an alternative for presentation of antigenic/therapeutic molecules to the immune system in order to trigger long lasting humoral and cellular responses [11]. These vaccines are based on constructed bacterial-derived plasmid that contains a eukaryotic gene expression cassette, responsible for coding the immunogenic or immunomodulatory molecule of interest in mammalian cells, thus, allowing their *in vivo* generation. DNA vaccine plasmids can be divided into two main structures: (i) the plasmid backbone for prokaryotic propagation, which contains a bacterial origin of replication that allows for maintenance and propagation of the plasmid in host cells; and a bacterial selectable marker like an antibiotic resistance gene that ensures stable plasmid inheritance during bacterial growth. The second structure is (ii) a transcriptional unit for eukaryotic expression, which includes the promoter, used to drive optimal and high expression of the ORF (open reading frame) of interest in mammalian cells; and the polyadenylation sequence (poly A), necessary for correct transcription termination of the ORF and export of the

stable mRNA from the nucleus to the cytoplasm. Besides this, the insertion in the ORF of interest of a specific consensus sequence present in the eukaryotic mRNA, signaling the *start* codon, called Kozak sequence (ACCATGG), is necessary to initiate protein synthesis in eukaryotic cells; and to ensure correct translation termination and prevent read through, it is also important to add soluble stop-codons (Figure 1).

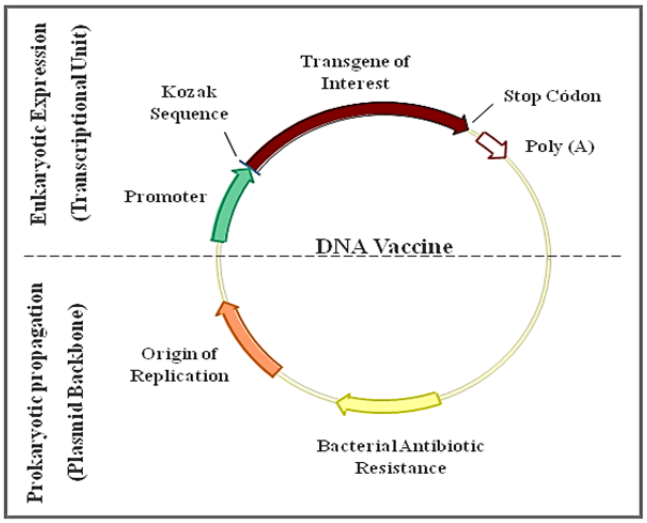

Fig. 1 Structure of DNA vaccine plasmids. The top of the figure shows the transcriptional unit (eukaryotic expression region), responsible for high-level expression of the antigen of interest in eukaryotic cells, containing the promoter, the transgene of interest with Kozak sequence, stop codon and the polyadenylation sequence (poly A). The lower part of the figure (prokaryotic region) is responsible for propagation and vaccine plasmid maintenance in bacterial cells, containing a prokaryotic replication origin and a selectable marker.

Various strategies have been developed to enhance the potential of DNA vaccines, such as i) codon usage adaptation, to maximize the expression of the ORF of interest based on generally available transfer RNA (tRNA) levels in host cells; ii) use of immunostimulatory sequences, namely unmethylated phosphodiester linked cytosine and guanine (CpG) motifs, that act through the Toll-like receptor 9 (TLR-9) to induce a series of immune stimulatory cytokines, enhancing both non-specific and antigen-specific responses; iii) use of leader sequences - enhancer elements and trans-activators of transcription can increase the activity of the promoter since they are responsible for regulation of genetic expression; iv) co-injection of immunomodulatory plasmids, like plasmids that code for cytokines - such as interleukines, interferons, stimulatory colony factors and tumoral necrosis factors, chemokines and costimulatory molecules - with the objective of increasing the quantity and potential of antigen presenting cells (APCs); and v) DNA nuclear targeting sequences (DTS), that consist on recognition sequences for endogenous DNA-binding proteins for the transport into the nucleus, which, in turn, may lead to an increase on the expression of the sequence of interest, optimizing the DNA vaccine [12-15].

