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Engineering of Lactic Acid Bacteria strains modulating immune response for vaccination and delivery of therapeutics

Marcela Azevedo

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Engineering of Lactic Acid Bacteria strains modulating immune response for vaccination and delivery of therapeutics

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ABSTRACT

The use of Lactic Acid Bacteria (LAB), such as *Lactococcus lactis* (LL), as DNA delivery vehicles represents an interesting strategy as they are regarded as safe. Wild type (wt) LL or recombinant invasive LL, were able to trigger DNA expression by epithelial cells both *in vitro* and *in vivo*. However, important information about how LL can transfer DNA plasmids is still missing. Therefore, we decided to construct a new recombinant invasive LL strain expressing mutated Internalin A (mInIA) from the pathogen *Listeria monocytogenes* to understand the manner by which the DNA is transferred to mammalian cells. mInIA expression was detected by FACS analysis and LL-mInIA strain showed to be more invasive than the wt strain after co-incubation assays with non-confluent or polarized intestinal epithelial cells (IECs). Confocal microscopy confirmed the invasive status of LL-mInIA which demonstrated to deliver more efficiently the eukaryotic expression vector coding the allergen β -lactoglobulin, pValac:BLG, *in vitro* to IECs and to dendritic cells (DCs). LL-mInIA was also capable to transfer pValac:BLG to DCs across a monolayer of differentiated IECs. *In vivo*, invasive lactococci tended to increase the number of mice expressing BLG. Moreover, noninvasive or invasive LL-mInIA stimulated the secretion of the pro-inflammatory cytokine IL-12 in DCs and, *in vivo*, after oral or intranasal immunization trials, non-invasive LL polarized the immune response more in the type 1 direction while invasive LL generated a Th2-type response in immunized animals. All these data gives new insights on the mechanism of lactococci uptake for delivery of therapeutics.

LIST OF ABBREVIATIONS

aa	Amino acids
Ads	Adenoviruses
APCs	Antigen presenting cells
B cells	B lymphocytes
BALB/c	Laboratory-bred strain of the House Mouse Jackson Laboratory
BALT	Bronchial/tracheal-associated lymphoid tissue
BGH	Growth Hormone
BLG	β -Lactoglobulin
CD	Crohn's disease
cDNA	Complementary DNA
CFU	Colony Forming Unit
CpG motifs	Cytosine-phosphate-guanine unmethylated
CTL	Cytotoxic T lymphocytes
DapD	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase DapD
DCs	Dendritic cells
DEAE-dextran	Poly-lysine, diethylaminoethyl-dextran
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DSS	Dextran sulfate sodium
EMA	European Agency for the Evaluation of Medicinal Products
EP	Electroporation
ER	Endoplasmic reticulum
FAE	Follicle-associated epithelium
FDA	US Food and Drug Administration
FnBPA	Fibronectin Biding Protein A
GALT	Gut associated-lymphoid tissue
GC	Guanine-cytosine
GF	Germ-free
gfp	Green fluorescent protein
GI	Gastrointestinal
GRAS	Generally regarded as safe

hAAT	Human α 1-antitrypsin
hCMV	Human cytomegalovirus
HGF	Hepatocyte growth factor
hGH	Human growth hormone
HIV-1	Human immunodeficiency virus type - 1
HK-1	Hemokinin-1
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN γ	Interferon- γ
Ig	Immunoglobulin/antibody
IgA	Immunoglobulin A antibody isotype
IgE	Immunoglobulin E antibody isotype
IgG1	Immunoglobulin G type 1 antibody isotype
IgG2a	Immunoglobulin G type 2a antibody isotype
IHNV	Infectious hematopoietic necrosis/Aquatic rhabdovirus
IL-12	Interleukine-12
IL-8	Interleukine-8
InIA	Internalin A
InIB	Internalin B
ISS's	Nucleotide hexamers
LAB	Lactic acid bacteria
LGG	<i>Lactobacillus</i> GG
LLO	Cytolysin listeriolysin O
LPS	Lipopolysaccharides
LRRs	leucin-rich repeat motif
LT	Lymphotoxin
LTA	Lipoteichoic acid
M cells	Microfold cells
MALT	Mucosa-associated lymphoid tissue
MAMPs	Microbial-associated molecular patterns
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II

mRNA	Messenger RNA
MRP	Maximum representation with parsimony
NALT	Nose-associated lymphoid tissue
NF- κ B	NF- κ B nuclear factor kappa B
NICE	Nisin Controlled Gene Expression
NK	Natural killer cells
NL	Netherlands
NLR	NOD-like receptors
NLRs	NLRs Nod-like receptors
NLS	Nuclear localization signal
OD	Optical density
ori31	replication origin of ϕ 31
ϕ 31	Bacteriophage ϕ 31
PAMPs	Pathogen-associated molecular patterns
PEI	Polyethyleneimine
PGN	Peptidoglycan
PM	Plasma membrane
polyA tail	Polyadenylation sequence
PPH	Baculovirus
PRRs	Pattern recognition receptors
PRSV	Rous sarcoma virus
PTK	Thymidine kinase promoter
pValac	Vaccination using Lactic acid bacteria
RLRs	RIG-I like receptors
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SARS	Severe Acute Respiratory Syndrome
SCFA	Short chain fatty acid
SDS-PAGE	Gel denaturing polyacrylamide
SIgA	Secretory IgA
SlpA	S-layer protein
ssRNA	Single-stranded RNA
SV40	Simian virus 40

T CD4+	T helper lymphocytes
T CD8+	Cytotoxic T lymphocytes
T cells	T lymphocytes
T3SS	Type III secretion system
Th	T helper lymphocytes
Th1	T helper cells type 1
Th2	T helper cells type 2
TJ	Tight junctions
TLR	Toll-like receptor
TLR1	Toll-like receptor type 1
TNF α	Tumor necrosis factor- α
Treg	Regulatory T cells
TTFC	Fragment C of tetanus toxin
UC	Ulcerative colitis
US	United States
US	Ultrasound
Usp45	Unknown Secreted Protein of 45 kDa
VALT	Vulvovaginal-associated lymphoid tissue
WHO	World Health Organization
wt	Wild type
XIES	Xylose-Inducible Expression System

GENERAL INTRODUCTION

I. COLLABORATIONS

This thesis is a part of a co-tutelle PhD program offered jointly by two higher education institutions; one in Brazil named Universidade Federal de Minas Gerais (UFMG), and the other one in France called Université Paris-Sud 11. This program allows the students to get a double/joint PhD degree delivered and recognized by both institutions besides exposing students to the international research community. The major experimental part of this work was conducted in France at Institut National de la Recherche Agronomique (INRA) facilities located in Jouy en Josas under the supervision of Dr. Jean Marc Chatel and the co-supervision of Dr. Philippe Langella, both researchers at ProbiHote Team from MICALIS unit (Microbiologie de l'Alimentation au Service de la Sante). The work was also supervised by Dr. Anderson Miyoshi and co-supervised by Dr. Vasco Azevedo; both of them professors at Instituto de Ciências Biológicas developing research at Laboratório de Genética Celular e Molecular (LGCM) from UFMG. This research project was financed in the last year by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) and, before that, by Cross talk European project during three years (april 2009-2012). Cross-Talk was a part of a Marie Curie Initial Training Network (ITN) focusing to study the interaction between microbiota and the human host. The network gathered 13 partners and 14 young scientists from more than 10 European and non-European Universities.

The Research Project (RP) comprising this thesis was the Cross Talk RP number 6 (RP6) (for more details see: <http://www.cross-talk.eu/index.php?id=63>) which aimed to understand the relationship between immune cells and epithelial cells with recombinant lactic acid bacteria (LAB) used as DNA delivery vehicle. Training of the fellows was the core activity of Cross Talk project. For this reason it supported mobility periods (maximum 9 months) abroad to enlarge recently established collaborations between laboratories with a view to improve students skills and promote the cross-border transfer of knowledge. Therefore, RP6 was also carried out during seven months in collaboration with Dr. Jerry Wells, a Professor at Host Microbe Interactomic Group (HMI) from Wageningen University – WUR Research Center located in The Netherlands. One of the deliverables established by Cross Talk network was the “Thesis committee” which was held once per year at Institut Pasteur situated in Paris. Thus, expertise scientists, such as Dr. Catherine Grillot-Courvalin (MD, PhD, Associate Professor at the Pasteur Institute, Unit of Antibacterial Agents) and Dr. Karine Adel-Patient (Researcher at INRA, UR, Unité d'Immuno-Allergie Alimentaire), were also involved in the project with the intent to give advices and propose actions to improve future publications.

II. WORK TOPIC

Lactococcus lactis (LL) is a bacterium traditionally used for food production and preservation. Therefore they are safe for human consumption and for this reason they harbor the GRAS (Generally Regarded As Safe) status. Due to this fact, a number of new applications have been proposed and LL was extensively engineered to function as a cell factory for many proteins of health interest, such as antigens for the development of new vaccines (Miyoshi and Azevedo, 2004; Wells and Mercenier, 2008). It was successfully demonstrated that LL is able to express antigenic proteins in different cells compartments (intracellularly, anchored to the cell wall or expressed to the extracellular medium) and deliver them at mucosal surfaces (for reviews see Wells, 2011; Pontes et al., 2011; Bermúdez-Humarán et al., 2011). Two great examples are the as β -lactoglobulin (BLG), one of the major cow's milk allergen, for the treatment of allergic diseases (Chatel et al., 2001) and E7 antigen from HPV virus for the treatment of colon cancer (Bermúdez-Humarán et al., 2005). More recently it was demonstrated that this bacterium is able to deliver DNA vaccines at mucosal surfaces (Chatel et al., 2008). This work, therefore, seek to exploit, understand and underline the mechanisms by which LL can deliver DNA vaccines to mammalian cells.

Briefly, a DNA vaccine consists of a circular DNA plasmid encoding antigenic proteins under the control of a mammalian promoter. After taking up the DNA, mammalian cells are able to drive the expression of the vaccine antigens. The plasmid contains an origin of replication that allows its replication inside bacterial hosts and a resistant marker that permits the selections of clones that harbors the plasmid DNA (Lichtor and Glick, 2012). DNA immunization can produce stronger and wider types of immunity within the organism – including antibody, T helper cell (CD4+ T cell), and cytotoxic T lymphocyte (CTL, CD8+ T cell) mediated immunity – to cover multiple diseases (Glenting and Wessels, 2005). The DNA vaccine can be applied directly to the human body (“naked DNA vaccination”); it can be carried by some viral vectors or formulated with liposomes or proteoliposomes (Gurunathan et al., 2000). Bacterial vectors are also widely used as it can protect the DNA against the attack by endonucleases and it can stimulate the mucosal immune system associated with the epithelium. Furthermore, it shows a better tropism into the body after immunization trials when compared to the other approaches. Moreover, there is no need for further steps of plasmid amplification and purification because the plasmid can replicate itself inside the bacteria. Therefore, this platform in vaccinology is considered to be very low cost (Glenting and Wessels, 2005). The use of LL as a DNA vector turns to be very interesting over the use of attenuated pathogenic bacteria, which are more traditionally used to deliver DNA, because they have GRAS status (Wells and Mercenier, 2008).

Guimarães et al. (2006) demonstrated that wild type (wt) LL could deliver DNA vaccines *in vitro*. Later, Chatel et al. (2008) observed that this bacterium was also able to transfer DNA plasmids *in vivo* to murine intestinal epithelial cells (IECs). Even though being confirmed the capacity of lactococci to transfer DNA vaccines, it still remains unclear how LL can deliver DNA to mammalian cells *in vivo*. Basically, there are at least three manners by which antigens, plasmids or bacteria in general can gain access to mammalian cells interior and reach the lamina propria. This material diluted in the intestinal lumen can be recognized by some specialized epithelial cells named Microfold cells (M cells) and transfer them to the underlying Peyer's patches (aggregations of lymphoid tissue that are usually found in the lowest portion of the small intestine). Antigen Presenting Cells (APCs) such as Dendritic cells (DCs) associated with the IEC monolayer can also extend their dendrites through the tight junctions and sample bacteria or antigens that are localized in the lumen (Rescigno et al., 2001). Finally, some vectors based on attenuated pathogenic species can express invasive proteins which are recognized by epithelial cells and actively invade the IEC monolayer reaching the lamina propria (Gewirtz and Madara, 2001). Once inside the cell, bacteria localized in the phagolysosome are normally target for degradation. Some attenuated pathogenic vectors such as *Listeria monocytogenes* are able to secrete cytolysins (Lysteriolysin O – LLO) which can make pores into the phagosomal membrane helping the plasmid to escape from the phagolysosome to the nucleus of the cell. Using the mammalian cell transcriptional machinery components, the antigen of interest can be produced (Faurez et al, 2010; Liu, 2010).

Based on this knowledge two hypotheses were designed to explain how LL can transfer DNA vaccines to epithelial cells: 1- Bacteria could be lysed in the intestinal lumen releasing the DNA vaccine which could be sampled by IECs, M cells and/or DCs. 2- Bacteria itself could be sampled by IECs, DCs and/or M cells. Our research group decided to test the second hypothesis by constructing recombinant invasive *L. lactis* strains thus improving the interactions between bacteria and IEC monolayer. One strain was able to express *Staphylococcus aureus* Fibronectin Binding Protein A (FnBPA) (LL-FnBPA+) (Innocentin et al., 2009). *In vitro* assays demonstrated that it was capable to deliver a eukaryotic expression vector to IECs (Caco-2 cell line) more efficiently than the wt strain. However, the use of this strain in immunization trials presented some limitations as the binding of FnBPA depends on the availability of fibronectin *in vivo*. Another strain was the one producing *Listeria monocytogenes* Internalin A (InIA) (LL-InIA+) (Guimarães et al., 2005). It was able to deliver more DNA to IEC (Caco-2 cell line) *in vitro* when compared to the wt strain; nevertheless, its use *in vivo* is very limited because InIA cannot bind to its receptor in mice, the murine E-cadherin. Even though LL-FnBPA+ and LL-InIA+ strains have been constructed and presented to be better tools to

deliver DNA vaccines *in vitro* when compared to wt lactococci, the process by which LL can transfer DNA vaccines remains unknown.

Therefore, strains of *L. lactis* expressing mutated InIA (LL-mInIA+), an invasin able to bind to murine E-cadherin, were constructed in this work and their ability to deliver cDNA from the major cow's milk allergen, β -lactoglobulin (BLG), was studied in different experimental models. It was also examined the capacity that LL-mInIA+ strain has to deliver BLG cDNA to murine epithelial cells after immunization trials. Furthermore, immune responses elicited after *in vivo* administration of invasive and noninvasive strains were measured, and the capacity of these strains in delivering DNA vaccines to antigen presenting cells (DCs) was evaluated as well.

The second chapter of this manuscript, where we present the first results, is related to the characterization of LL-mInIA+ strain as a vehicle to deliver the eukaryotic expression vector, pValac:BLG, previously constructed by Pontes and collaborators (2012), either *in vitro* or *in vivo* to IECs. Firstly, *L. lactis* NZ9000 wt strain was transformed with pOri253:mInIA plasmid harboring the gene of mInIA from *Listeria monocytogenes*. Flow cytometry analysis confirmed that the new strain was able to successfully express mInIA at its surface. Moreover, it presented to be almost 1000 times more invasive than the wt strain after co-incubation assays with Caco-2 cells. Confocal images confirmed this invasive status as recombinant LL-mInIA+ were found attached to Caco-2 cells being found in the interior of the cells. Afterwards, *L. lactis* producing mInIA was transformed with pValac:BLG plasmid and its capacity to transfer the cDNA of BLG was then measured. LL-mInIA+BLG (invasive strain) and LL-BLG (noninvasive strain) were co-incubated with Caco-2 cells and after three days BLG expression by the mammalian cells were evaluated by ELISA. It was observed that the invasive characteristic increased plasmid transfer as Caco-2 cells incubated with LL-mInIA+BLG produced more BLG when compared to the cells incubated with LL-BLG. After the *in vitro* studies we moved to *in vivo* assays in which mice were intragastrically administrated with *L. lactis*, LL-BLG or LL-mInIA+BLG for three consecutive days, and the small intestine removed for isolation of IECs. BLG DNA delivery efficiency *in vivo* is slightly improved by the production of mInIA. However, no significant advantages were observed by using LL-mInIA+BLG compared to LL-BLG. Based on this result we hypothesize that most likely, plasmid transfer *in vivo* is a combination of two mechanisms, bacteria and released plasmid captures by specialized epithelial cells (Microfold cells, M) or IECs as *L. lactis* is a transient specie and cannot survive within the gastrointestinal tract (TGI).

The third chapter is dedicated to studies performed with the intent to investigate the immune response elicited after oral or intranasal immunization with the noninvasive (LL) or invasive *L. lactis* (LL-FnBPA+ and LL-mInIA+) carrying pValac:BLG. It is known that the administration of a plasmid DNA encoding antigens usually elicits a T helper cell type 1 (Th1) immune response. In order

to check if the same immune profile could be obtained after immunization trials with noninvasive or invasive *L. lactis*, conventional mice were orally or intranasally administered with the strains and BLG-specific primary immune response were monitored. We have demonstrated that after oral or intranasal administration with invasive *L. lactis* FnBPA+, mice elicited a Th2 immune response characterized by the detection of IL-4 and IL-5 cytokines. Differently, the oral or intranasal administration of noninvasive *L. lactis*-BLG elicited a Th1 immune response characterized by the detection of IFN- γ . To test the capacity of the strains to avoid allergic immune responses, mice intranasally pre-treated with both strains were sensitized with BLG and then immune response was measured. After sensitization, we detected a significantly lower concentration of BLG specific IgE in mice immunized with noninvasive *L. lactis*. This result was expected as Th1 immune cells, which were induced by noninvasive *L. lactis*, can suppress the generation of allergic immune responses (Romagnani, 2004). IL-4 and IL-5 cytokines were again found in a higher amount in mice sensitized and previously immunized by intranasal or oral route with the invasive lactococci. To check if the Th2 induced profile was due to the expression of invasins at *L. lactis* cell wall, another recombinant invasive *L. lactis*, LL-mInIA+BLG, was intranasally administered in conventional mice and immune response was evaluated. We observed again a polarization to the Th2 immune profile by using the invasive lactococci. In order to explore if the alteration of some component of the bacterial cell wall could be the cause of this immune polarization, the peptidoglycan composition from the cell wall of invasive and non-invasive strains were analyzed and no differences could be observed. In conclusion, we demonstrated that the expression of invasins at the surface of *L. lactis* modifies its immunomodulatory properties.