DNA vaccines have a wide range of attributes that confer them many advantages over other vaccine technologies in terms of safety, ease of fabrication, stability and mobility (Table 1). Due to their highly flexible genetic design and simple structure, DNA plasmids are easily manipulated and modified in a short period of time, a critical attribute for production of vaccines against emerging pandemic threats. Furthermore, there is the possibility of encoding multiple proteins in a single construct and of adding an adjuvant to enhance their potency. Cheap and large-scale production of DNA vaccines within a short frame of time is possible too, since these are easily replicated and amplified in bacteria, besides being highly stable and easily stored. Beyond that, this vaccine platform technology represents an attractive tool due to its ability to induce all three arms of adaptive immunity: antibodies, helper T cells (T_H) and cytotoxic T-lymphocytes (CTLs), as well as innate immune responses [16].

Table 1 Advantages and Characteristics of DNA vaccines

Advantages	Characteristics
Immunogenicity	- Induction of T and B cell-specific antigen as well as live vaccines
Safety	- Inability to revert to virulent forms as live vaccines - Unlike some inactivated vaccines, the efficacy does not depends of toxic treatments - No significant adverse effects in clinical trials so far
Construction	- Modifications in transgene construct and vaccine can be made in short time - Optimization of plasmids and transcript possible
Stability	- Increased thermal stability compared to other vaccines - Longer life time
Mobility	- Easy storage and transport compared to other vaccines - There is no need for constant refrigeration
Time manufacturing	- Rapid production and formulation - Reproducible in large-scale production

Concerns exist relating to the possibility of their integration into the host's genome, development of auto-immunity, as well as resistance to the antibiotic used for selection of plasmids in bacterial cells. However, currently tested DNA vaccines have not presented relevant integration into the genome of host cells and no convincing evidence for auto-immunity development in response to a DNA vaccine exists in pre-clinical and clinical studies. Moreover, DNA vaccines show little risk of either reversion to a disease-causing form or secondary infection, as these are i) non-live, ii) non-replicating, iii) non-spreading, and iv) show fewer incidences of systemic adverse effects [17].

1.1. Routes of administration

Initially, genetic immunization consisted in the direct administration of a DNA plasmid (so-called "*naked DNA*") into tissues capable to internalize and express an immunogenic antigen, for the development of DNA vaccines, or a gene to treat or prevent a disease, for gene therapy proposals [9]. Moreover, to achieve significant levels of immunity in humans and large animals, DNA delivery methods often require very high doses of plasmids and multiple boosts [18-20]. One reason for low effectiveness of DNA vaccines in humans could be insufficient membrane permeability and cellular uptake of plasmid DNA (pDNA), resulting in poor gene expression. Therefore, more potent transfection methods for DNA vaccines and/or more effective means of delivery and potent adjuvants must be developed for the technology to realize its potential [21].

The most widely employed method to administer DNA vaccines was the intramuscular injection. Currently, many works has been published using this route of immunization with success, whether for prophylaxis or immunotherapy. As an example, a DNA vaccines for human papillomavirus type 16 (HPV-16), able to induce antigen-specific CD8(+) T-cell responses, and to confer preventive resistance to transplantable murine tumor cells and therapeutic anticancer effects in mice previously challenged with transplantable murine tumor cells [22,23]. Moreover, in another work, in human HIV vaccine trial, HIV-1 gag gene DNA was administered intramuscular route either alone or in combination with IL-12 or IL-15 plasmid cytokine adjuvants, but there were minimal responses to HIV gag DNA alone, and no apparent augmentation with either IL-12 or IL-15 plasmid cytokine adjuvants [24]. So, newer formulations or methods of delivery are being required to increase their immunogenicity.

The intramuscular route does not appear to be particularly efficient at inducing immune responses in humans, as plasmid DNA administered by this route is poorly distributed, inefficiently expressed and rapidly degraded [25]. Several studies have demonstrated the importance of direct transfection of antigen-presenting cells (APCs) to a high immune response [26,27]; however, following intramuscular immunizations, the pre-dominant cell type transfected with the DNA vaccines are myocytes [28].

Intradermal, subcutaneous, intraperitoneal, sublingual, intrarectal, ocular, intravenous and intranodal injections or application to mucosal surfaces (vaginal, nasal and oral) are other possible routes of administration, however used less frequently at the beginning of DNA vaccine research [29,30]. Even though intradermal application has been the most extensively used method to deliver DNA vaccines, a study conducted by Lechardeur and colleagues (1999) demonstrated that 90 minutes after plasmid injection only 0.1% of the injected material is able to reach the cell nucleus. Endonucleases have been implicated in degrading injected plasmids favoring this rapid elimination [31].

Thereby, to achieve signficant levels of immunity, naked DNA delivery often requires very high doses of plasmids and multiple boosts [20]. Actually, DNA degradation represents a fundamental problem for genetic immunization, as destruction of incoming genes translates into loss of gene expression [32]. Therefore, several strategies have been designed to avoid DNA clearance, which would lead to a higher number of antigen-expressing cells, for instance DCs and epidermal cells, thus increasing specific T and B cell responses against the antigen.

1.2. Different cellular transfection methods

The low immunogenicity of early DNA vaccines is hypothesized to stem, in part, from inefficient uptake of the plasmids by cells due to inefficient delivery [33]. Nonetheless, the reasons for the failure of DNA vaccines to induce potent immune responses in humans have not been completely elucidated [34]. Therefore, research has focused on developing novel strategies to enhance transfection efficiency and improve other facets of the DNA vaccination platform using several strategies [10,33]. It has been shown that plasmid modifications (i.e. altered transcriptional elements, gene of interest codon optimized or use of a dual stop codon) can significantly improve either antigen transcription or translation by the host cell [10,35]. Another important effort to improve gene expression includes the delivery method employed to introduce the DNA vaccine in the organism, which have been shown to protect the DNA against degradation, facilitating its uptake by mammalian cells [34].

Several different methods for gene delivery have been proposed. They are classified in two main categories: no biological and biological methods. The first one includes physical methods, such as gene gun and electroporation [36,37] and chemical approaches, comprising the use of cationic polymers or cationic lipids (liposomes) [38].

Gene gun is a ballistic needlefree injection device that promotes cellular and humoral immunity to the antigens encoded by the DNA delivered in murine and human hosts [39-42]. This method is ideal for gene transfer directly into intracellular spaces of targeted tissues, and has several advantages, as the requirement of small amounts of DNA, the DNA is bombarded directly into cells, high transfection efficiency, high immunogenicity, and high antibody production. The disadvantage of this delivery method is the need of special device and formulation, the Th2 response may not be the response required, and it requires inert particles as carrier [43,44].

Electroporation is an electrically meditated injection technique, which induces the permeabilization of the plasma membrane creating pores and, allowing the introduction of plasmids containing foreign genes into the cells, which will subsequently express the desired genes. The reclosing of the cell membrane occurs naturally; however, the cell membrane can irreparably damaged if high voltages are applied [45,46]. This technique is rapid, simple and an efficient method, widely used for DNA delivery, membrane protein insertion and other macromolecules delivery into various types of cells, with numerous applications in the medical field and conventional biological laboratories [46].

For chemical approaches, the most studied strategy is the use of cationic polymers and cationic lipids. In these methods, the DNA is transferred to the cells through intracellular vesicles, from which a small fraction of the DNA is released into the cytoplasm and migrates into the nucleus, where transgene expression takes place. In a comparative work, animals immunized intranasally with the combination of DNA plasmids of a multiclade/multigene HIV-1 formulated with a cationic lipid adjuvant showed significantly higher IgA levels in fecal pellets, and a enhanced IgG systemic responses occurred both when the DNA was delivered mucosally and into the muscle. Animals immunized with plasmid DNA alone (naked) did not develop measurable vaccine specific systemic IgG responses and very low or no mucosal humoral responses [47]. Thus, it can be noted a need for methods that increase the levels of cell transfection, which consequently increases the level of the immune response generated by this vaccines.