The fourth chapter presents *in vitro* studies which were made with the goal to give new insights on the mechanisms of plasmid transfer by using noninvasive or invasive lactococci. All the works about plasmid transfer using *L. lactis* performed by our research group and others used IECs as a model to evaluate plasmid transfer. In this work we evaluated DNA transfer capacity of LL to dendritic cells (DCs) as these cells are the major antigen presenting cells of the organism that are in direct contact with diluted antigens of the TGI. We have shown that noninvasive *L. lactis*, and invasive *L. lactis* strains expressing either *S. aureus* FnBPA, or *L. monocytogenes* mInIA can transfect bone marrow-derived DCs (BMDCs) and deliver the cDNA of BLG. The invasive status appeared to be advantageous facilitating the bacteria-cell contact and, thus, allowing a higher translocation of the pValac:BLG plasmid. Moreover, BMDCs co-cultured with non-invasive or invasive Lactococci were able to secrete elevated levels of the pro-inflammatory cytokine IL-12, suggesting that this immune response is due to MAMPs (Microbe Associated Molecular Patterns) naturally found in *L. lactis* and not related to BLG expression by the BMDCs or with mInIA or FnBPA invasins. In order to understand

how lactococci can interact with IECs or DCs from the intestinal epithelium to deliver therapeutic plasmids, a monolayer of differentiated Caco-2 cells were used as a model that could mimic the situation encountered *in vivo*, where bacteria and antigens face a protective monolayer of epithelial cells. Firstly, we checked whether the invasive *L. lactis* strains (LL-FnBPA+ and LL-mInIA+) were able to internalize the monolayer more efficiently than the wt strain. Co-incubation of bacteria with polarized Caco-2 cells showed that LL-mInIA+ is 100 times more invasive than LL and equally invasive compared to LL-FnBPA+ strain. Additionally we also investigated the cross-talk between differentiated IECs, BMDCs and bacteria using an *in vitro* Transwell co-culture model. Co-incubation of strains with the co-culture model has shown that DCs maintained with LLmInIA-BLG strain was able to express significant higher levels of BLG. This data was not observed for LL-FnBPA+BLG or noninvasive LL-BLG strain. The fact that *L. lactis* producing mInIA was the only strain capable to mediate plasmid transfer may be due to the expression of E-cadherin receptor by BMDCs, which could facilitate the contact and capture of the bacteria.

The use of *L. lactis* as a tool for DNA delivery has proved to be an interesting alternative approach for the design of new mucosal vaccines. Taken together, we believe that this work brings new information regarding the mechanisms by which *L. lactis* can transfer DNA plasmids to eukaryotic cells. We believe that all this data can facilitate its use as a vehicle for immunization proposals in near future. We also believe that all these tools and models designed or used in this work may lead to the construction of new food-grade live vaccines based on *L. lactis*. Such uses for vaccination purposes are promising for future therapeutic use of this bacterium.

III. THESIS OUTLINE

This manuscript was divided into six chapters and two appendices. Below, a brief description of the content covered in each chapter/appendices:

Chapter 1: Thesis introduction about DNA vaccines, immunological principles of genetic immunization and the vectors used to deliver plasmid vaccines with a focus on the use of bacteria, particularly lactic acid bacteria (BL), native or recombinant, as vehicles for gene vaccination. Also describe the main mechanisms of interaction between BL and the immune system associated with the intestinal epithelium for transferring cDNA, for example the cDNA of the major allergen of cow's milk, β -lactoglobulin.

Chapter 2: Aim of the study

Chapter 3: Presented in a scientific paper form, it describes the use of a new recombinant *L. lactis* strain expressing an invasin derived from *Listeria monocytogenes* as a vehicle for DNA delivery in vitro or to epithelial cells from BALB/c mice, which has been accepted for publication.

Chapter 4: Also presented as a scientific article, it describes the different immune responses obtained after immunization trials with native or recombinant strains expressing on its surface invasins derived from pathogenic bacteria. This chapter is in preparation to be submitted for publication.

Chapter 5: Presented in a scientific paper form, it presents the ability of invasive *L. lactis* strains to deliver plasmids for antigen presenting cells such as dendritic cells, in vitro. This chapter is in preparation to be submitted for publication.

Chapter 6: General discussion of the results, which were presented in Chapters 2, 3 and 4 and obtained conclusions and future directions of the work.

Chapter 7: References.

Appendix 1: Presented in a scientific paper form, this section describes the use of an *L. lactis* strain expressing the mutated Internalina A and listeriolysin O, two proteins derived from *L. monocytogenes*, as a potential vehicle for genetic immunization or against listeriosis.

Appendix 2: A literature review on the use of probiotics or recombinant probiotic as immunotherapy for the treatment of allergic diseases.

CHAPTER 1
THESIS INTRODUCTION

1. DNA VACCINES

1.1 Historical perspective of DNA vaccines

The advent of molecular biology and genetic engineering has had a dramatic effect on vaccine development, providing greater opportunities for construction of inactivated antigens, attenuation of pathogenic organisms through direct mutation, and more recently, the construction of DNA vaccines (Plotkin, 2005). They are plasmids that contains a transgene encoding the sequence of a target protein under the control of a eukaryotic promoter at the beginning of the gene (Srivastava and Liu, 2003; Ingolotti et al., 2010; Liu, 2011). Following *in vivo* administration, the plasmid DNA can transfect mammalian cells which will be then capable to express the protein of interest *in situ* (Ingolotti et al., 2010; Liu, 2011) (Figure 1).

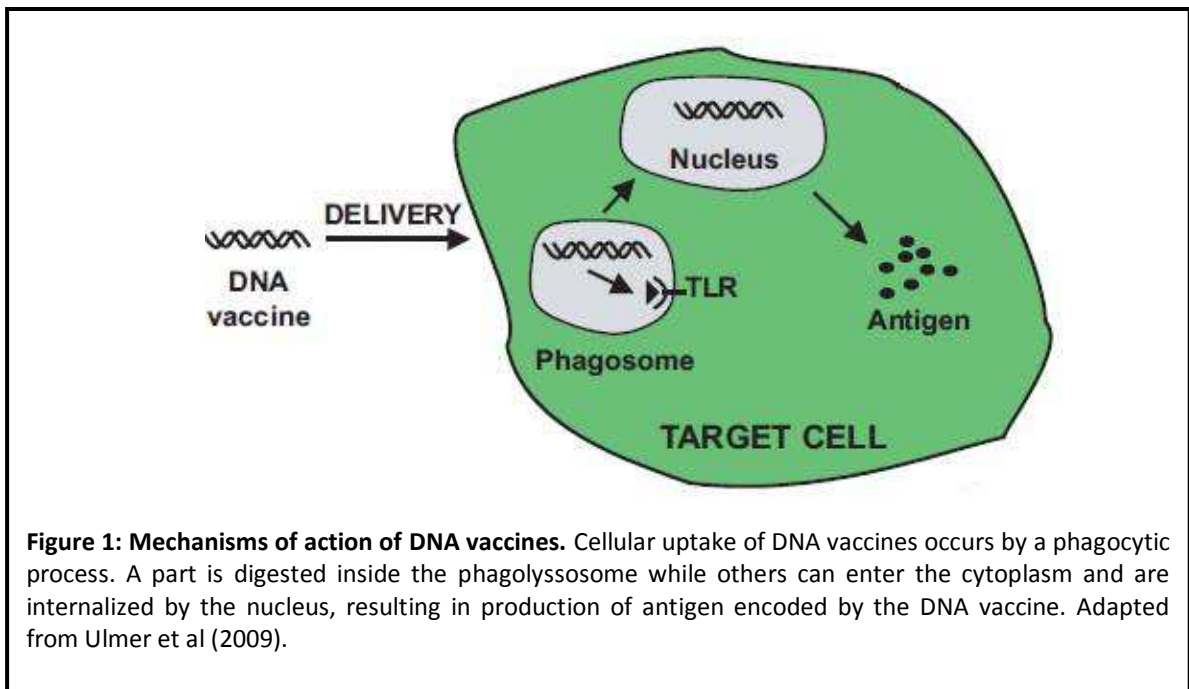
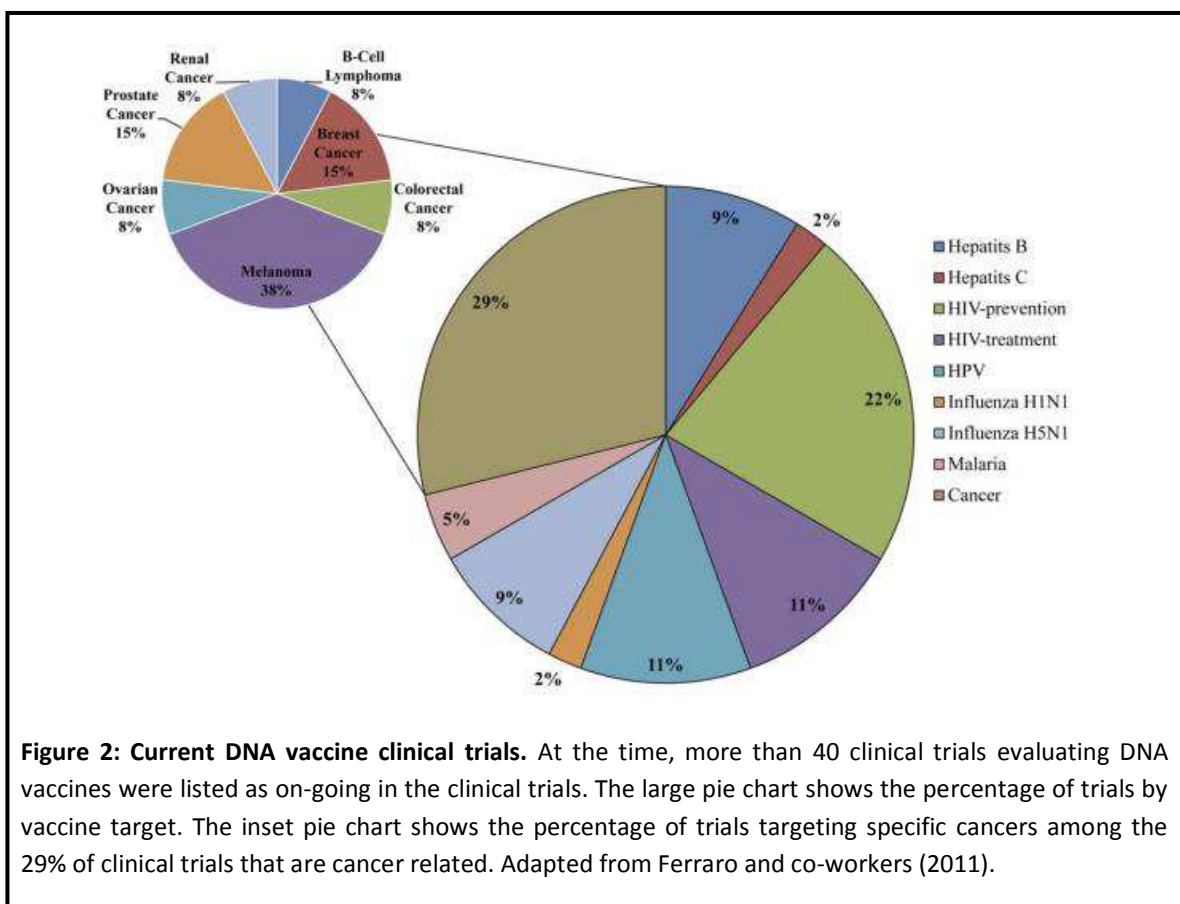


Figure 1: Mechanisms of action of DNA vaccines. Cellular uptake of DNA vaccines occurs by a phagocytic process. A part is digested inside the phagolysosome while others can enter the cytoplasm and are internalized by the nucleus, resulting in production of antigen encoded by the DNA vaccine. Adapted from Ulmer et al (2009).

The first study found in the literature about DNA vaccination started in the early 1990s when Wolff and collaborators observed that injection of a “*naked*” plasmid DNA encoding foreign antigens in mice has made their muscle cells capable of expressing these same antigens (Wolff et al., 1992). Later, Tang, Stephen A Johnston and Michael DeVit, three scientists from University of Texas, Dallas, USA, demonstrated that the injection of gold microspheres coated with human growth hormone (hGH) DNA into the skin of a mouse was able to generate both hGH- and human α 1-antitrypsin (hAAT)-specific antibodies (Tang et al. 1992). At the same time, three other research groups reported on a meeting held at Cold Spring Harbor Laboratory (NY, USA) that the

injection of a DNA in immunizations trials against influenza or HIV-1 was able to drive both humoral and cellular specific immunity. Later, Margaret Liu and colleagues published similar results, as well as other researchers (Fynan et al., 1993; Ulmer et al., 1993; Ingolotti et al., 2010) confirming the immunogenic and protective capacity of DNA vaccines (Ingolotti et al., 2010; Liu, 2011; Li et al., 2012).

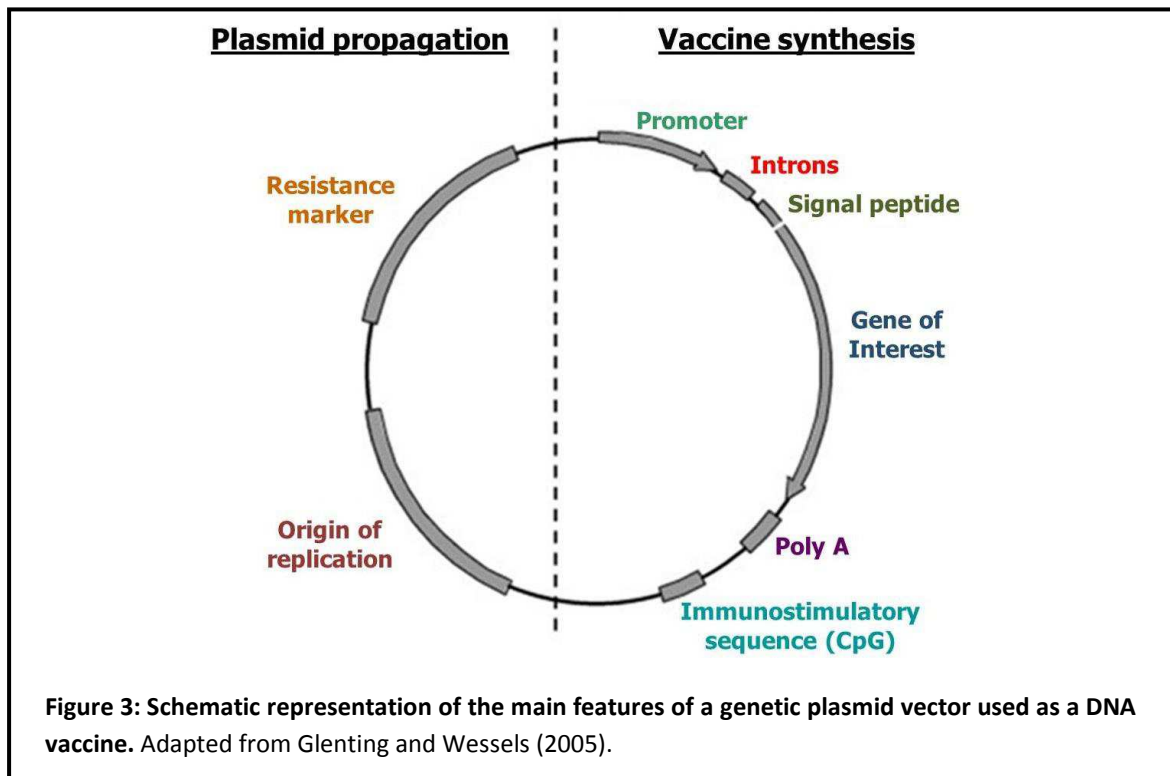
The use of DNA as a strategy for vaccination has progressed very rapidly. Within twenty years since the first publication demonstrating the ability of a plasmid to generate protective immune responses, DNA vaccines have reached enticing results. This vaccine platform has entered into a variety of human clinical trials for prophylactic vaccines against viral, bacterial or parasitic infections and also as a potential therapy to treat infectious diseases, cancers, Alzheimer disease, allergy and autoimmune disorders (for a review see Liu, 2010 and Liu, 2011) (Figure 2).



1.2 Structural features of DNA vaccines

A DNA vaccine plasmid can be divided into two main structures: the plasmid backbone and the transcriptional unit. The plasmid backbone, also referred as prokaryotic system, is usually

composed of (i) a prokaryotic origin of replication that allows the plasmid propagation into the host bacterium and (ii) a resistance marker, necessary to enable a selective growth of the DNA vaccine in bacteria. The transcriptional unit, also known as eukaryotic system, contains the promoter and a gene encoding the antigen of interest followed by a transcript termination/polyadenylation sequence named polyA tail. The first promoters used were from a viral origin and they are still widely used. Two great examples are the cytomegalovirus (hCMV) and the Simian virus 40 (SV40) that can drive the expression of the transgene/antigen in a vast variety of mammal cells types, enabling the expression of antigens *in situ* (Ingolotti et al., 2010). Other common promoters are the polyhedrin promoter from baculovirus (PPH), the thymidine kinase promoter from Herpes simplex virus (HSV)-1 (PTK), the 5'LTR promoter from human immunodeficiency virus (HIV; PLTR) and the promoter of the Rous Sarcoma Virus (PRSV) (Becker et al., 2008). Due to the fact that some cytokines may differentially regulate the CMV promoter altering transgene expression, the use of nonviral promoters is presently the topic of intensive research. For example, major histocompatibility complex (MHC) class II promoter is being investigated as possible alternatives (Vanniasinkam et al., 2006; Ingolotti et al., 2010). Another structure that can be present in the plasmid vaccine are segments of DNA not needed to create the protein (introns), usually inserted after the promoter and before the gene of interest. Their presence can increase the promoter activity (Kano et al., 2007). Moreover, the insertion of introns is also a strategy to avoid the antigen expression by the prokaryotic machinery in bacteria, thereby ensuring that expression is only possible through the eukaryotic system (Becker et al., 2008). A multiple cloning site allowing the insertion of the gene is present as well as the signal peptide allowing the secretion of the target protein. Another component is the gene of interest which is usually codon optimized to match it with the target organism and is terminated by a dual stop codon. A polyadenylation signal (polyA) necessary for transcriptional termination, stability, processing and translation of eukaryotic mRNA is also present and is commonly derived from the bovine growth hormone, SV40, or rabbit β -globin gene (Williams et al., 2009). DNA vaccines may further contain endogenous adjuvants and "CpG motifs" (cytosine-phosphate-guanine unmethylated), responsible for increasing the magnitude of the immune response as they can enhance T lymphocyte recruitment or expansion (Glenting and Wessels, 2005; Kano et al., 2007; Becker et al., 2008; Williams et al., 2009). Major structures of DNA vaccines are illustrated in figure 3.