Biological methods include viral and bacterial DNA delivery. Attenuated or non-pathogenic viruses have demonstrated the feasibility of gene therapy/antigen delivery. The classes most widely used for gene therapy applications are Oncoretrovirus and Lentivirus as their genome integrates into the host cellular chromatin. On the other hand, viruses like adenoviruses and herpes viruses, able to persist in the cell nucleus predominantly as extrachromosomal DNA molecules, are more preferably used for DNA vaccination. Although viral vectors have been widely used in clinical trials, their inconvenience is that they may cause genetic diseases or favor the development of cancerous cells. Actually, severe adverse effects have occurred during two independent gene therapy trials raising serious safety concerns about the use of viral vectors [48].

2. Use of bacteria as a vehicle to mucosal delivery of DNA vaccines

The use of bacterial system for DNA delivery has been extensively explored [49]. Several different bacteria can transfer vaccine plasmids to mammalian host cells becoming an active plasmid manufactures. In 1980, Schaffner observed for the first time a gene transfer from bacteria to mammalian cells when tandem copies of SV40 virus genome, carried by *E. coli* laboratory strains, were transferred into co-cultured mammalian cells [50]. Since then, many bacterial transfer systems have been developed and improved to transfer plasmid DNA into eukaryotic host cells.

The use of bacteria as vehicles for the delivery of DNA vaccines has several advantages when compared to other types of vaccinates such as naked DNA vaccination, vaccination with viral carriers or vaccination with purified or carrier-based protein antigens. Live recombinant bacteria or attenuated bacterial pathogens attend some necessary

characteristics proposed in an ideal system such as i) capacity to protect the DNA vaccine from physical elimination and enzymatic digestion, ii) ability to target inductive sites of the body allowing stimulation of the immune system and iii), capacity to appropriately stimulate innate immune system to generate effective adaptive immunity [51].

Bacterial carrier strains are easy to manufacture and allow for the maintenance of plasmids with a high cloning capacity. Stable replication of vaccine plasmids by different bacterial carrier species can be further ensured by introducing bacterial genes essential for survival or virulence within the host into the vaccine plasmids and thereby circumventing the need to co-administrate plasmid selection markers [49]. Furthermore, in contrast to immunization with naked plasmid DNA, no further plasmid amplification and purification steps are needed, thereby reducing cost and labor extensively [52,53].

Another attractive feature is their potential for oral administration, which may stimulate both mucosal and systemic immune responses [54]. Considering that oral and nasal mucosa represent the first line of defense against many pathogenic microorganisms, mucosal immunization may play a critical role in host defense against pathogens that are in direct contact with mucosal surfaces [55], which are protected by a large and highly specialized innate and adaptive immune system. Furthermore, the World Health Organization, (WHO) recommends the use of mucosal vaccines because of economic, logistic and security reasons [51,56,57]. Therefore, the development of effective strategies for the delivery of DNA vaccines to the mucosal tissues has received considerable attention over the past decade as well as the use of recombinant bacteria as carrier systems.

2.1. Immunological aspects of bacterial DNA delivery to the mucosa

After oral inoculation, bacteria carrying plasmids are recognized by immune cells and intestinal epithelial cells (IECs) lining mucosal surfaces. Some attenuated pathogenic vectors can invade IECs via the expression of some proteins termed invasins to deliver DNA vaccines, or they can either be sampled by intestinal lamina propria dendritic cell subsets (DCs) or by some specialized epithelial cells named Microfold cells (M cells) overlying Peyer's patches (PPs) [58]. The PPs are isolated lymphoid follicles in draining gut mesenteric lymph nodes, considered more accessible to antigens and bacteria present in the luminal compartment. The M cells, presents in the Gut-associated lymphoid tissue, take up particulate antigens and specific binding proteins by endocytosis and transport them to the underlying immune cells [57].