Another advantage consist on the fact that DNA plasmids are easy to handle and rapid to construct, an interesting attribute for making vaccines against an emerging pandemic threat (Liu, 2011). As DNA vaccine plasmids are non-live, non-replicating and non-spreading, there is little risk of either reversion to a disease-causing form or secondary infection (Kutzler and Weiner, 2008). Additionally, plasmids are quite stable at room temperature, are easily stored and the genes in the plasmids can be made with fidelity to the wild type (wt) protein (Liu, 2011; Li et al., 2012).

1.3 Safety issues

Despite all its assets, when the first studies describing the use of DNA vaccines for treating infectious diseases appeared in the scientific community, a number of concerns associated with their use were raised. The possibility that they could integrate into the host genome, thus increasing the risk of malignancy (by activating oncogenes or inactivating tumor suppressor genes) or to induce responses against self-antigens thereby triggering the development of autoimmune disease were the major worries. Besides that, it was not known whether DNA vaccines could induce a local inflammatory response against cells producing the vaccine-encoded antigen (such as muscle or skin cells), induce tolerance rather than immunity (Klinman et al., 1997) or spread antibiotic resistance genes to the environment after clinical trials (Kutzler and Weiner, 2008). In fact, the introduction of plasmid DNA into humans requires special considerations which have

been addressed in several regulatory draft guidance established by the World Health Organization (WHO), US Food and Drug Administration (FDA), or European Agency for the Evaluation of Medicinal Products (EMA) (EMA, 2006; FDA, 2007; WHO, 2007; Williams et al., 2009).

According to Wang and co-workers, the plasmid should contain no significant homology with the target organism genome in order to reduce chances of chromosomal integration (Wang et al., 2004). No observable integration of the DNA into the host genome occurred and there was no observed tolerance to the antigen or autoimmunity (Liu and Ulmer, 2005). It was calculated that DNA integration into the host genome occurs at rates that are orders of magnitude below the spontaneous mutation frequency. However, plasmids that are modified or adjuvanted with the goal of increasing immunogenicity could increase the chances of integration (Liu, 2011). Additionally, oncogene activation was not observed in preclinical or clinical evaluation of DNA vaccines (Kutzler and Weiner, 2008). Another issue regarding DNA vaccination involved antibiotic resistance. As mentioned before, antibiotic-resistant marker is needed to select bacteria harboring the DNA vaccine. Usually, the antibiotic gene present in vaccine plasmids is restricted to those that are not commonly used to treat human infections, for instance kanamycin. As the development of antibiotic-free selection systems is desirable from both cost and safety perspective (Bower and Prather, 2009), alternative strategies that do not use antibiotic selection are being explored (Glenting and Wessels, 2005; Kutzler and Weiner, 2008). Several researchers successfully modified the vector, host, or both to develop alternative plasmid selection systems to ensure efficient killing of plasmid-free cells. For example, Cranenburgh and collaborators chose to target *dapD*, an essential gene in *Escherichia coli* responsible for diaminopimelate and lysine biosynthesis. The endogenous *dapD* locus was disrupted, and a copy of *dapD* under the control of a *lac* promoter was integrated into the bacterial chromosome. This strain was then transformed with a high copy plasmid containing the *lac* operator, necessary to drive the expression of the diaminopimelate and lysine biosynthesis gene. As a result, only cells containing the plasmid DNA with the *lac* operator sequence survived in culture (Cranenburgh et al., 2001; Bower and Prather, 2009).

1.4 Immunological aspects of DNA vaccines

1.4.1 Routes of administration

Genetic immunization initially consisted in the direct administration of a DNA plasmid (so-called "*naked DNA*") into tissues capable to internalize and express the immunogenic antigen, mimicking thus a natural infection (Liu, 2010; Liu, 2011). Basically, there are two main routes for DNA vaccines administration: intramuscular injection and intradermal injection (parenteral

routes). Subcutaneous injection, intraperitoneal, sublingual, intrarectal, ocular, application to mucosal surfaces (vaginal, nasal and oral), intravenous and intranodal injections are other possible routes of administration, but they were used less frequently at the beginning of DNA vaccine research (McCluskie et al., 1999; Faurez et al., 2010).

The most widely employed method to administer DNA vaccines for many years was the intramuscular injection. Naked DNA resuspended in saline buffer could be injected directly into skeletal muscle tissue using a hypodermic needle. Nevertheless, it has been shown that from 95% to 99% of intramuscularly injected plasmids found in the interfibrillar space are degraded in the muscle tissue within 90 min post-administration (Barry et al., 1999). A study conducted by Lechardeur and colleagues demonstrated that 90 minutes after the plasmid injection, only 0.1% of the injected material was able to reach the cell nucleus. Endonucleases have been implicated in degrading injected plasmid favoring its rapid elimination (Lechardeur et al., 1999). Actually, DNA degradation represents a fundamental problem for genetic immunization, as destruction of incoming genes translates into loss of gene expression (Barry et al., 1999).

It was also noticed that tissues differ for their efficiency to present antigens to the immune system (Fynan et al., 1993; McCluskie et al., 1999). Therefore the route of administration dramatically influences the strength and nature of immune responses to a plasmid-encoded viral antigen (McCluskie et al., 1999). Some works demonstrated that tissues, such as the skin and the mucosal linings of the respiratory tract and gut are more suitable routes for plasmid inoculation as they are associated with lymphoid tissues and provide high levels of local immune surveillance (Fynan et al., 1993). Hence, administration of DNA to mucosal surfaces can provide systemic immunity as well as specialized surveillance for major portals of pathogen entry (Fynan et al., 1993; McCluskie et al., 1999; Azizi et al., 2010).

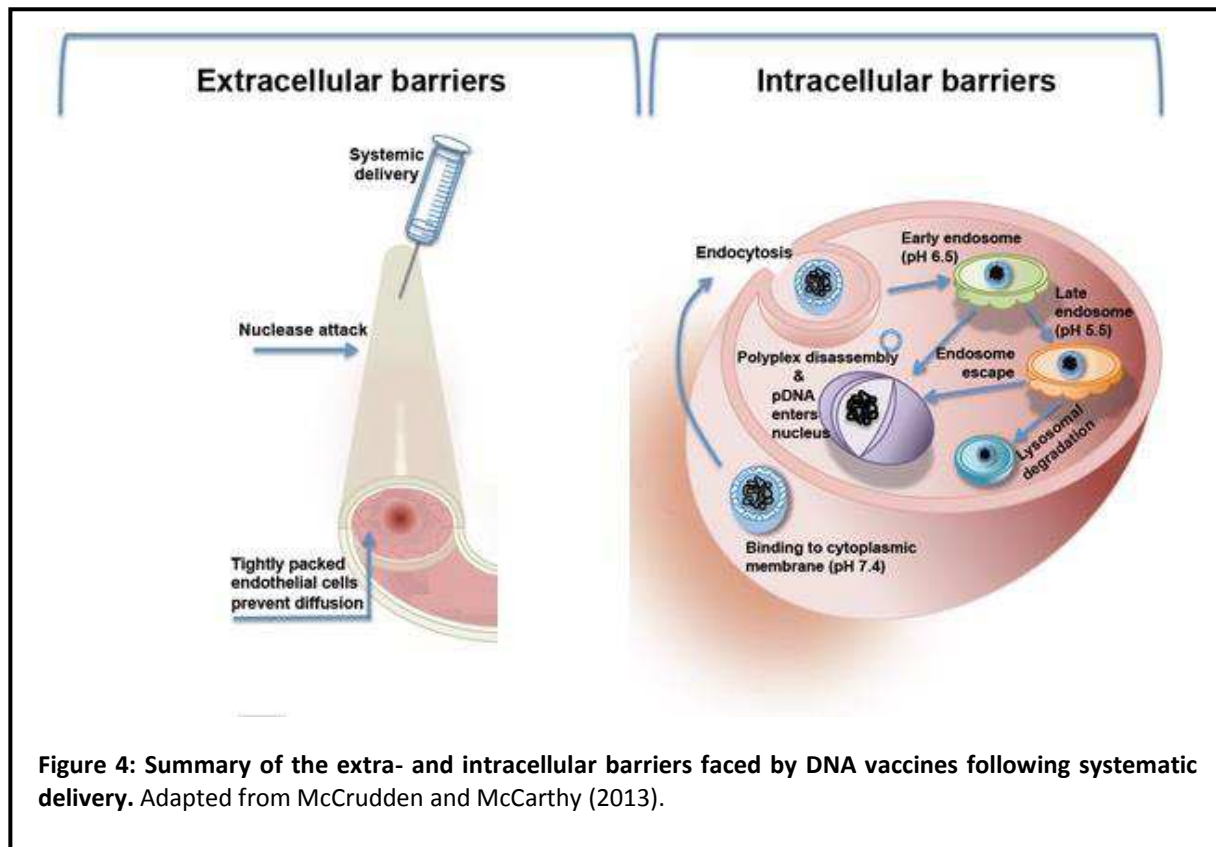
1.4.2 Fate of plasmid DNA after administration

After intramuscular or intradermal injection, the plasmid DNA will transfect somatic cells, such as myocytes and keratinocytes, and/or resident Antigen Presenting Cells (APCs) like dendritic cells (DCs) and macrophages located in the lamina propria (Kutzler and Weiner, 2008; Liu, 2011). DNA enters the cell through two main mechanisms. One is known as fluid-phase endocytosis (not mediated by a receptor), which involves the ingestion of small molecules and/or fluids surrounding the cell (pinocytosis) (Levy et al., 1996; Budker et al., 2000; Varkouhi et al. 2011). Another is known as adsorptive endocytosis (not mediated by a receptor as well) in which the DNA is taken in by a cell by splitting off small vesicles from the cell surface (Budker et al., 2000; Faurez et al., 2010; Varkouhi et al. 2011). The plasmid sequence can also be recognized

simultaneously by different receptors located at the plasma membrane (PM) from eukaryotic cells (Lehmann and Sczakiel., 2005).

Once inside the cell, DNA faces another obstacle: it needs to migrate to the cell cytoplasm. The endocytic vesicles containing the plasmid vaccine fuses with the lysosome, whose function is to digest molecules originally incorporated into the endosome through the activity of many hydrolytic enzymes (Varkouhi et al., 2011). It is still not well understood how the DNA escape from the phagolysosome and reaches the cell cytoplasm (Faurez et al., 2010). Continuously, once within the cytoplasm, vectors that survive from the endonucleases "attack" will reach the cell nucleus and, finally, initiate transcription of the gene of interest. Vacik and colleagues demonstrated that DNA located in the cytoplasm binds to both microtubules and microfilaments that form the cytoskeleton of cells through adapter proteins named dynein, and thus reach the cell nucleus (Vacik et al., 1999). It has been also suggested that DNA molecules located in the cytoplasm can create associations with polypeptides, such as transcription factors, that contains a nuclear localization signal (NLS) required to enter the nucleus (Hebert, 2003).

The third and last obstacle blocking the expression of the antigenic protein is the nuclear membrane. Some studies demonstrated that the plasmid vaccine is able to cross the nuclear membrane by two different ways: (i) passage of the DNA by diffusion through nuclear pore complexes, and (ii) during mitosis, when nuclear envelope is disassembly in dividing cells (Faurez et al, 2010). The DNA located in the nucleus can have access to the transcription machinery turning possible the transcription of the gene of interest. Later, the RNA transcript can be translated into protein in the cytoplasm of the cell (Faurez et al, 2010; Liu, 2011). The host cell provides necessary post-translational modifications mimicking a real infection, this feature being one of the biggest advantage of the genetic immunization (Kutzler and Weiner, 2008; Liu, 2011). Figure 4 illustrates both intra and extracellular barriers that the DNA vaccine needs to face before reaching the cell nucleus.



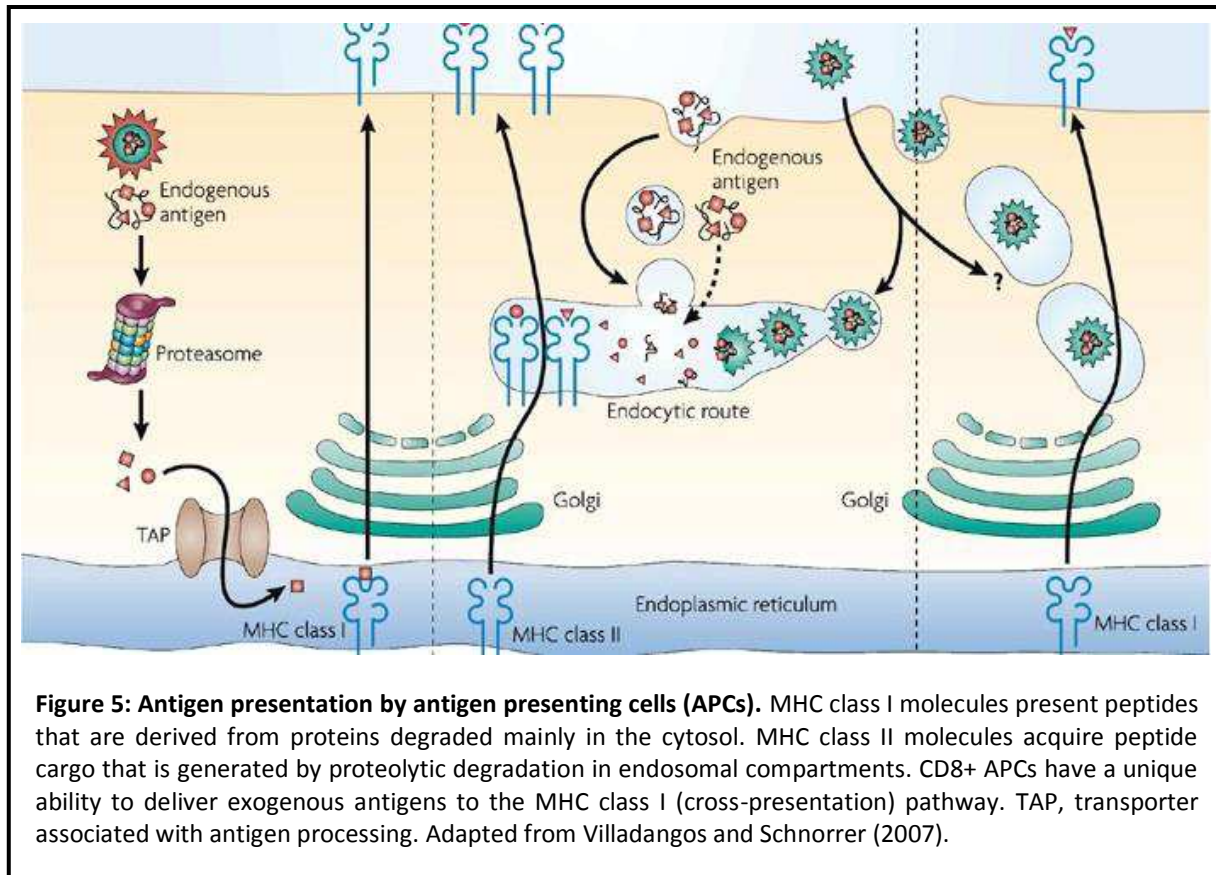
1.4.3 Antigen presentation

The idealized model by which host cells express the antigen, present them to naïve T cells and generate specific immunity is presented in figure 5 and 6. The host-synthesized antigens located in APCs are processed by the proteasome; the resulting peptides are translocated into the endoplasmic reticulum (ER) and rapidly gain access to the MHC class I molecules (Ackerman et al., 2003; Srivastava and Liu, 2003; Liu 2010). Antigen-loaded APCs can, subsequently, travel to the draining lymph nodes where they ‘present’ antigenic peptide–MHC I complexes in combination with signaling by co-stimulatory molecules to naïve T cells. The presentation of peptides derived from endogenous proteins (synthesized in the cell) through MHC class I molecules induces the activation of cytotoxic CD8+T cell responses (CTL responses), which function is to eradicate cells harboring intracellular infections (Neefjes and Sadaka, 2012). Considering that all nucleated cells express MHC I molecules, myocytes and keratinocytes can also expose antigenic peptide–MHC I complexes, however, they cannot migrate to draining lymph and present them to naïve T cells. Only macrophages and DCs can stimulate adaptive immune responses by presenting antigens to naïve T cells located in the draining lymph nodes.

Even though they are not capable of presenting peptides, myocytes and keratinocytes can secrete antigens to the extracellular environment. Secreted proteins can be endocytosed by

several types of APCs and ultimately presented via MHC class II molecules as they are considered extracellular proteins and therefore are processed by the endocytic or exogenous pathway (Ackerman et al., 2003; Kutzler and Weiner, 2008; Joffre et al., 2012). APCs containing antigenic peptide–MHC II complexes can travel to the draining lymph nodes and ‘present’ them in a combination with co-stimulatory molecules to naïve T cells, stimulating the generation of CD4+ T helper cells (Th cells). The effector mechanism of these lymphocytes is to eliminate extracellular antigens (detailed later, topic 1.4.3 of this manuscript) (Donnelly et al., 2000).

It is important to mention that exogenous antigens located in apoptotic or necrotic transfected cells can be presented through another way called cross-presentation (Ackerman et al., 2003; Kutzler and Weiner, 2008; Joffre et al., 2012; Neefjes and Sadaka, 2012). Some subtypes of dendritic cells have developed the ability to efficiently present peptides derived from exogenous antigens on MHC class I molecules. It was suggested that internalized protein somehow gain access to the cytosol, where they are degraded by the proteasome. Proteasome-generated peptides can then reach the classical MHC class I-mediated antigen presentation pathway, which involves the transport of peptides into the endoplasmic reticulum. This via is called cytosolic pathway (Joffre et al., 2012). On the other hand, through the vacuolar pathway, antigens do not need to escape from the phagosome to the cytoplasm. Some works demonstrated that early phagosomes resemble the endoplasmic reticulum in composition, before being fused with lysosomes. Because of this, some components localized in the endoplasmic reticulum interacts with the early phagosome recruiting transporters associated with antigen processing. Therefore, exogenous antigens are degraded into peptides in the phagosome, where they are then loaded on MHC class I molecules (for a review see Joffre et al., 2012; Neefjes and Sadaka, 2012 and Dresch et al., 2012).