Behind bacterial invasion, it has been shown that innate immune receptors expressed by IECs and DCs, such as pattern recognition receptors (Toll-like and Nod-like receptors), can respond to some bacterial components known as microbe-associated molecular patterns (MAMPs) which serve as natural antigens after host cell invasion [59]. The bacterial recognition by the immune system modulates innate immune response, therefore, promoting a robust and lasting adaptive response [60]. After invading mammalian cells, the bacterium is usually involved by a primary vesicle, named phagosome. This vesicle then fuses to a lysosomal compartment where bacterial lysis occurs, allowing, then, the release of the plasmid DNA, which may reach the host cytosol. Therefore, the eukaryotic vector can be capable to migrate to the nucleus of the cell, where the ORF of interest is transcribed for subsequent protein synthesis by the host cellular machinery [52,61]. Exogenous antigens, presented on the surface of epithelial cells or secreted to the extracellular medium can be phagocytized by antigen presenting cells (APCs), particularly DCs residing in the subephitelial dome region of the lymphoid follicles, migrating to the nearest mesenteric lymph nodes where the antigen is presented to naïve T cells, inducing cellular and humoral immune responses specific against the encoded antigen (Figure 2).

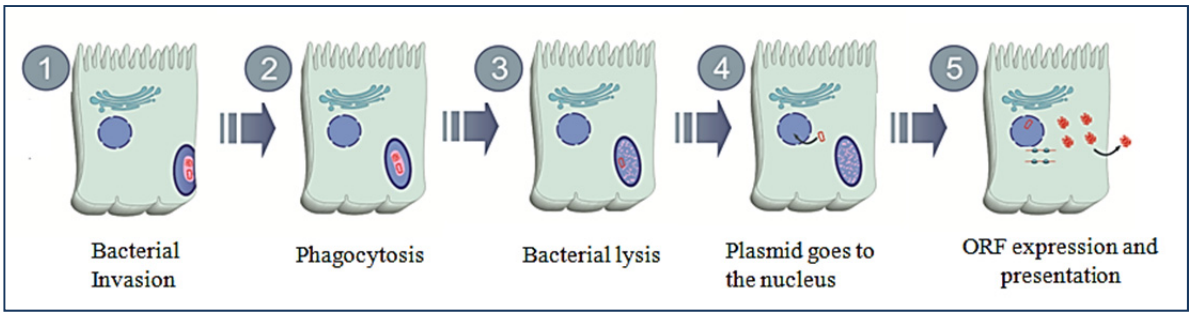

Fig. 2 Schematic representation of bacteria-mediated transfer of plasmid DNA into intestinal cells. The bacteria harboring a plasmid containing an eukaryotic expression cassette designed to express the ORF of interest entry the target cells and in the phagolysosome occurs bacterial lysis. Thus, the plasmids are released in the cytoplasm and then can be transferred into the nucleus of the host cell, occuring the expression of the ORF of interest and protein synthesis by host cells machinery (Adapted from [5]).

Overall, PPs facilitate induction of mucosal immunity, with antigen stimulated B- and T-cells able to migrate to the systemic blood supply via the lymph to be seeded back to mucosal sites around the body [62]. This enables communication from one mucosal surface to the other mucosal surfaces of the body [63]. Besides this, another

advantage of using bacteria as mucosal delivery vehicles for DNA vaccines is their potential to elicit antigen-specific secretory immunoglobulin (Ig) A responses. It is generally accepted that mucosal vaccines that elicit both secretory IgA and effective systemic immune responses could have advantages over many existing vaccines [59].