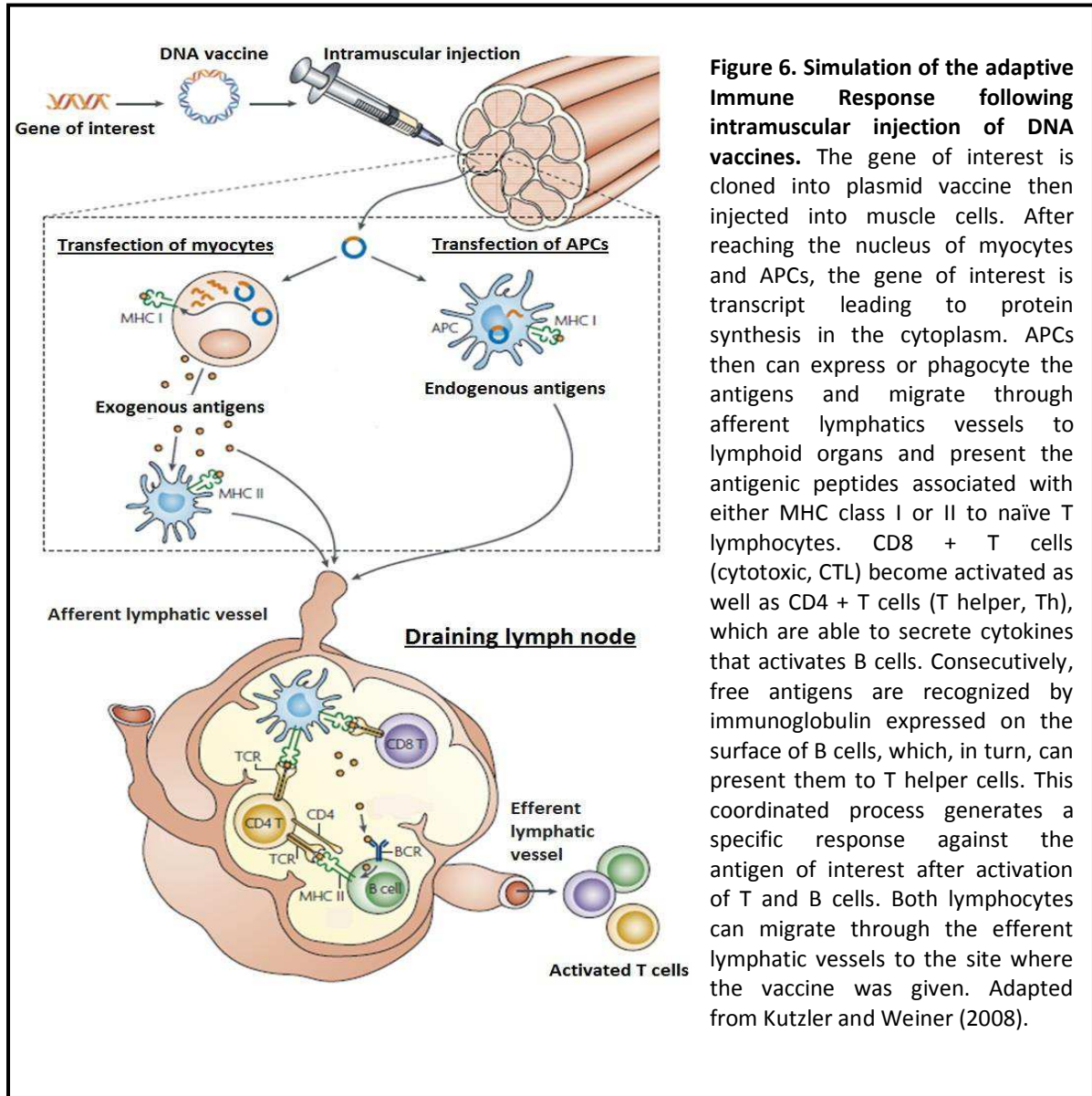


1.4.4 Adaptive immune response: Cellular and Humoral Immunity

The mammalian immune system comprises of innate and adaptive branches that mount integrated protective responses against intruding foreign antigens. The innate immune system includes DCs, macrophages, granulocytes, and natural killer (NK) cells that mediate fast but nonspecific responses after recognizing generic microbial structures. In contrast, the adaptive immune system includes T and B cells that mediate specific but temporally delayed responses after recognizing antigenic epitopes (Cerutti et al., 2012). Therefore, adaptive immune responses depend on antigen activation of B and T lymphocytes into antibody-producing plasma (humoral immunity) and T effector (cellular immunity) cells, respectively (Malek and Castro, 2010). Antigens administered in the form of DNA can stimulate both humoral and cellular immunity as they have been shown to be protective against viral, bacterial, and tumor challenge (Howarth and Elliott, 2004).

In order to stimulate both types of adaptive immune responses, APCs firstly present antigens through different pathways, as described in the previous section, activating CD8+ T cells (cytotoxic lymphocytes, CTLs) and CD4+ T helper cells (T helper lymphocytes). Inside the draining lymph nodes, B cells in contact with protein antigens, are stimulated by certain cytokines, such as IL-21, IL-4, and IL-10, released mainly by CD4+ T cells (T-dependent B cell activation) (Cerutti et al., 2012).

The contact with non-protein antigens can stimulate B cells as well in the absence of T cells (T-independent B cell activation). Afterwards, activated T (CD8+ or CD4+) and B cells are able to migrate to the site where the vaccine was given and exert their effector functions (Kutzler and Weiner, 2008). Figure 6 contains a summary of the steps by which DNA vaccines can induce the activation of T and B lymphocytes after intramuscular injection.



While the role of CTLs is principally to kill infected cells, T helper cells perform their key roles in controlling humoral, CTL-mediated and inflammatory immune responses through the release of various cytokines (Sandberg and Glas, 2001; Howarth and Elliott, 2004). CD4+ T cells may differentiate into subsets of effector cells that produce different sets of cytokines, which are

responsible for their distinct functions. In the injection site, activated T helper cells can be differentiated in the presence of interleukine-12 (IL-12) and IL-18 (both produced by local DCs) into type 1 T helper cells (Th1) that secrete IL-2, IL-12, IFN- γ and lymphotoxin (LT). On the other hand, in the presence of IL-4 (expressed by DCs) these cells are differentiated into type 2 helper T cells (Th2) that secrete mainly IL-4, IL-5 and IL-10. This initial polarization of the response is self-perpetuating as Th1 cytokines enhance further Th1 responses and down-regulate Th2 cytokines, and vice versa. It is still not clear how the initial polarization towards either Th1 or Th2 is controlled, but it is thought to involve a number of factors including the route of immunization and the type of APC that presents the antigen (DCs, macrophages or B cells), the pattern of cytokines released by cells from the innate immune system, and the antigen epitope/density/affinity (Abbas et al., 1996; Mosmann and Sad, 1996). Through the release of cytokines, Th1 cells can activate "infected" macrophages expressing antigens to produce, for instance, reactive oxygen intermediates and induce inflammation, favoring pathogen elimination. Differently, Th2 cells express cytokines that activates for example eosinophils, cells associated with allergy and asthma and responsible for combating multicellular parasites (Mosmann and Sad, 1996).

Different vectors and delivery modes of plasmid DNA were also found to stimulate different T helper responses. Thus, there are increased efforts to develop specific vectors to promote the type of T cell response (Th1 or Th2) for diverse applications, such as for prevention of infectious diseases or asthma and diabetes (Liu, 2010; Liu, 2011). Naked DNA vaccines have been shown to induce either Th1 or Th2 responses in experimental animals, depending on the method of administration. When delivered by intramuscular injection, DNA vaccine tends to induce Th1 responses. It was proved that CpG motifs in the bacterial DNA plasmids induce a local inflammatory response, leading to the accumulation of pro-inflammatory cytokines, such as IL-12, at the vaccination site.

B cells also confer immune protection after DNA vaccination by producing antibody molecules, also known as immunoglobulins (Igs), which can recognize antigen through either low or high affinity binding modes. After antigen recognition events in the lymph nodes (figure 6), B cells are activated resulting in the proliferation of antigen-specific B cells (a process known as clonal expansion). Depending on the stimuli, subsets of mature B lymphocytes can express different surface Ig isotypes (i.e IgG1, IgG2a, IgE or IgA), migrate to the local site where the vaccine was given and become immunoglobulin-secreting plasma cells (plasmocytes), capable of secreting Ig (Mosmann and Sad, 1996; Kaplan et al., 2001). Antibody isotype is governed by the cytokine environment and Th1-Th2 balance. For example, it was demonstrated that IL-4 favors the production of IgG1, while IFN- γ favors the production of IgG2a (Nagabhushanam and Cheers,

2001; Kaplan et al., 2001). Antibodies can exert their effector function by neutralizing toxins, opsonizing or lysing circulating microorganisms, inducing cytotoxicity in cells infected or expressing the antigen (Janeway, 2001).

1.4.5 Immune memory

After the primary immune response, a population of lymphocytes that mediate long-lived immunological memory is produced. These lymphocytes, termed memory cells have, for many years, been considered a dormant population, lacking the effector functions displayed by cells during the acute phase of infection. However, immediately upon antigen contact, they can clone rapidly and restore their effector functions (i.e B cells: secretion of antibodies; CTLs: lytic activity; T helper: secretion of cytokines). The precise changes that distinguish naive, effector, and memory lymphocytes are now a field of intense research. Protective immunity against reinfection is one of the most important consequences of adaptive immunity. This means that secondary exposure to an antigen produces a much more rapid immune response, turning the individual not as badly affected compared to the first time the antigen appeared in the organism. Actually, it has been demonstrated that if the preexisting memory cells are sufficiently numerous, they can contain the infection immediately. A vaccine takes advantage of this effect, giving protection to subsequent exposure to the same antigen (Justewicz and Webster, 1996; Leifert and Whitton, 2000; Janeway, 2001).

Compared to other types of immunization, DNA vaccines offer a unique opportunity to enhance the duration of immune responses through their capacity for prolonged antigen expression, thus favoring the formation of immune memory cells. Some studies have shown that a single intramuscular DNA vaccination, when combined with electroporation (a delivery method, significantly enhanced the onset and duration of the primary antibody response and maintained immune memory (Tsang et al., 2007). Fazio and collaborators demonstrated that a single in utero DNA immunization with a DNA vaccine based on hepatitis B virus (HBV) at two-thirds of pig gestation produced, at birth, antibody titers considered protective in humans. The boost of antibody titers following recall at 4 and 10 months demonstrated the establishment of immune memory, illustrating the relevance of naked DNA-based vaccination aimed to prevent death among first week infants (Fazio et al., 2004). Another similar work was performed by Rigato and co-workers in which they shown that the intravenous injection led to in utero immunization. A DNA vaccine encoding LAMP-1 with Gag and other Human Immunodeficiency Virus type 1 (HIV-1) antigens administrated in BALB/c mice before conception or during pregnancy induced a long-lasting memory response (Rigato et al., 2012). In order to stimulate the development of immune memory cells, immunization of DNA

along with adjuvants molecules are being performed, as recently described. It was observed that the use of Hemokinin-1 (HK-1), a factor that activates B cells for proliferation, as an adjuvant molecule enhanced the immunogenicity of HBsAg DNA vaccines, resulting in stronger humoral and memory responses against hepatitis B infection (Chen et al., 2012).

1.5 Preclinical and clinical progress of DNA vaccines

To date, there have been several preclinical and clinical studies on DNA vaccines. Although US FDA still did not approved DNA vaccines for use 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 (Klinman et al., 2010). In addition to the initial demonstration of the efficacy of DNA vaccines to protect against infectious challenge in a mouse model of influenza virus, DNA vaccines have been shown to protect against influenza in ferrets, and primates; lymphocytic choriomeningitis virus; herpes simplex virus in guinea pigs and mice; rabies virus; cottontail rabbit papillomavirus; hepatitis B virus; malaria (*Plasmodium falciparum*); HIV in nonhuman primates; and against other several bacterial pathogens (Srivastava and Liu, 2003).

What is remarkable is that in the past 6 years, four DNA vaccines for larger animals have been licensed for use in the veterinary field. Two of them target infectious diseases such as West Nile virus in horses, authorized for use in the United States (US); and aquatic rhabdovirus (also termed as infectious hematopoietic necrosis, IHNV) in salmon, approved for use in Canada. Another one is a cancer vaccine for melanoma in dogs; and the last one has a therapeutic purpose in swine in which a plasmid encoded the growth hormone, when delivered before specific vaccination, demonstrated enhanced protection against *Mycoplasma hyopneumoniae*; both of them are authorized for use in the US and Australia. Although these animal disease models are not completely similar to humans, past success with DNA vaccines turns this vaccine platform very promising for use in humans (Faurez et al., 2010; Ingolotti et al., 2010; Findik and Çiftci, 2012) (Table 1).

Vaccine target	Product name	Company involved	Date licensed and country	Target organisms	Benefits
West Nile virus	West Nile Innovator	Centers for Disease Control and Prevention and Fort Dodge Laboratories	2005 USA	Horses	Protects against West Nile virus infection
Infectious haematopoietic necrosis virus	Apex-IHN	Novartis	2005 Canada	Salmon	Improves animal welfare, increase food quality and quantity
Growth hormone releasing hormone	LifeTide-SW5	VGX Animal Health	2007 Australia	Swine and food animals*	Increases the number of piglets weaned in breeding sows; significantly decreases perinatal mortality and morbidity
Melanoma	Canine Melanoma Vaccine	Merial, Memorial Sloan-Kettering Cancer Center and The Animal Medical Center of New York	2007 USA, conditional license	Dogs	Treats aggressive forms of cancer of the mouth, nail bed, foot pad or other areas as an alternative to radiation and surgery

*Refers to agricultural animals as opposed to pet animals — a higher level of stringency is required for approval for animals that are part of the food chain.

Table 1. Current licensed DNA therapies. Adapted from Kutzler and Weiner (2008).

Even though DNA immunization have evolved greatly over the last 20 years, the Achilles heel of DNA vaccines proved to be their poor immunogenicity in humans and other large animals, instead of the initial safety concerns. To date, the potency of the immune responses has been disappointing in humans; nevertheless, humoral and cellular (T helper and cytolytic T cell) responses were obtained (Srivastava and Liu, 2003; Bolhassani et al., 2011). Clearly regimens, plasmid dose, timing of doses, adjuvants and routes of vaccination have been considered as well, and wide variety of strategies will be developed to optimize DNA vaccine immunogenicity.

1.6 Improvement of DNA vaccines immunogenicity

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 (Ferraro et al., 2011). Nonetheless, the reasons for the failure of DNA vaccines to induce potent immune responses in humans have not been elucidated (Bolhassani et al., 2011). Therefore, research has focused on developing novel strategies to enhance transfection efficiency and improve other facets of the DNA vaccination platform using several strategies (Kutzler and Weiner, 2008; Ferraro et al., 2011).

Reasonably, vector modifications that improve antigen expression are highly correlative with improved immune responses. It has been shown that the use of altered transcriptional elements, like (i) the modified CMV promoters (chimeric SV40-CMV promoters), (ii) mRNA containing introns, (iii) gene of interest codon optimized to match with the target organism, (iv) the use of a dual stop codon to limit read through translation, and (v) transcription terminators/polyadenylation signals can significantly improve either antigen transcription or translation by the host cell (Kutzler and Weiner, 2008; Williams et al., 2009). Optimization of the initiation start site for protein synthesis (kozak consensus sequences) is also desirable as endogenous sites of viruses and bacteria might not be optimal for expression in mammalian cells (Kutzler and Weiner, 2008). The addition of leader sequences can also enhance the stability of mRNA and contribute to translational efficiency. Considering the commercialization of DNA vaccines, the use of high-efficiency origins of bacterial replication can also markedly improve the quantity of plasmid product (Williams et al., 2009).

Of course the right choice of antigens is crucial for obtaining a satisfactory immune response as well as the use of unmethylated CpG, which should be included in the plasmid backbone in order to enhance the potency of a DNA vaccine (Glenting and Wessels, 2005). Another strategy attempt to increase the magnitude of immune responses by co-expression of cytokines, chemokines or co-stimulatory molecules, that can have a substantial effect on the immune response (Liu, 2003; Kutzler and Weiner, 2008; Ferraro et al., 2011). Dam and dcm methylation performed by bacteria should also be taken into account as this may affect antigen

of sustained expression of the introduced genes in the target tissue as the DNA is superficially penetrated into the tissue (Niidome and Huang, 2002). Therefore, multiple administrations are required in many cases (Yang et al., 2001). More than just a fashion statement, tattoos can be used to introduce DNA vaccines in the epidermal and dermal layers. DNA plasmids delivered by tattooing have been shown to induce higher specific humoral and cellular immune responses compared to intramuscularly injection of naked DNA. However, this is a very cost-effective method that may be used when more rapid and more robust immune responses are required (Pokorna et al., 2008; Bolhassani et al., 2011).

Ultrasound (US) can be used to transiently disrupt cell membranes to enable the incorporation of DNA and this technology has been applied in clinical trials.

An alternative physical delivery system is a device that generates an electric current, *in vivo* electroporation, to create temporary holes in the cellular membrane (Srivastava and Liu, 2003). That was initially studied to enhance the efficacy of chemotherapy agents (Ferraro et al., 2011). Potent immune responses against hepatitis B surface antigen and HIV gag protein were obtained by electroporation of muscle after intramuscular injection of naked plasmid DNA (Niidome and Huang, 2002). However, the concerns about the chromosomal integration observed in a safety study of electroporation mean that this technology needs careful evaluation for humans (Liu, 2011).

In vitro studies have shown that laser beam can deliver a certain amount of energy onto a target cell, modifying permeability through a local thermal effect, thus facilitating DNA incorporation by the cell. This novel technology is currently being explored (Bolhassani et al., 2011). Another new method is the dermal patch delivery (DermaVir). It is a self-adhesive patch coated with multiple antigen or adjuvant encoding plasmids and a synthetic polymer that forms pathogen-like nanoparticles (Ferraro et al., 2011).

It is worth highlighting that the method of delivering a DNA vaccine can drastically influence the type of immune response induced by the vaccine. For instance, gene gun typically induces Th2 reactions whereas needle inoculation triggers a Th1 response (Bolhassani et al., 2011). Moreover, whilst each of these methods has contributed to incremental improvements in DNA vaccine efficacy, more is still needed for human DNA vaccines success commercially (Bolhassani et al., 2011; Liu et al., 2012). Nevertheless, these various delivery technologies merit continued development and evaluation (Liu, 2011).

1.7.2 Chemical vectors

Chemical vectors that are mainly based on engineered DNA nanoparticles produced with various range of macromolecules (Niidome and Huang, 2002). Generally, the major advantages

are (i) they can tightly compact and protect DNA, (ii) be recognized by specific cell-surface receptors expressed in DCs or macrophages, (iii) disrupt the endosomal membrane to deliver DNA plasmids to the nucleus after its destabilization when the pH of the medium is reduced to below 6 (Bolhassani et al., 2011). Usually, non-viral vectors include mainly DNA-liposome complexes and DNA-polymer complexes. Nowadays DNA-peptide complexes are also being used as DNA carriers. It has been shown that they are able to condense plasmid DNA, resulting in a highly stable complex with potent transfection activity *in vitro* (Niidome and Huang, 2002). These vectors are currently being evaluated in clinical trials (Zhang et al., 2012).