2.2. Bacteria as delivery vehicles of DNA vaccines

Currently, attenuated enteropathogenic species, such as *Salmonella typhi, Listeria monocytogenes, Shigella flexneri, Yersinia enterocolitica* and *Escherichia coli*, are the most widely used bacterial delivery systems of eukaryotic expression vectors into mammalian cells [52]. Many works has demonstrated its ability to deliver vaccine vectors to host cells and generate a significant immune response [64-68].

One great example of extracellular pathogenic bacteria that was used as a DNA vaccine vector is *Yersinia enterocolitica*. The use of this bacterium as a vector to deliver DNA was reported by Al-Mariri and co-workers. Following two intragastric immunizations in BALB/c mice, the attenuated *Yersinia* vectors harboring a DNA vaccine encoding *Brucella abortus* antigens elicited antigen-specific serum immunoglobulin and Th1-type responses among splenocytes [69]. *Listeria monocytogenes* represents another important example of a bacterial vector vastly used for DNA delivery. Several preclinical studies have demonstrated the ability of this bacterium for intracellular gene or protein delivery both *in vitro* and *in vivo* [70].

However, even though interesting, their use is limited as they present a potential risk of reversion to their wild-type (virulent) phenotype, associated risk of infection and sensitive public opinion about its use, not being completely safe for use in humans, especially in children and immunocompromised patients. Moreover, variation in the immunogenicity among the strains used has constituted a major problem as it has been difficult to reach the right balance between the level of attenuation (i.e., lack of disease symptoms) and immunogenicity (i.e., efficacy) [71].

In this regard, the use of non-pathogenic and food-grade bacteria, such as lactic acid bacteria (LAB), represent a more attractive alternative to the use of attenuated pathogenic bacteria as vehicles for mucosal delivery of DNA vaccine antigens and other therapeutic molecules because (i) they can be delivered safely at high doses and generate mucosal and systemic immune responses; (ii) some species are not immunogenic and therefore can be continuously used in immunization programs; (iii) they do not contain endotoxins such as lipopolysaccharides (LPS) on their cell wall, eliminating the risk of anaphylaxis to LPS; and (iv) they can be easily engineered to express multiple proteins and other molecules [72,73]. LAB includes mainly species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Streptococcus*. *Lactococcus lactis* is probably the most economically important LAB species standing as a model microorganism, is very easy to manipulate and was the first one to have its genome sequenced [74,75]. Moreover, traditional fermentation performed by *L. lactis* is being used from ancient times which turned this bacterium part of the cultural and traditional custom among the indigenous communities in most developing or developed countries [76]. Therefore, as they are used for centuries in food fermentation and preservation, the FDA has granted *L. lactis* as generally regarded as safe (GRAS) microorganism for human consumption [77].

2.3. Use of wild type *Lactococcus lactis* as a vehicle for DNA delivery

After the pioneering work of Wells and colleagues, increasing attention has been given to *L. lactis* as a vehicle for the presentation of exogenous antigens at mucosal surfaces [73,78-80]. Besides being considered safe, *L. lactis* has several characteristics that make them an ideal vector for immunization [81,82]. First, it can resist the acid environment of the stomach being able to survive into the gastrointestinal tract. Another attractive property of this bacterium is that their administration is capable of inducing high production of antigen-specific IgA. As *L. lactis* is not very immunogenic, unlike pathogenic microorganisms, they can be continuously used in immunization programs [83,84]. Finally, they are capable of expressing multiple proteins and therapeutic molecules at different cell compartments as they present several expression systems [85]. *L. lactis* has been therefore widely used as vehicles for the delivery of exogenous antigens at mucosal surfaces. This bacterium is one of the most advanced prototypes of non-invasive, non-colonizing bacterial vaccine vectors. *L. lactis* can be orally administrated [86] and has been shown to stimulate the phagocytic system of the host [84].