So far, lipid and polymer complexes are the most studied and used chemical vectors. Negatively charged, DNA neutralizes both cationic liposomes and polymers leading to the formation of electrostatic complexes with plasmid DNA. This condensation provides size reduction and protection against nuclease degradation (Pichon et al., 2010; Bolhassani et al., 2011). Moreover, the encapsulation of plasmid DNA into micro or nanospheres has proved to protect plasmids from the environment and to target the genetic material to a specific cell type. For example, it has been shown that some liposomes and polymers can effectively aid in directing antigens to APCs by efficiently trafficking through local lymphoid tissue (Lu et al., 2009). Liposome-based gene delivery comprises a large number of cationic lipids, such as quaternary ammonium detergents and lipid derivatives of polyamines. Even though being capable of accommodating large size plasmids and immunostimulatory agents, it was observed that the use of liposomes sometimes leads to a transient expression of the transgene (Fenske and Cullis, 2008). Polymers are also being explored as delivery vectors (Niidome and Huang, 2002). A very interesting work was performed by Roy and collaborators in 1999. Mice that received nanoparticles containing a dominant peanut allergen gene (pCMVArah2) produced secretory IgA and serum IgG2a. Compared with non-immunized mice or mice treated with 'naked' DNA, mice immunized with nanoparticles showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage (Roy et al., 1999). Nevertheless, the use of both liposomes and polymers has been shown to present some limitations as they exhibited great toxicity *in vivo* (Fenske and Cullis, 2008; Bolhassani et al., 2011). Consequently, nanoparticle-based vaccines require the development of increasingly safer formulations (Xiang et al., 2010).

1.8 Biological delivery systems for DNA vaccines

1.8.1 Virus as DNA delivery vehicles

To date, live vectors, such as attenuated or non-pathogenic virus, have demonstrated the feasibility of gene therapy/antigen delivery and remain one of the best vehicles to introduce

genes into cells (Pichon et al., 2010). Virus represents ideal nanoparticles due to their regular geometries, well characterized surface properties and nanoscale dimensions (Bolhassani et al., 2011).

The five main classes of viral vector can be categorized in two groups according to whether their genomes integrate into host cellular chromatin (oncoretroviruses and lentiviruses) or persist in the cell nucleus predominantly as extrachromosomal DNA molecule or episome (AAVs, adenoviruses and herpes viruses) (Thomas et al., 2003). The basis for selecting a particular viral vector includes how well the heterologous gene is expressed as the quantity of antigen may affect the immune response. In addition, the type of cells “infected” by the virus vector that will produce the antigen, may affect the nature and potency of immune response. Another factor influencing the choice of vector is the persistence of the virus, which might be useful for prolonging immunity (Liu, 2011). Figure 8 illustrates how a viral particle can transfect a mammalian cell and deliver a gene of interest.

Oncoretrovirus vectors were the first class of viral vector to be developed and have, so far, been the most widely used in clinical trials. The inconvenient is that they may cause genetic diseases or favor the development of cancerous cells because they can integrate their genetic material into host cellular genome (Thomas et al., 2003). Recombinant adenoviruses (Ads) are also being extensively used for gene therapy because they are extremely efficient at delivering DNA to target cells and have low potential for oncogenesis (Bolhassani et al., 2011; Liu, 2011). Nonetheless, a severe fatal adverse effect have occurred during a gene therapy trial raising serious safety concerns about the use of viral vectors (Pichon et al., 2010). In 1999 administration of a recombinant adenoviral vector generated adverse reactions in a patient causing his death, and in 2000 another study found that administration of retroviral vector induced leukemia in 2 of 11 patients who received the treatment (Check, 2002; Check, 2003). Besides these two tragic events, viral vectors have several manufacturing issues as they are occasionally developed from pathogenic species. Thus, the scientific community has boosted efforts to pursue an ideal non-virus live vector to improve the effects of DNA vaccination (Kano et al., 2007; Pichon et al., 2010).

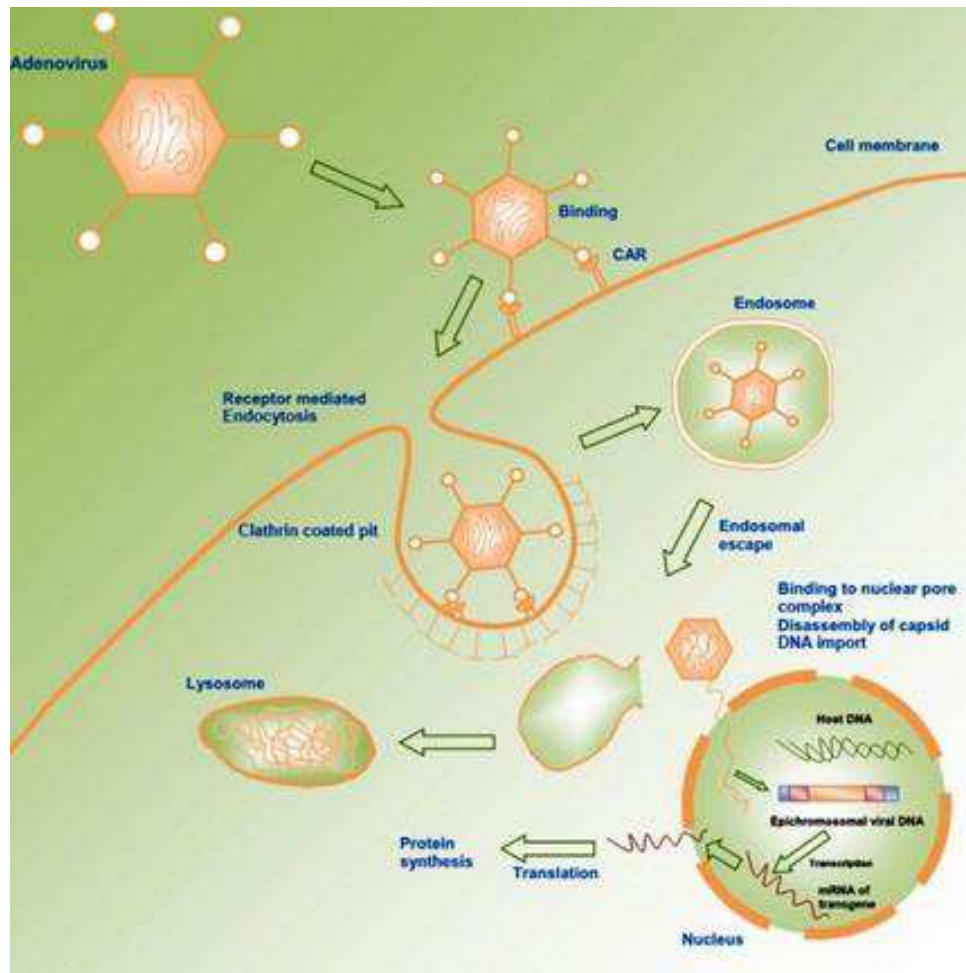


Figure 8: Transduction using adenoviral vectors. Recombinant adenovirus enters cells via CAR-mediated binding allowing internalization via receptor-mediated endocytosis through clathrin-coated vesicles. Inside the cytoplasm, the endocytosed adenoviral vector escapes from the endosomes, disassembles the capsid and the viral DNA enter into the nucleus through the nuclear envelope pore complex. The viral DNA is not incorporated into the host cell genome, but rather assumes an epichromosomal location, where it can still use the transcriptional and translational machinery of the host cell to synthesize the gene of interest. Adapted from Pankajakshan and Agrawal (2013).

1.8.2 Bacteria-based vectors

Studies conducted in 1980 by Walter Schaffner demonstrated that the bacteria are capable of transferring genetic material into mammalian cells *in vitro*. For this reason they have been proposed as new vectors for plasmid vaccines transfer (Courvalin et al. 1995; Sizemore et al. 1995; Vassaux et al. 2006). Later, it was also demonstrated that Gram-positive bacteria, such as *Listeria monocytogenes* was able to deliver plasmid DNA (Becker et al., 2008). Ever since, attenuated or artificially engineered to be invasive bacteria have been tested as a vehicle for transgene delivery (Seow and Wood, 2009).

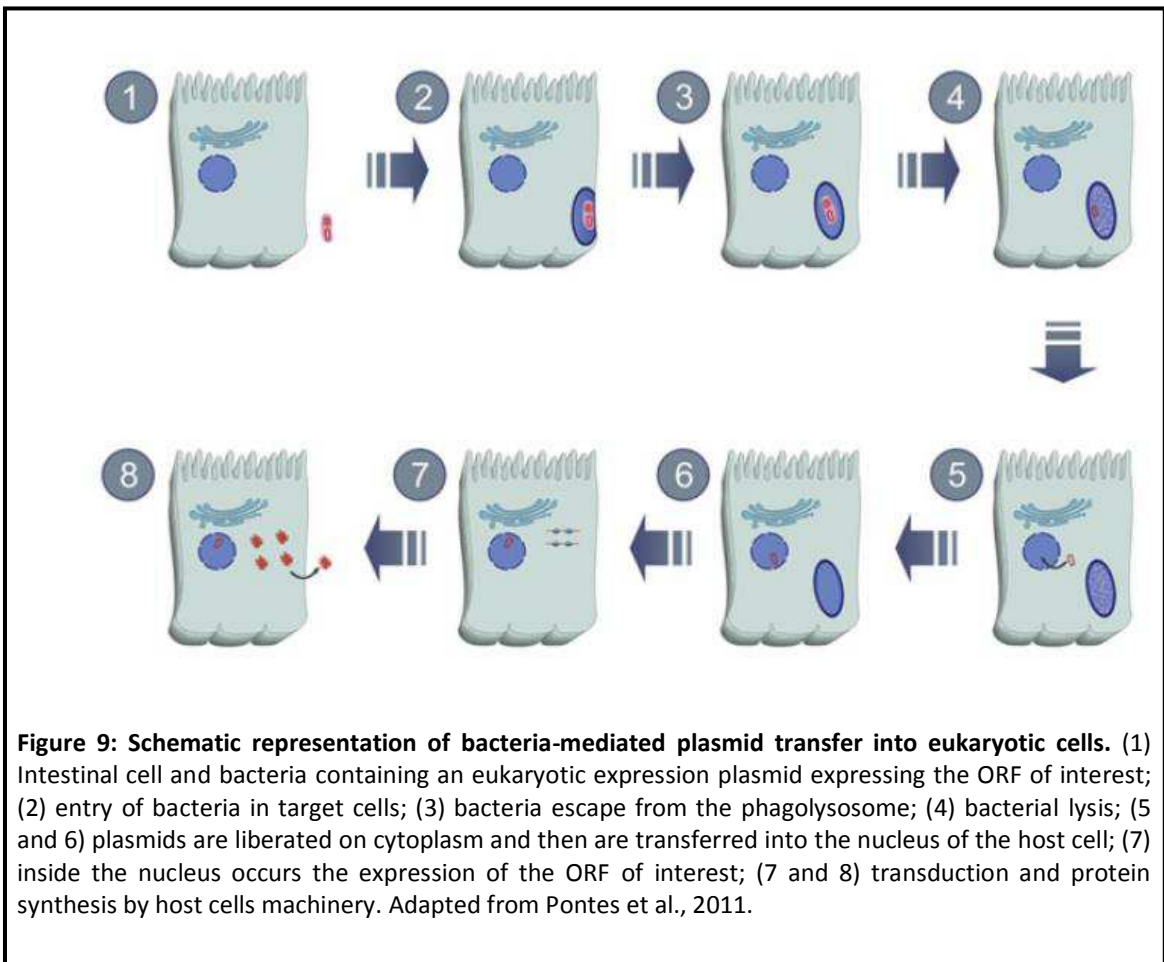
2. BACTERIAL VECTORS AS DNA DELIVERY VEHICLES

The use of bacterial systems for gene therapy/antigen delivery (also known as bactofection), is especially interesting as some vectors based on pathogenic strains has an innate tropism for specific tissues of the host which are indispensable for the development of an immune response (Pilgrim et al. 2003). One attractive feature of bacterial vectors is their potential for oral administration, which may stimulate both mucosal and systemic immune responses (Izadpanah et al. 2001; Srivastava and Liu, 2003). Oral and nasal mucosa represents the first line of defense against many pathogenic microorganisms (Seow and Wood, 2009). Mucosal immunization can lead to the proliferation of T helper cells that stimulates the secretion of IgA antibodies in the epithelium. Secretory IgA (SIgA) represents the most abundant immunoglobulin in our body which plays a critical role in host defense as it is capable of neutralizing pathogens or toxins that are in direct contact with the mucosal surfaces (Lamm, 1997; Macpherson et al. 2001). Furthermore, WHO recommends the use of mucosal vaccines because of economic, logistic and security reasons (Neutra and Kozlowski 2006; Wells, 2011). Furthermore, bacterial carrier can specifically target and transfer main APCs, such as DCs. After bacterial uptake by DCs, they can act as adjuvants due to the presence of some molecules unique to bacterial cell walls known as microbe-associated molecular patterns (MAMPs) (Becker et al., 2008). The recognition of MAMPs by the host can modulate innate immune responses, promoting, as a consequence, an efficient and adaptive long-lasting immune response (Schoen et al., 2004). Another advantage consists on the fact that bacteria are able to accommodate large-size plasmid, which allows the insertion of multiple genes of interest, protecting the DNA plasmids against endonuclease degradation (Hoebe et al., 2004; Seow and Wood, 2009). Finally, bacteria-based vectors are considered low cost since plasmids can be amplified by the simple growth of bacterial cultures turning purification steps unnecessary (Hoebe et al. 2004).

2.1 Basic Principles for Bacteria-Mediated DNA delivery at mucosal surfaces

Vectors based on attenuated pathogenic species can actively invade the mucosal epithelium and translocate across the epithelial barrier turning possible the expression of the transgene. Actually, it has been reported that some vectors, such as *Agrobacterium tumefaciens*, are able to transfer expression cassettes even without invading the target cells (Weiss and Chakraborty, 2001). Once inside the cells, bacterial vectors are able to escape from phagolysosome vesicles by the secretion of a variety of phospholipases and pore-forming cytolysins and enter into the cytoplasm of host cells. A part of bacteria that are degraded inside the phagolysosome release the plasmid vaccine, which can reach the cytoplasm through the pores made by the phospholipases and pore-forming cytolysins (Hoebe et al., 2004; Schoen et al., 2004).

Carrier bacteria are lysed inside phagocytic vacuole as the result of either metabolic attenuation (auxotrophy), an inducible autolytic mechanism or simply from treatment with antibiotics (Weiss and Chakraborty, 2001). In the cytoplasm, the transgene product can be secreted via host cell-mediated expression of the genetic cargo released by the bacteria (Seaow and Wood, 2009). Details on how the antigen of interest can be expressed by the mammalian cell are found in section 1.4.2. Figure 9 summarizes the steps of plasmid delivery by using a bacterial vector.



2.1. 1 Intestinal mucosa

The intestinal mucosa has very interesting particularities at the immunological point of view since it evolved to tolerate compounds from commensal flora and to recognize antigens from pathogenic species and induce long-lasting immune responses against them (Macpherson and Harris, 2004). Organized lymphoid tissues called GALT, the largest immune organ in the human body, can be found associated with the intestinal mucosal (Neish, 2009). It comprises the Peyer's patches in the small intestine and aggregates of lymphoid follicles in the lamina propria scattered throughout the intestine. Peyer's patches are composed by aggregated lymphoid follicles surrounded by a particular epithelium, the follicle-associated epithelium (FAE) that forms

the interface between the GALT and the luminal microenvironment (Perdigón et al. 2001; Perdigón et al. 2002; Hobson et al. 2003; Jung et al., 2010). Among the pathogenic bacteria with a digestive tropism such as *Escherichia coli*, *Yersinia*, *Mycobacterium avium paratuberculosis*, *Listeria monocytogenes*, *Salmonella typhimurium* and, *Shigella flexneri*, all of them have been reported to invade the host by adhering with FAE M-cells (Jung et al., 2010).

Additionally, special mucosal DCs located in the lamina propria of the small intestine can extend their dendrites between tight junctions (TJ) of epithelial cells and capture antigen directly from the intestinal lumen (Rescigno et al. 2001). The binding between DCs and epithelial cells occurs through the expression of TJ proteins by DCs which can establish TJ-like structures with neighboring epithelial cells. Interestingly, lamina propria DCs can discriminate between pathogenic and nonpathogenic bacteria. In fact, when the intestine is infected with nonpathogenic bacteria, DCs do not receive any migratory signal and stay *in situ*, whereas infection with pathogenic bacteria induces a large migration of DCs from the mucosa to the mesenteric lymph nodes. Thus, DCs play an active role in bacterial handling across mucosal surfaces (Rimoldi et al., 2004). After interacting with antigens, DCs can present them to naïve T cells in the mesenteric lymph nodes, establishing a cross-talk between innate and adaptive immunity. It has been shown that lamina propria DCs stimulated with commensal bacterial antigens can induce T cell-independent IgA class switching and soluble IgA (SigA) production by intestinal B cells (Barbosa and Rescigno, 2010). The IgA is the main class of immunoglobulin found in intestinal secretions. Its role is to protect the intestinal epithelium against enteric pathogens and toxins (Mantis and Forbes, 2010). Recent studies demonstrated that IgA are also implicated in the regulation of gut commensal bacterial communities as it is able to control microbiota expansion while maintaining gut homeostasis (Barbosa and Rescigno, 2010; Suzuki et al., 2010). In conclusion, through a coordinated manner, T and B cells together with epithelial cells are capable of creating a powerful line of defense against infectious agents and exert an important role in regulating immune responses in the intestinal mucosa (Hobson et al. 2003; Izadpanah et al. 2001; Kutzler & Weiner, 2008). The major components of the immune system associated with the gastrointestinal tract are described in Figure 10.

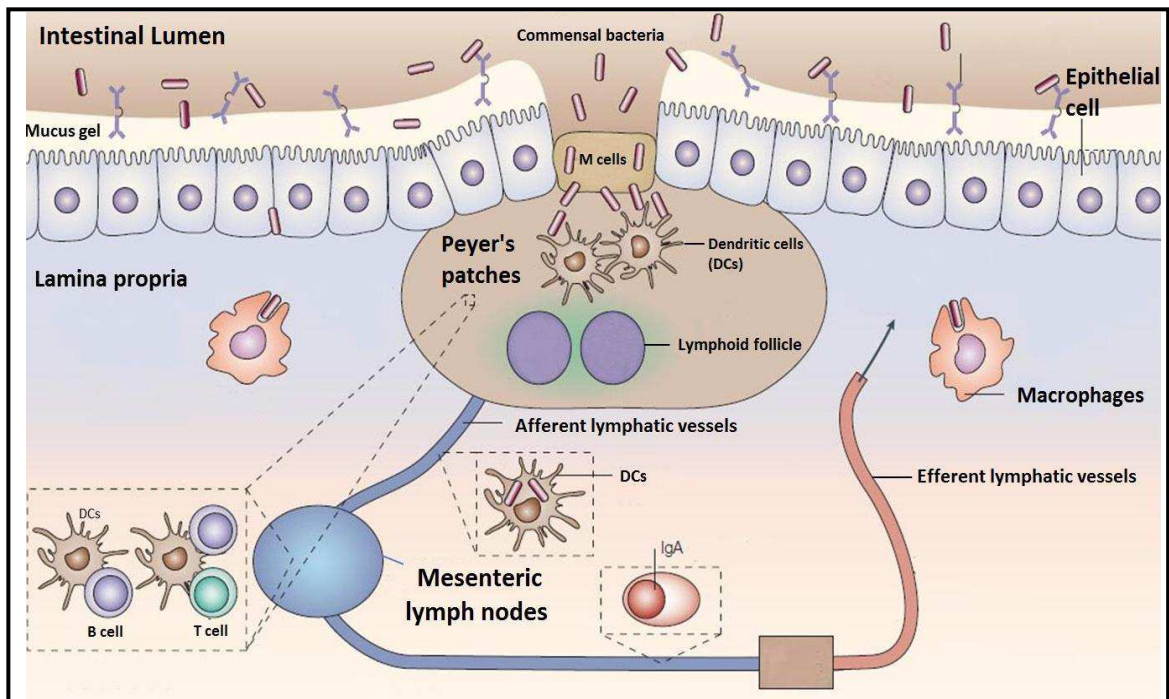
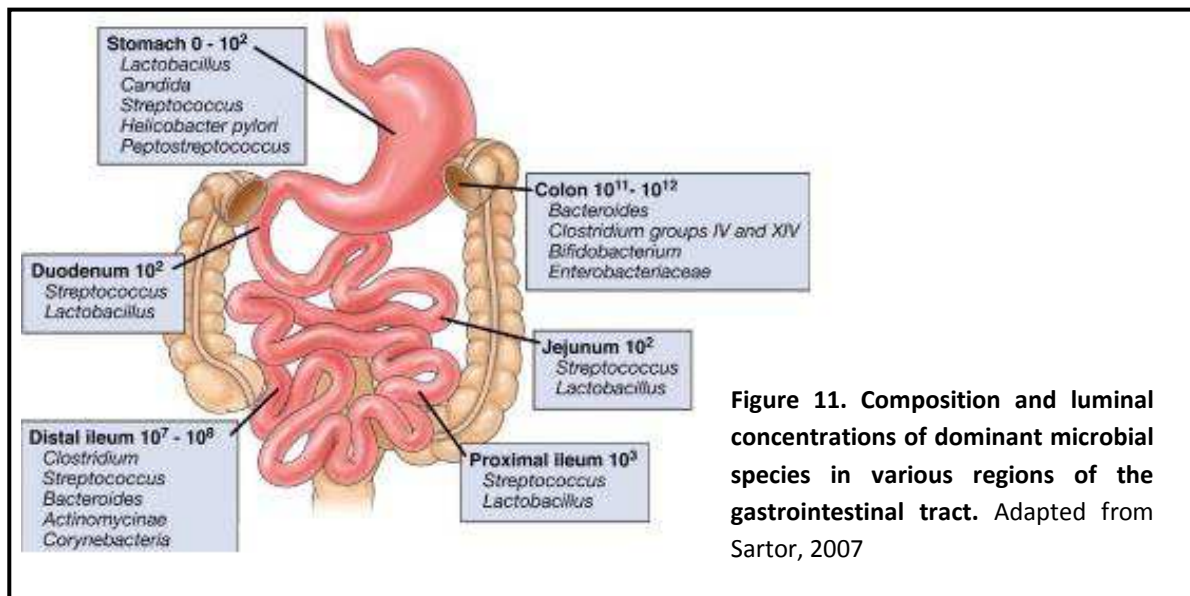


Figure 10. Intestinal epithelium and the gut associated immune system. The IECs form a barrier that prevents biochemical and physical invasion of microorganisms into the body. M cells transports antigens from the intestinal lumen to antigen presenting cells such as DCs, which are located in Peyer's patches. DCs containing antigens travels through the afferent lymphatics vessels to the mesenteric lymph nodes and present them to naïve T cells, activating adaptive immune responses. Activated T cell and B lymphocytes are able to migrate from the lamina propria via the efferent lymphatic vessels. Figure adapted from Macpherson and Harris, 2004.

2.1.2 Commensal bacteria and the intestinal mucosa

Commensals colonize the human gut soon after birth. Bacteria from the birth canal and from maternal milk are the first colonizers and their persistence depends on the ability of strains to interact among them and with the thick mucus layer that lines the intestinal lumen (Barbosa and Rescigno, 2010). The trillions of commensal microorganisms that constitute the intestinal microbiota are primarily composed of five bacterial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. *Bacteroidetes* and *Firmicutes* predominate and represent approximately 90% of the total gut microbiota (Figure 11). Some of these microorganisms can exert many benefits to the human health as they can promote plant polysaccharide digestion and uptake of nutrients by host intestinal cells. Moreover, in addition to nutritional benefits, commensal bacteria may protect their host through antimicrobial activity against pathogenic bacteria and are able to regulate intestinal immune homeostasis (Yan and Polk, 2004; Jung et al., 2010; Kosiewicz et al., 2011).



Commensals are not ignored by the intestinal immune system. Recent evidence shows that they can interact with IECs and deliver tolerogenic signals that are transmitted to the underlying cells of the immune system (Rescigno, 2009). Immunological tolerance against non-pathogenic bacteria and antigens is a phenomenon observed along the gastrointestinal mucosa, which avoids reactions against proteins and commensal bacteria. Oral tolerance is an active process, leading to the generation of antigen-specific T lymphocytes that suppress further immune stimulation. Consequently, mucosal tolerance protects the mucosa from detrimental inflammatory immune responses (Jung et al., 2010). It has been shown that resident DCs constantly traffic between mesenteric lymph nodes and gut mucosa, even in the absence of inflammatory stimuli, and this recirculation is considered important to maintain tolerance to oral antigens and to the microbiota (Iliev et al., 2007). Actually, exposure of immune cells to bacteria has important consequences for gut health: excessive contact promotes exaggerated pro-inflammatory immune responses, whereas limited bacterial or no bacterial exposure, such as in germ-free conditions, can dramatically impair immune development and function (Kelly and Mulder, 2012). A break of this steady state is the major cause of inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD) (Guarner and Malagelada, 2003). Some commensal bacteria may induce intestinal inflammation while others control it. The commensals capable of controlling inflammation in the gut mediate their effect either by balancing the immune response in favor of regulation or by controlling bacteria that may directly mediate intestinal inflammation. In addition to inflammatory diseases, there are other chronic diseases that may be impacted by the gut microbial community. Actually, allergic disease

development, cancer, especially colon cancer, has been associated with alterations in the intestinal microbiota (Kosiewicz et al., 2011).

2.3 Bacterial vectors used for gene transfer

2.3.1 Pathogenic bacterial DNA delivery

Enteropathogenic species are the most widely used bacterial delivery systems (Schoen et al., 2004). The majority of the works describe the use of *Shigella*, *Salmonella* and *Listeria* strains, being these very well-known examples of bacterial vectors. With respect to their localization during the infective process, enteropathogenic bacteria employed as DNA vaccine carriers can be subdivided into extracellular pathogens, such as some *E. coli* strains or *Yersinia* spp., intraphagosomal pathogens like *Salmonella* spp., and intracytosolic pathogens, *Listeria monocytogenes*, *Shigella* spp (Loessner et al. 2007). In all cases, the bacteria used for such proposed vaccines are attenuated strains. Their use is very interesting since they have a natural tropism for dendritic cells and macrophages in the lymphoid tissue of the intestinal mucosal surface, and thus naturally targets these inductive sites allowing the exploitation of this property in the development of mucosal DNA vaccines (Becker et al. 2008). Attenuated pathogenic bacteria as carriers for DNA vaccines has been applied in the prophylaxis and therapy of several infectious diseases (Schoen et al. 2004) and tumors (Yu et al. 2004). Promising results have been obtained in animal models, however, none of these strains were licensed for use in humans.

There are several strategies available to attenuate strains. Most of the vectors used for genetic immunization contains a deletion of an essential gene in bacterial metabolism (auxotrophic mutants), limiting their growth *in vivo*. Other attenuated bacteria are engineered to express a phage lysine upon their entry into eukaryotic cells (Pilgrim et al. 2003; Loessner et al. 2007), turning them unable to perpetuate within the host and thereby cause an infection. There are many enteropathogenic strains currently being used as DNA delivery vehicles and the main examples are described below.

2.3.1.1 Extracellular pathogens

One great example of extracellular pathogenic bacteria that was used as a DNA vaccine vector is *Yersinia enterocolitica*. It is a pleomorphic gram-negative bacillus that belongs to the family Enterobacteriaceae. *Yersinia's* survival is mediated by a virulence plasmid named pYV encoding a series of virulence genes (Novoslavskij et al., 2012). 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 (Al-Mariri et al., 2002).

2.3.1.2 Intraphagosomal pathogens

Salmonella genus stands out among other intraphagosomal pathogens used to deliver vaccine plasmids. *Salmonella typhimurium*, for example, has long served as a model organism for genetic studies and are probably the most widely used bacteria for antigen delivery applications. It is a gram-negative rod-shaped bacillus and approximately 2000 serotypes cause human disease (Ohl and Miller, 2001; Parsa and Pfeifer, 2007). It has been shown that *Salmonella* pathogenicity island 1 (SPI-1) encodes genes necessary for invasion of IECs and induction of intestinal secretory and inflammatory responses. Differently, *Salmonella* pathogenicity island 2 (SPI-2) encodes genes essential for intracellular replication (Ohl and Miller, 2001).

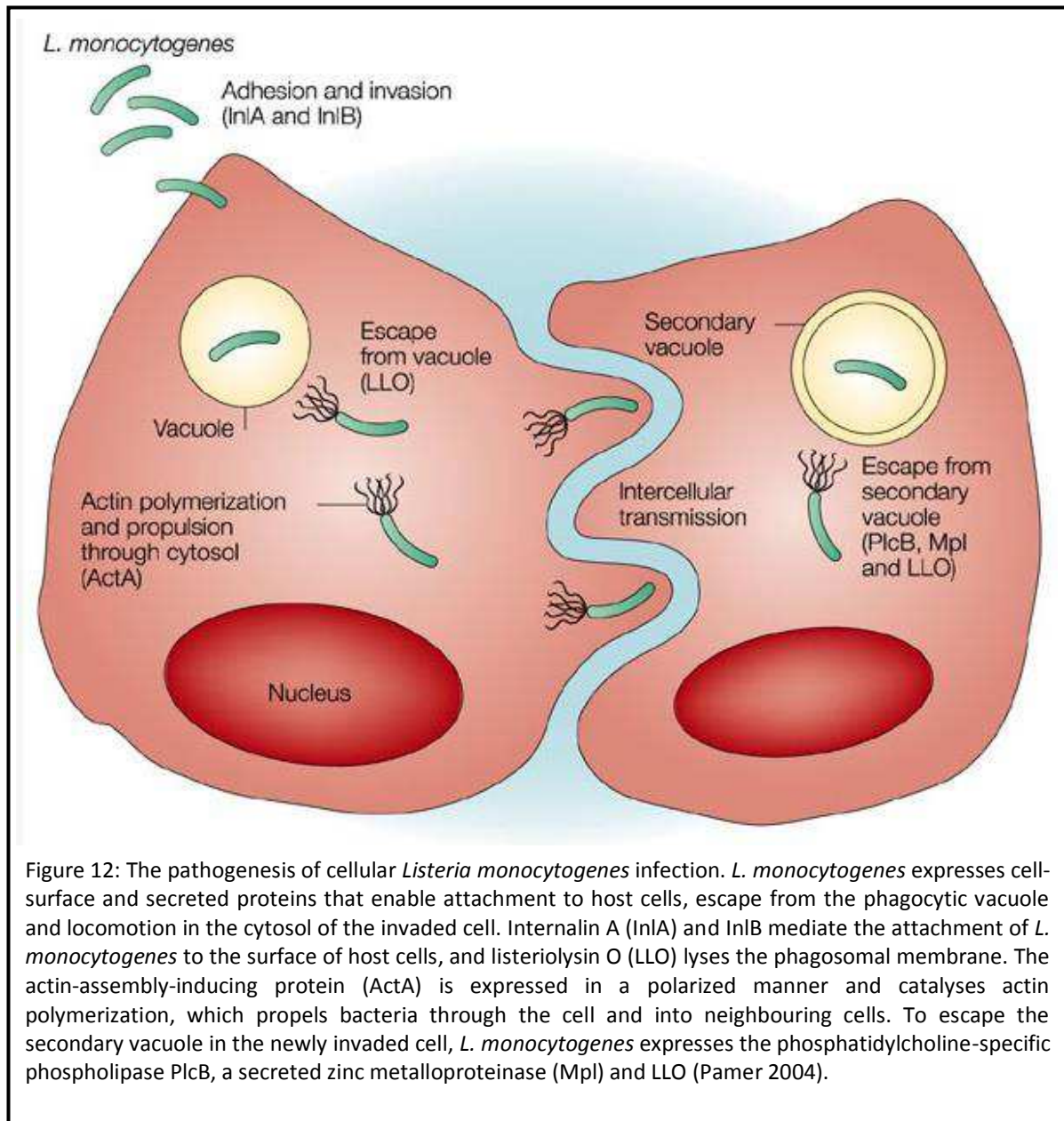
Recently, it was reported that the administration of an attenuated *Salmonella typhimurium* strain delivering a DNA vaccine encoding duck enteritis virus UL24 could induce systemic and mucosal immune responses in ducks, conferring a good protection against challenge (Yu et al., 2012). Another interesting work was performed by Wen and collaborators (2012) where they found that *S. typhi* loaded with an HIV gp140 DNA vaccine were readily taken up *in vitro* by murine macrophage cells, and gp140 antigen was efficiently expressed in these cells. Peripheral and intestinal mucosal anti-gp140 antibody responses in mice vaccinated were significantly higher than those in mice immunized with naked DNA vaccine, demonstrating the bacteria carrying gp140 plasmid could be used as a new strategy for development of HIV vaccines (Wen et al., 2012).

2.3.1.3 Intracytosolic pathogens

Listeria monocytogenes represents another important example of a bacterial vector vastly used for DNA delivery. This gram-positive bacterium is responsible for listeriosis, a severe human infection with an overall 30% mortality rate. Similar to the gram-negative enteropathogenic bacteria *Salmonella*, *Shigella*, and *Yersinia* spp., *L. monocytogenes* has the capacity to induce its own uptake into nonphagocytic mammalian cells (Lecuit et al., 1997). Three tight barriers (the intestinal, blood-brain, and placental barriers) can be crossed by this pathogen (Dussurget et al., 2004) by the expression of two surface proteins, internalin A (or InlA) and InlB, encoded by two genes (inlA and inlB) organized in an operon (Braun et al., 1997; Lecuit et al., 1997). The targets of InlA and InlB on the surface of the host cell are the adhesion molecule E-cadherin and the hepatocyte growth factor (HGF) receptor Met, respectively. Met is ubiquitously expressed, allowing InlB to mediate bacterial internalization in a large number of cell types, whereas InlA shows a more stringent cell tropism, as E-cadherin is only expressed by a limited number of cells

of epithelial origin (Bonazzi et al., 2009). InlA is a sortase anchored, cell wall protein which contains 800 amino acids (aa) in length and composed of seven distinct domains and it is considered one of the major virulence genes in *L. monocytogenes* (Monk et al., 2010). Recently, it was demonstrated that E-cadherin is accessible from the lumen around mucus-expelling goblet cells and around extruding enterocytes at the tip of villi. Thus these cells are preferentially invaded by *L. monocytogenes*, which is rapidly transcytosed across the intestinal epithelium, and released in the lamina propria by exocytosis from where it disseminates systemically (Nikitas et al., 2011). Once inside the cell this bacterium is capable to resist early intravacuolar killing. Phagosomal escape is largely mediated by the cholesterol-dependent cytolysin listeriolysin O (LLO), which is essential for *Listeria* virulence. Rupture of the *Listeria*-containing phagosome is a dynamic multistep process. After internalization, an LLO expression is up-regulated at low pH inside the phagosome. The acidifying phagosome leads to LLO oligomerization of cholesterol-bound monomers into a prepore complex, followed by insertion into the lipid bilayer. Maximizing LLO activity in the vacuole but not in the cytosol is a critical aspect of the intracellular lifestyle of *Listeria* because LLO mutations with increased expression or pore-forming activity destroy the host cell and decrease virulence. Once in the cytosol, *L. monocytogenes* is capable of actin-based motility and cell-to-cell spread without an extracellular phase (Cossart and Toledo-Arana, 2008; Radtke et al., 2011). All this biological properties make *L. monocytogenes* a promising platform for development as a vector for gene therapy, anti-cancer vaccine vector or antigen delivery vehicle. Several preclinical studies have demonstrated the ability of this bacterium for intracellular gene or protein delivery *in vitro* and *in vivo*, and this vector has also displayed a relatively safety and efficacy in clinical trial (Tangney and Gahan, 2010) (Figure 12).

However, even though attractive, there is always the insecurity that attenuated vectors might restore the ability to replicate and cause disease in the patient. This fact should be taken into further consideration when it comes to the administration in infants and immunocompromised individuals. Actually, reversion to virulence, preexisting immunity, and reactogenicity will always remain major concerns (Schoen et al., 2004). Thus, the scientific community has recently been exploring the use of non-pathogenic bacteria, such as lactic acid bacteria (LAB), as vectors for genetic immunization (Mercenier and Wells, 2008).



3. LACTIC ACID BACTERIA (LAB)

3.1 Taxonomy and characteristics

Phylogenetically, LAB belongs to the clostridial branch of the Gram-positive bacteria that produce lactic acid as the major product of sugar fermentation. Species of *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Bifidobacterium*, *Vagococcus*, and *Weissella* belong to the group of LAB. The most representative is the *Lactobacillus* genus which contains approximately 80 species (Carr et al. 2002; Price et al., 2011). The individual species and strains of *Lactobacillus* differ by the profiles of fermenting saccharides; however this property is not sufficient for the identification at the species

level. Some representatives of the genus are included in probiotic preparations with regard to their beneficial health effects on the human and animal organisms (described later in this manuscript). For example, they are able to interact with the immune system, to reduce the manifestations of lactose intolerance by consuming fermented milk products and to produce bacteriocins (antimicrobially active substances) (Bilková et al., 2011; Masood et al., 2011). Two species, belonging to lactic bacteria, play a role in the production of fermented milks and dairy products: *Streptococcus thermophilus* and *Lactococcus lactis*. However, not all LAB species have a positive impact on human health or food production. Some can cause food spoilage, while several important (human) pathogenic species are included in the LAB group (Felis and Dellaglio, 2007; Price et al., 2011). The genus *Streptococcus*, for instance, contains about 60 species and a number of them are known for their pathogenicity. The species *S. thermophilus*, however, is constituted by non-pathogenic microorganisms, very essential in the dairy manufacturing (Felis and Dellaglio, 2007).