Recently, the use of LAB as DNA vaccine delivery vehicles has been evaluated as an alternative strategy for vaccination [80,87]. Delivery of DNA into mammalian cells was demonstrated, by Guimarães and colleagues in 2006, using a wild type (wt) *L. lactis* strain harboring a DNA plasmid coding for bovine ß-lactoglobulin (BLG), a major cow's milk allergen, under the transcriptional control of the viral promoter CMV (cytomegalovirus). Production and secretion of BLG was observed in the intestinal epithelial cell line Caco-2 after incubation with the recombinant *L. lactis*, demonstrating that this bacterium could efficiently deliver a fully functional plasmid into epithelial cells [86]. In order to evaluate the capacity of this bacterium to transfer DNA *in vivo* to mice IECs, Chatel and co-workers orally immunized mice with *L. lactis* carrying the same eukaryotic expression plasmid and the strain seemed to be able to translocate the intestinal membrane as BLG cDNA was detected in the epithelial cells of the immunized mice [87]. Even though these studies successfully demonstrated that *L. lactis* can be used as vector for genetic immunization, the ratio of gene transfer observed was low. Several strategies that have been shown to increase DNA delivery *in vitro* are currently being used, such as chemical treatments to weaken bacterial cell wall. *L. lactis* NZ3900 when treated with

glycine showed a higher frequency of eukaryotic expression encoding red fluorescent protein (RFP) transfer to Caco-2 cells. Treatment with penicillin and lysozyme was showed to be more effective in *S. gordonii* [88]. Recently, another very interesting approach based on the use of recombinant invasive *L. lactis* strains was developed and it has been currently being tested either *in vitro* or *in vivo*.

2.3.1. Recombinant invasive *Lactococcus lactis* as plasmid DNA delivery vehicles

Pathogenic bacteria have evolved sophisticated strategies to overcome host defenses, to interact with the immune system and to interfere with essential host systems. In order to explore the concept 'patho-biotechnology' and to increase the capacity of lactococci to persist in the gastrointestinal tract to deliver DNA vaccines, some strains of *L. lactis* expressing invasins have been reported and are thought to contain improved biotechnological and clinical applications [89,90]. One very interesting strategy performed by Guimarães and collaborators was to engineer *L. lactis* to express InlA (LL-InlA+) from *Listeria monocytogenes*. LL InlA+ is a cell wall-anchored protein and major invasin of *L. monocytogenes*, which binds to an extracellular domain of E-cadherin, its receptor [91,92]. InlA gene was cloned and expressed under transcriptional control of the native promoter. Western blot and immunofluorescence assays revealed that recombinant lactococci efficiently displayed the cell wall anchored form of InlA. Moreover, invasion rates of LL-InlA+ strain in Caco-2 cells was approximately 100-fold higher than the wild type (wt) lactococci. The recombinant strain also proved to be able to enter intestinal cells *in vivo*, after oral inoculation of guinea pigs. After internalization, LL-InlA+ was able to deliver a functional eukaryotic gfp gene (green fluorescent protein) into epithelial Caco-2 cells [89].

The results mentioned above were obtained through the use of a large plasmid called PLIG (10 kb), resulted from the co-integration of two replicons: one from *E. coli* and the other from *L. lactis*. After several attempts to insert antigens into this plasmid, it was concluded that its structure and size was hindering cloning procedures. Therefore, in order to circumvent this difficulty, a new plasmid termed pValac (Vaccination using Lactic acid bacteria), containing a smaller size (3742 bp), was constructed. The pValac is formed by 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 messenger 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. In order to evaluate pValac functionality, the gfp ORF was cloned into pValac (pValac:gfp) and fluorescence was analyzed by transfection in PK15 cells. The applicability of pValac was demonstrated by invasiveness assays of *L. lactis* inlA+ strain harbouring pValac:gfp into Caco-2 cells. After transfection assays with pValac:gfp, it was observed that PK15 cells were able to express GFP. Moreover, *L. lactis* inlA+ strain were able to invade Caco-2 cells and deliver pValac:gfp into epithelial cells [93]. Due to its small size, pValac permit the cloning of large gene fragments representing, thus, a promising tool for genetic immunization.