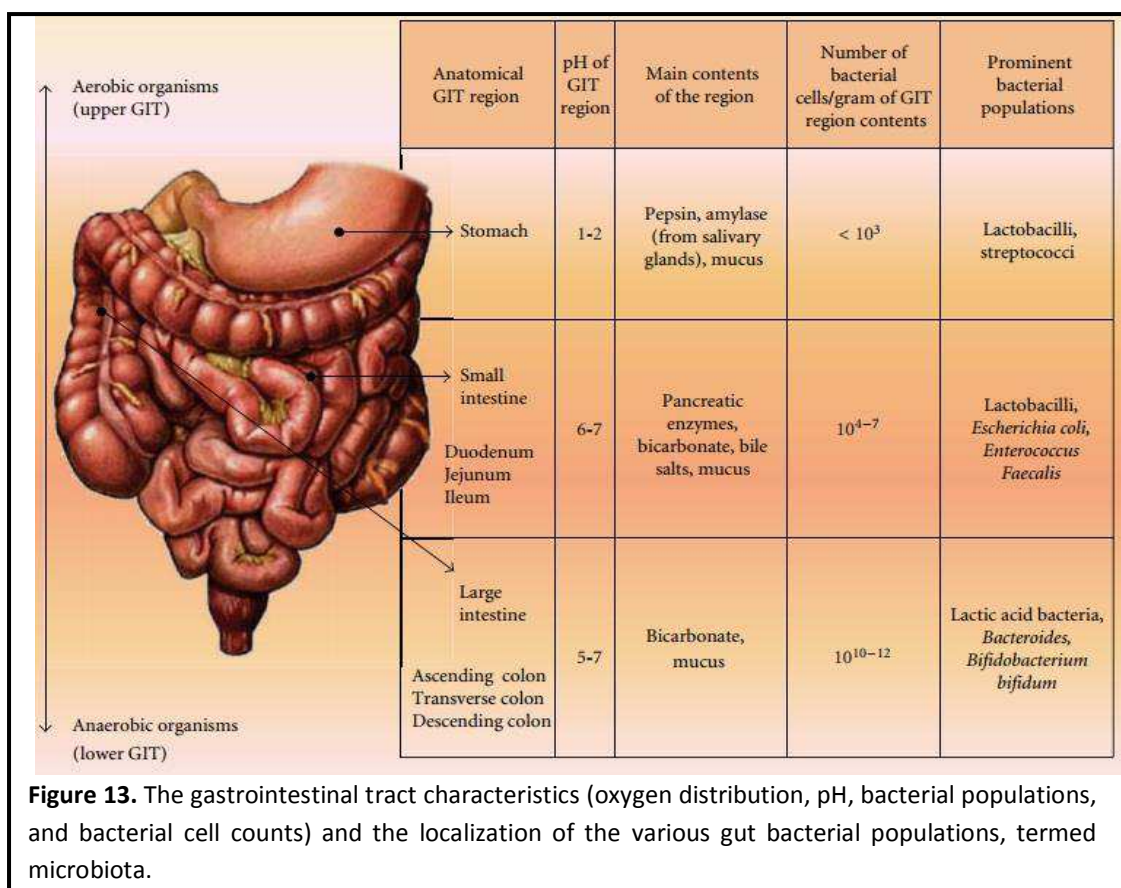
Lactic bacteria can tolerate mild acidic conditions of around pH 4 for several weeks and are generally classified as facultative anaerobic, catalase-negative organisms. Their shapes are primarily cocci (spheres), bacilli (rods), or ovoid. They may also be γ -shaped, as in the case of bifidobacteria, and usually they have less than 55 mol% G+C content in their DNA (Masood et al., 2011). However, even though species from the *Bifidobacterium* genus present a high G+C content in their DNA (55-57 mol%) and are not genetically closely related to LAB from the taxonomic point of view, recently they have been grouped as LAB bacteria since they are able to produce lactic and acetic acids (Felis and Dellaglio, 2007).

Lactic acid bacteria are distributed in different ecological niches; they are found in plants, in fermented foods, and in the gastrointestinal tract of many animals, including humans, where some species can live as commensal microorganisms (Steidler & Rottiers, 2006). Human beings since ancient times and without understanding the scientific basis, use these bacteria for the manufacture and maintenance of various types of foods such as cheese, wine, yogurt, fermented milks, pickles, kefir, butter, koumiss among others. Nowadays it is known that food preservation is due to acidification of the medium (pH 3.5 to 4.5), production of bacteriocins and organic compounds. Due to the fact that they are used for centuries in food fermentation and preservation, the FDA has granted this bacteria as generally regarded as safe (GRAS) for human consumption (Van de Guchte et al. 2001).

3.1.1 Physiology of lactic acid bacteria into the human gastrointestinal tract

The physiology of lactic acid bacteria used for food fermentation is well studied and their behavior for food production has received considerable attention. Nowadays, more attention has

been paid on the metabolic functions and phenotypic activities of enteric lactic bacteria during their life into the human gastrointestinal tract as they have a significant impact on host metabolism, participating in microbial-mammalian co-metabolism (Prakash et al., 2011). Species of the genera *Lactobacillus* and *Bifidobacterium* are some of the most important taxa involved in human nutrition. The upper portion of the gastrointestinal tract, made up of the stomach and the duodenum, harbours very low numbers of microorganisms, with less than 1000 bacterial cells per gram of contents, with the predominant microorganisms present being *Lactobacilli* and *Streptococci* (Prakash et al., 2011), while bifidobacteria are largely present into the colon (Price et al., 2011). Figure 13 illustrates some characteristics of the gastrointestinal tract and the localization of various gut bacterial populations, termed microbiota.



3.1.2 The probiotic action

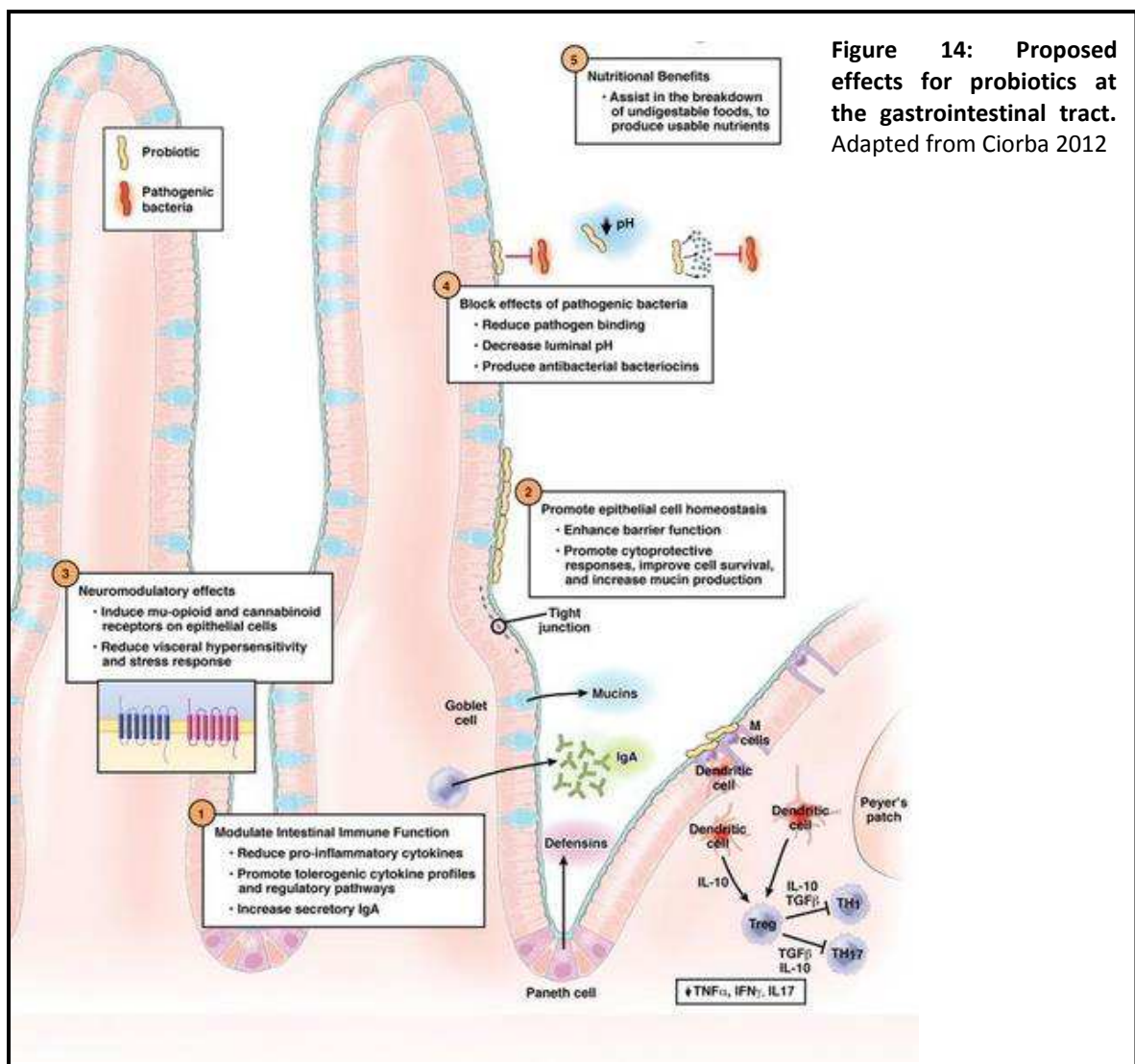
Probiotics are defined as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (An Interpretive review of Recent Nutrition Research, 1997). The beneficial effects of LAB were revealed by a Russian Scientist E. Metchnikoff

(1845–1919) who proposed that extended longevity of people of Balkan could be attributed to their practice of ingesting fermented milk products (Metchnikoff, 1907; WHO, 2002; Masood et al., 2011). It was vastly demonstrated that LAB can produce these beneficial effects by restoration of normal intestinal flora, elimination of intestinal pathogens, reinforcement of intestinal barrier capacity to foreign antigens, stimulation of nonspecific immunity such as phagocytosis, stimulation of humoral immunity and production of antiinflammatory products (WHO, 2002; Masood et al., 2011). Moreover, the deliberate ingestion of certain *Lactobacillus* strains has been associated with prevention and/or recovery from various illnesses, ranging from gastrointestinal infections to allergy (Vaughan et al., 2006; Prakash et al., 2011). Thus, nowadays the effect of LAB as useful probiotics is being extensively explored.

Studies have shown that some species of LAB are able to compete with potential pathogens for nutrient fixing sites, preventing in this way, infections. These bacteria can also impede pathogens proliferation through the synthesis of bacteriocins, volatile organic acids and hydrogen peroxide. The anticarcinogenic effects are attributed to inhibition of pro-carcinogens enzymes or to the stimulation of the host immune system (Ewaschuk et al. 2008; Zoumpopoulou et al., 2009). Karczewski and coworkers (2010) demonstrated that *Lactobacillus plantarum* is capable of modifying the tight junctions of epithelial cells keeping them more closely together, thereby strengthening the epithelial barrier. Two probiotic commensal bacteria, *Lactobacillus acidophilus* and *Streptococcus thermophilus* were able to prevent enteroinvasive *Escherichia coli* to disrupt the intestinal epithelial barrier *in vitro* (Resta-Lenert and Barrett, 2003). Recently, a very interesting work was performed by Rocha and co-workers in which they observed that several *L. delbrueckii* strains showed anti-inflammatory effects *in vitro*, to an extent that varied between strains. These effects observed was attributed to bacterial surface exposed proteins that affected the central part of the NF- κ B activation, a pathway that plays a key role in regulating pro-inflammatory immune responses. One of the selected strains could significantly reduce the macroscopic and microscopic symptoms of DSS-induced colitis in the mouse intestinal tract, diminished body weight loss, and improving survival (Santos Rocha et al., 2011).

Several probiotic strains or commensals, such as *Lactobacillus* GG (LGG), *Bifidobacterium breve*, *Streptococcus thermophilus*, *B. bifidum* and *Ruminococcus gnavus*, secrete metabolites that inhibit apoptosis and promote cell growth contributing for intestinal homeostasis (Ewaschuk et al., 2008). Certain Lactobacilli have immune regulatory properties, including interactions with immune cells of the intestinal mucosa, modulation of immune responses and induction of humoral and cellular immunity. For example, *Lactobacillus casei*, *L. paracasei*, *L. rhamnosus*, *L. reuteri*, *L. plantarum* and *L. johnsonii* may influence the maturation of dendritic cells, which plays an important role in orchestrating the immune responses (Delcenserie et al. 2008).

However, despite the various results obtained in this research field, several mechanisms still remain to be discovered given the enormous complexity of the relationships between prokaryotic and eukaryotic cells from the intestine (Zoumpopoulou et al., 2009). A variety of LAB strains are being evaluated for their potential to adhere to the epithelium in vitro and in vivo and several models have been recently developed, which will undoubtedly help to better understand the relationship between LAB, intestinal mucosa and activation of the immune system. This knowledge is essential to the development and optimization lactic bacteria as a therapeutic tool (Wang et al., 2011; Wells, 2011). Figure 14 summarizes the effects that probiotic species may exert at the gastrointestinal tract.



3.2 The model LAB: *Lactococcus lactis*

Lactococcus lactis is probably the most economically important LAB species and also a very important model organism for low GC gram-positive bacteria. It is still stimulating extensive research efforts which have resulted in the genome sequencing of three strains of the species

(Bolotin et al., 2001; Felis e delagio 2007). The Lactococci have been used primarily as starter cultures for various dairy products (yogurt, Cheddar, and hard cheeses). They are non motile and form ovoid cells with the tendency to chain in one direction. They are facultatively 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, where it contributes a highly prized flavor (Carr et al., 2002). *L. lactis* has the GRAS status (Generally Regarded as Safe), although there are few case reports of being an opportunistic pathogen in small animals (Mannion and Rothburn, 1990).

3.3 *Lactococcus lactis*: From cheese making to Heterologous protein Delivery

Among all LAB, *L. lactis* is the most well characterized species and stands as a model organism not only because of its economic importance, but also due to the fact that: (i) *L. lactis* is a microorganism very easy to manipulate, (ii) they are "GRAS", (iii) *L. lactis* was the first lactic bacterium to have its genome sequenced (Bolotin et al. 2001), (iv) thus, they have a large number of efficient genetic tools already developed and (v) does not contain Lipopolysaccharide (LPS), an endotoxin commonly responsible for anaphylactic shock, in its outer membrane such as *E. coli* (de Vos & Simons, 1994; Duwat et al. 2000; Mercenier et al., 2000; Bolotin et al. 2001).

Due to the fact that in the last two decades several genetic tools have been developed for *L. lactis* such as, transformation protocols, cloning-or screening-vectors, mutagenesis systems, protein expression and targeting-systems, novel applications have been proposed for this bacterium. These systems have been used to engineer *L. lactis* for the intra- or extra-cellular production of numerous proteins of viral, bacterial or eukaryotic origins (Le Loir et al., 2005). Therefore, *L. lactis* has moved from being exclusively considered as a microorganism for food fermentation to a useful resource to develop biopharmaceuticals (Villatoro-Hernández et al., 2012). To date, *L. lactis* is increasingly used for modern biotechnological applications such as the production of recombinant proteins for food, feed, pharma and biocatalysis applications.

Besides being quite acid resistant, *L. lactis* has many others interesting properties that makes them ideal for the production of heterologous molecules. This bacterium does not produce endotoxins or other toxic metabolic product (Bolotin et al., 2001), contains few secreted proteins, of which only one, Usp45 (Unknown Secreted Protein of 45 kDa), is secreted in sufficient quantities to be detected by gel denaturing polyacrylamide (SDS-PAGE) staining technique "Commassie blue" (van Asseldonk et al. 1990). This fact is very interesting as it simplifies purification step after bacterial growth in fermentors (Le Loir et al., 2005). Furthermore, the most commonly used laboratory strains (IL1403 and MG1363) are devoid of plasmid and do not

produce extracellular proteases (Gasson et al. 1983; Chopin et al. 1984). Nowadays, there are many recombinant *L. lactis* strains being used for high scale production of heterologous proteins (Mierau et al., 2005). Recently this bacterium became famous as the first genetically modified organism to be used alive for the treatment of chronic intestinal disease. Patients with Crohn's disease received with genetically modified *L. lactis* (LL-Thy12) in which the thymidylate synthase gene was replaced with a synthetic sequence encoding mature human interleukin-10. Treatment with LL-Thy12 decreased disease activity and fecally recovered LL-Thy12 bacteria were dependent on thymidine for growth and interleukin-10 production, indicating that the containment strategy was effective (Braat et al., 2006).

3.3.1 Gene expression systems and heterologous protein production in *Lactococcus lactis*

The expression of heterologous proteins in *L. lactis* was achieved by the development of genetic knowledge, the improvement of molecular biology techniques and the studies focusing on the regulatory elements of gene expression, such as promoters, inducers and repressors (Miyoshi et al., 2004). Through this combination, high levels of a wide variety of heterologous proteins of different origins were cloned in *L. lactis* by using several vectors containing constitutive or inducible promoters (Langella and Le Loir, 1999; Pontes et al., 2011). Among all expression systems available for use in *L. lactis* certainly Nisin Controlled Gene Expression (NICE) is the most used (Figure 15). NICE system was developed at NIZO Food Research, Netherlands (NL), and has played an important role in the development of recombinant *L. lactis* strains. It can be used for over expression of homologous and heterologous genes for functional studies and to obtain large quantities of specific gene products. The major advantages of this system over some others are the expression of membrane proteins, secretion of proteins into the medium, tightly controlled gene expression and simple scale up and downstream processing. NICE system is based on a combination of the PnisA promoter with the regulatory genes NisRK (Wells et al. 1993). In the presence of Nisin the promoter can induce transcription of the molecule of interest (Bermúdez-Humarán et al. 2004). Nisin is a 34 amino anti-antimicrobial peptide that binds to lipids and then forms small pores in the cytoplasmic membrane leading to subsequently to cell death. Because of its broad host spectrum, it is widely used as a preservative in food. For the signal transduction system the 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 the strain NZ9000 (nisin positive) (Kleerebezem et al., 1997; Mierau and Kleerebezem, 2005). When a gene of interest is subsequently placed behind the inducible promoter PnisA on a plasmid or on the chromosome, expression of the gene of interest can be induced by the addition of sub-inhibitory

amounts of nisin (0.1 – 5 ng/ml) to the culture medium. Depending on the presence or absence of the corresponding targeting signals, the protein is expressed into the cytoplasm, into the membrane or secreted into the medium. Many exogenous proteins have been expressed in *L. lactis* through this system (Kuipers et al., 1993; de Ruyter et al. 1996).