Nevertheless, even though interesting, the use of LL-InlA+ strain presented some bottlenecks: InlA cannot bind to its receptor in mice, murine E-cadherin, thus impeding *in vivo* experiments in these animals. Therefore, it is only possible to study the effect of LL-InlA+ strain in guinea pigs or transgenic mice, which may be laborious and/or expensive [94]. For this reason, a new recombinant *L. lactis* strain expressing Fibronectin Biding Protein A (FnBPA) (LL-FnBPA+) of *Staphylococcus aureus* [95], was constructed and therefore evaluated with the goal of improving the delivery of DNA to mammalian cells [96]. FnBPA naturally mediates adhesion of *S. aureus* to the host tissue and its entry into non-phagocytic cells [97]. Thus, FnBPA utilization could facilitate the interaction of *L. lactis* with IECs leading to a more efficient delivery of DNA vaccines. *L. lactis* FnBPA+, and *L. lactis* InlA+ showed comparable internalization rates in Caco-2 cells and conventional or confocal fluorescence microscopy demonstrated big clusters of *L. lactis* FnBPA+ and *L. lactis* InlA+ which were uptaken by Caco-2 cells. Invasive lactococci were then used to deliver a eukaryotic GFP expression plasmid (pValac:gfp) in Caco-2. After 3 hours co-incubation, invasive *L. lactis* were capable of transferring gfp to Caco-2 cells more efficiently than the non-invasive strains [96]. Recently, another work was performed with LL-FnBPA+ strain in which they used the BLG allergen and GFP to characterize the potential of this strain as an *in vivo* DNA vaccine delivery vehicle. LL-FnBPA+ carrying the plasmid pValac:BLG (LL-FnBPA+ BLG) showed to be more invasive than LL-BLG noninvasive strain, after co-incubation with Caco-2 cells. Moreover strain LL-FnBPA+ BLG presented to be as invasive as LL-FnBPA+ strain. Then *in vitro* experiments demonstrated that Caco-2 cells co-incubated with LL-FnBPA+ BLG could produce up to 30 times more BLG than cells co-incubated with the noninvasive LL-BLG. Furthermore, *in vivo* it was demonstrated that, in order to effectively deliver DNA, LL-FnBPA+ requires a pre-coating with Fetal Calf Serum before oral administration. A second interesting observation concerns the fact that enterocytes were able to express cDNA of GFP or BLG without regard to the strains used (invasive or not). Finally, the use of LL-FnBPA+ could increase the number of mice producing BLG, but not the level of BLG produced. Nevertheless, the observed increase was not statically significant [98]. Since FnBPA requires an adequate local concentration of fibronectin to bind to integrins [99,100], the interaction between FnBPA and epithelial cells may be a complex process in vivo, being this fact one of the inconvenient of this approach. Another very interesting work was performed by De Azevedo and collaborators in 2012, when a mutated form of Internalin A (mInlA), which can bind to murine E-cadherin thus allowing in vivo studies in conventional mice, was expressed successfully at *L. lactis* surface (LL mInlA+). It have been shown that this novel invasive strain was capable to invade Caco-2 cells more efficiently

than the wt strain and consequently deliver more plasmid coding for cBLG. Furthermore, *in vivo* studies demonstrated that LL mInlA+ tended to increase the number of mice producing BLG [101].

All strains that have been developed to be used as vaccine DNA delivery vectors are promising for mucosal immunization and the strategy to use *L. lactis* as a tool to deliver therapeutic plasmids proved to be valuable. Therefore, in order to improve this DNA delivery, our research group are currently seeking for other invasin gene that can easily bind to murine epithelial cells from conventional mice as well as testing other LAB specie that can stay longer than *L. lactis* in the gastrointestinal tract. Both strategies are being considered feasible as it could allow the use of LAB as efficient DNA-vaccine delivery vehicles in near future [80,102].

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