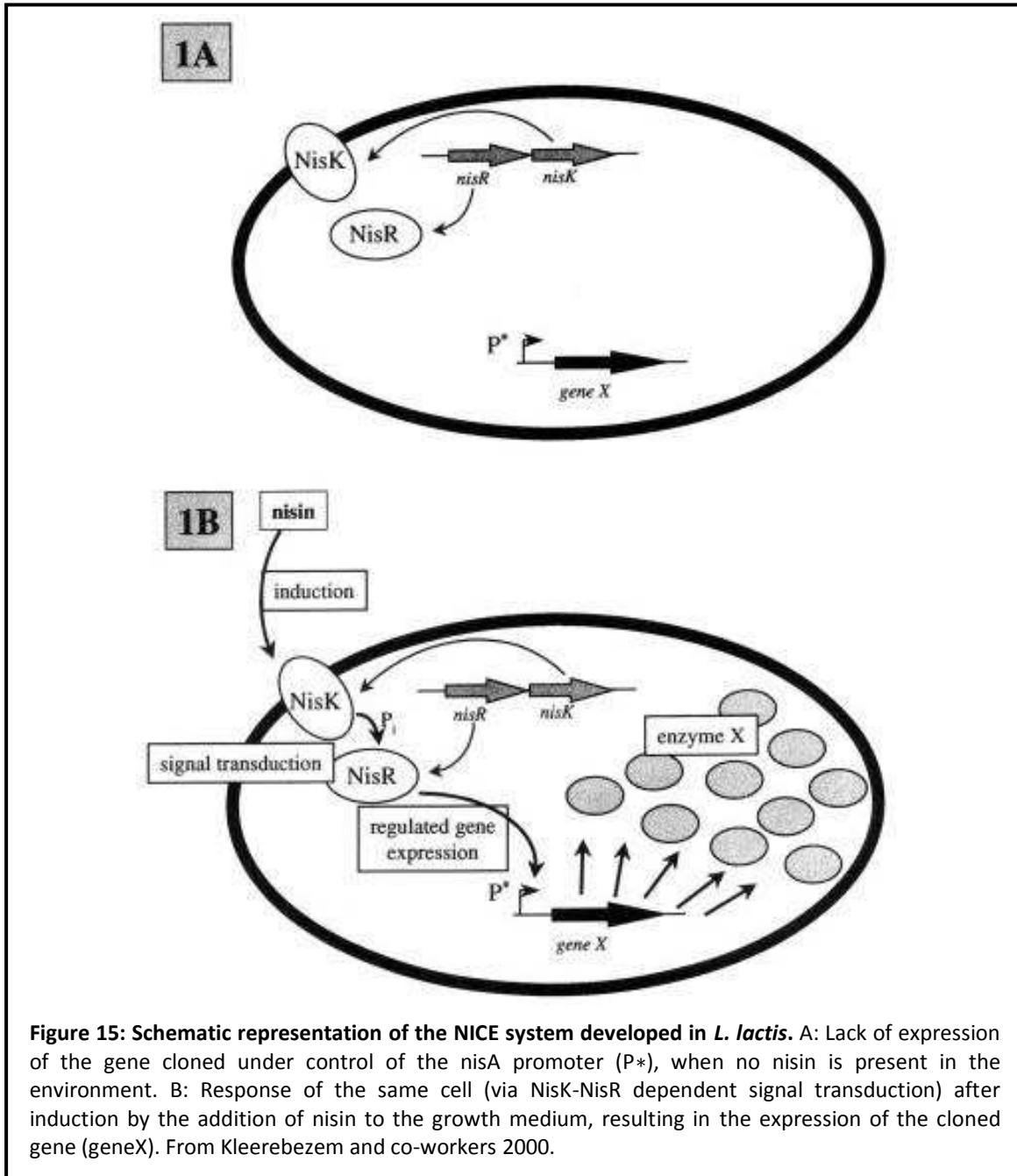
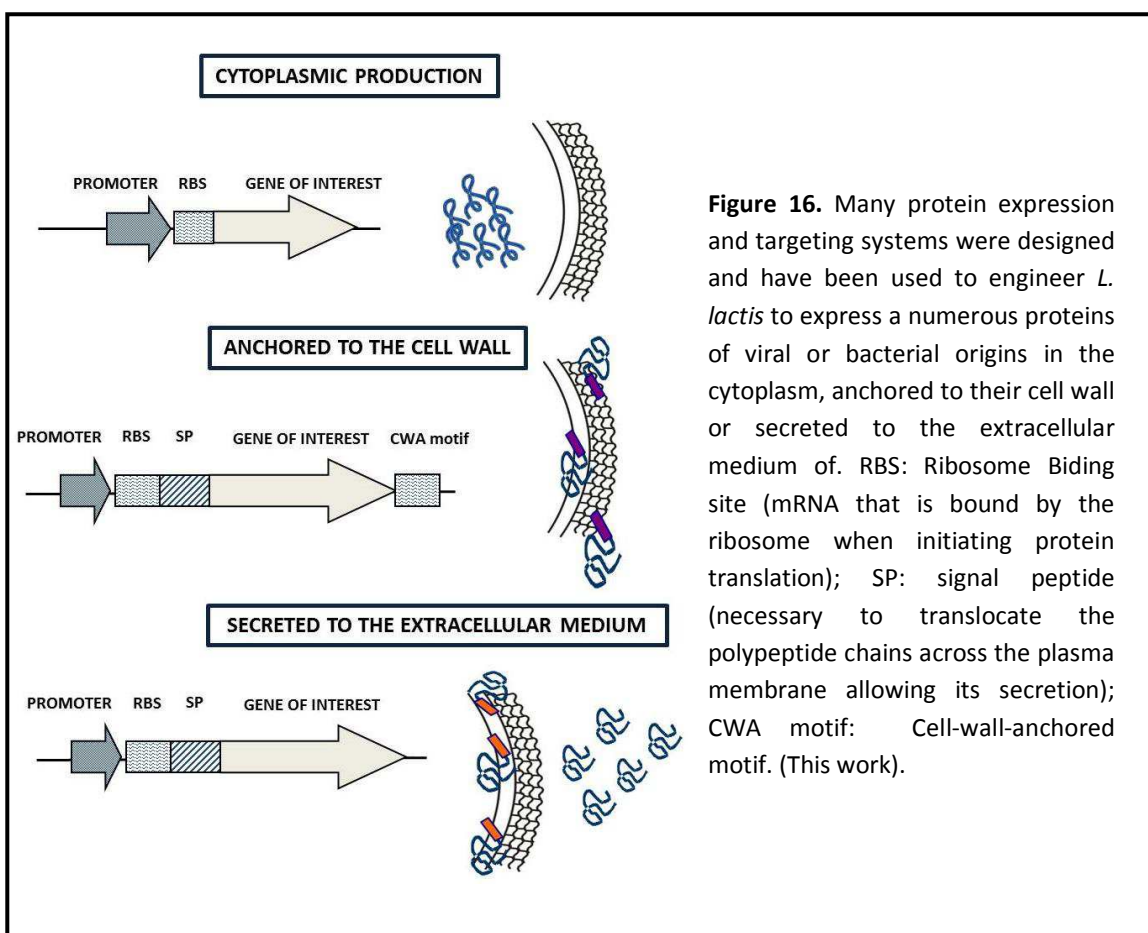


Figure 15: Schematic representation of the NICE system developed in *L. lactis*. A: Lack of expression of the gene cloned under control of the *nisA* promoter (P^*), when no nisin is present in the environment. B: Response of the same cell (via NisK-NisR dependent signal transduction) after induction by the addition of nisin to the growth medium, resulting in the expression of the cloned gene (*geneX*). From Kleerebezem and co-workers 2000.

In order to obtain a desired biological activity or depending on the nature of the target protein expressed by *L. lactis*, it is necessary that, after their synthesis, they are modified, sorted and dispatched towards their correct final destinations: (i) cytoplasm (ii) anchored to the cell wall

or (iii) secreted to the extracellular medium (Figure 16). The choice of the protein localization depends on the application proposed for the strain. For example, the intracellular production protects the recombinant protein from adverse environmental conditions (for example, low pH of the stomach); however, cell lysis is required for protein delivery. The antigen/enzyme expressed at the cell wall is also very interesting approach as it allows interaction with the environment (digestive tract, for instance) but also limits their possible degradation by proteases. Finally, the antigen released to the external medium is a nice strategy when it comes to the commercialization of recombinant proteins without the contamination with LPS, as it facilitates purification steps (Bermúdez-Humarán et al. 2004; Pontes et al., 2011).



Besides NICE, various expression systems that allow different cellular locations of the gene of interest were developed for use in *L. lactis*, and other LAB strains as well (Norton et al. 1995; Dieye et al. 2001). Sugar-inducible expression systems have been developed and some of them represent an alternative laboratorial tool for heterologous proteins production in *L. lactis*. Among them, Xylose-Inducible Expression System (XIES) stands out as it can correctly address

recombinant proteins expressed by *L. lactis* to the intracellular medium, anchored to the cell wall or secreted to the environment. Developed by Miyoshi and collaborators (Miyoshi et al., 2004), this very interesting system is based on the use of a xylose-inducible lactococcal promoter, P(xylT) from *L. lactis* NCDO2118 strain. In the presence of transported sugars (as glucose, fructose and/or mannose), P(xylT) was shown to be tightly repressed; differently, in the presence of xylose, P(xylT) is transcriptionally activated (Lokman et al., 1994; Jamet, 2001). The capacities of this system to produce cytoplasmic and secreted proteins were tested using the *Staphylococcus aureus* nuclease gene (*nuc*) fused or not to the lactococcal Usp45 signal peptide. Xylose-inducible *nuc* expression was shown to be tightly controlled and resulted in high-level and long-term protein production leading to a correct targeting either to the cytoplasm or to the extracellular medium. Furthermore, this expression system demonstrated to be very versatile as it can be switched on or off easily by adding either xylose or glucose, respectively (Miyoshi et al., 2004). Moreover, considering that nisin is quite expensive, XIES is a very feasible system as it make use of two simple sugars, xylose and glucose, facilitating its use in large-scale production. XIES system has been extensively employed in the biotechnology field for production of different heterologous protein. In order to study the effect of pure heat shock proteins (HSPs) on the immune system, recently De Azevedo and co-workers (De Azevedo et al., 2012) constructed a recombinant *L. lactis* strains able to produce and properly address the *Mycobacterium leprae* 65-kDa HSP (Hsp65) antigen to the cytoplasm or to the extracellular medium, using the XIES system. Approximately 7 mg/L recombinant Hsp65 was secreted and no protein degradation, related to lactococcal HtrA activity, was observed. The Limulus amoebocyte lysate assay demonstrated that the amount of LPS in the recombinant Hsp65 preparations was 10-100 times lower than the permitted levels established by the FDA, demonstrating the potential of these strains for a variety of biotechnological, medical and therapeutic applications. Recently, the ORF encoding the S-layer protein (SlpA) of *Lactobacillus brevis* was cloned into *L. lactis* under the transcriptional control of the XIES system. SlpA was correctly secreted into the extracellular medium, as determined by immunoblotting. Assays on the kinetics of SlpA production revealed the presence of glucose was sufficient to block the transcription, even if xylose was present into the medium. The successful use of XIES to express S-layer proteins in *L. lactis* opened new possibilities for an efficient production and isolation of SlpA S-layer protein for its various applications in biotechnology and importantly as an antigen-carrying vehicle (Hollmann et al., 2012).

There are other controlled expression systems besides NICE and XIES. An elegant system has been developed on the basis of the middle promoter of the lactococcal bacteriophage ϕ 31, which is induced by infection with ϕ 31. An expression plasmid containing the replication origin of ϕ 31 (*ori*31), which is induced upon bacteriophage infection, results in runaway plasmid

replication. The protein of interest is then produced also after bacteriophage infection, resulting in the lysis of *L. lactis*. Because of the lysis that is ultimately realized, this system was termed explosive expression. A drawback is the phage-dependent induction which may be difficult to realize on an industrial scale. Moreover, there are the Thermoinduction and pH-dependent systems, both very interesting but also presents some disadvantages (de Vos, 1999; Pontes et al., 2011).

Still being GRAS organisms, the use of genetically modified *L. lactis* raises legitimate concerns about their survival and propagation in the environment, and about the dissemination of antibiotic selection markers or other genetic modifications to other microbes. Therefore, an auxotrophic *L. lactis* strain was engineered to lack the thymidine synthase (*thyA*) gene. Thymine starvation results in activation of the SOS repair system and DNA fragmentation, leading to bacterial death (Lee, 2010). To accompany this purpose, a food-grade host/vector expression system for *L. lactis* was recently constructed. It uses the alanine racemase gene (*alr*) as the complementation marker. The alanine racemase auxotrophic mutant *L. lactis* NZ9000 Δ *alr* was obtained by double-crossover recombination using temperature-sensitive integration plasmid *pG(+)**host9* and a food-grade vector *pALR*, containing entirely lactococcal DNA elements (lactococcal replicon, nisin-inducible promoter *PnisA* and the *alr* gene from *Lactobacillus casei* BL23) as a complementation marker. The green fluorescent protein and capsid protein of porcine circovirus type II were successfully overexpressed under the nisin induction by using the new food-grade host/vector system. This strain could be further used in as an excellent antigen delivery vehicle, and could be also suitable for the use in the manufacture of ingredients for the food industry as it is safe (Lu et al., 2012).

3.3.2 *Lactococcus lactis* as mucosal delivery vectors for therapeutic proteins

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 (for a review see (Wells and Mercenier, 2008; Bermúdez-Humarán et al., 2011 and Pontes et al., 2011).

Iwaki and collaborators in 1990 were one of the first to attempt to use *L. lactis* as live vaccines. Mice immunized orally with *L. lactis* strain expressing the Pac antigen of *Streptococcus mutans* were able to produce both IgG and IgA Pac-specific antibodies. This result showed that *L. lactis* could be used as a carrier for both presentation and delivery of antigens to the immune system. Since then, several antigens derived from bacteria and viruses have been expressed in *L. lactis* and immunization with these recombinant strains generated prophylactic and therapeutic effects in several animal models (Wells & Mercenier, 2008; Wells, 2011).

Most studies concerning *L. lactis* as live vaccine were performed using the fragment C of tetanus toxin (TTFC), a highly immunogenic antigen. Norton and colleagues (1995) found a significant increase in the levels of IgA after oral immunization of mice with recombinant *L. lactis* strains producing TTFC. Other studies have shown that animals vaccinated with *L. lactis* producing the intracellular form of the antigen developed high levels of IgG and IgA antibodies specific for TTFC. Subsequently, all animals became resistant to challenge with tetanus toxin (Wells et al. 1993; Robinson et al. 1997). Other several works confirmed the ability of *L. lactis* to present antigens to the mucosa, generating specific immune response (Chatel et al. 2001). Encouraging results were obtained after animal immunization with *L. lactis* expressing E7 antigen, derived from human papillomavirus type-16 (HPV-16) virus, anchored to the cell wall. Vaccinated animals showed humoral and cellular immune responses specific against the antigen (Bermúdez-Humarán et al. 2004). Later, the administration of mice with live Lactococci expressing both E7 antigen and IL-12 induced systemic and mucosal immune responses and protected the animals against HPV-16 induced tumors (Bermúdez-Humarán et al., 2005). Cortes-Perez and collaborators also developed a safe mucosal vector for HPV-16 prophylactic vaccination based on *L. lactis* strains expressing human papillomavirus type-16 L1 protein (Cortes-Perez et al., 2009). Table 2 summarizes some studies that were performed in different laboratories around the world corroborating the ability of *L. lactis* in inducing long-lasting immune responses.

ANTIGENS EXPRESSED BY <i>L. LACTIS</i>	IMMUNE RESPONSE OBSERVED	APPLICATION	REFERENCE
EDIII antigen from dengue virus type 2	Neutralization of the virus <i>in vitro</i>	Dengue virus (DENV) control strategy	Yes et al., 2008
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
Antigenic protein of <i>Proteus mirabilis</i> HBPM	Protection to the animals against challenge with <i>P. mirabilis</i> virulent strain	Control of urinary tract infections	Scavone et al. 2007
PspA antigen derived from <i>Streptococcus</i>	Better protected against challenge with	Vaccine against pneumonia	Hanniffy et al., 2007

<i>Pneumoniae</i>	the virulent strain		
Antigen IcrV from <i>Yersinia pseudotuberculosis</i>	Humoral and cellular immune responses were observed conferring protection against challenge	Vaccine against Far East scarlet-like fever	Daniel et al., 2009
EspB antigen from the type III secretion system (T3SS) of <i>E. coli</i> serotype O157:H7	Significant levels of specific serum Ig and faecal IgA were detected	New strategie to fight against enterohemorrhagic <i>E. coli</i>	Ahmed et al., 2012
<i>Leishmania</i> antigen LACK and the proinflammatory cytokine IL-12	Protection against challenge	Vaccine to combat Leishmaniasis	Hugentobler et al., 2012
Mature murine IFN-gamma	--	Adjuvant tool	Bermúdez-Humarán et al., 2008
Antigen involved with LPS transport (wzm) from <i>Vibrio cholerae</i> O1 strain	Augmentation of both systemic and mucosal immunity	Vaccine against cholera	Zamri et al., 2012
Catalase-producing <i>L. lactis</i>	Inhibition of chemically induced colon cancer	Cancer therapy	De Moreno de LeBlanc et al., 2008
IL-10	Anti-inflammatory effects controlling intestinal inflammation	Treatment of allergy and inflammatory bowel diseases (IBD)	Cortes-Perez et al., 2007; Braat et al. 2006 ; Marinho et al., 2010

There are many others protection studies performed with recombinant *L. lactis* strains and results are very encouraging as protection or partial protection after challenge was observed (Pontes et al., 2011; Bermudez-Humaran et al., 2011; Tarahomjoo, 2012). A summary of the current published applications of *L. lactis* delivery is illustrated in Figure 17. Nevertheless, even though interesting, the strategy to express recombinant antigenic proteins in *L. lactis* presents one problem associated with post-translational modifications performed on recombinant proteins

by *L. lactis* that might affect their bioactivity (Marreddy et al., 2011). Therefore, a novel technology has emerged to circumvent this problem: delivery of cDNA encoding antigenic proteins by *L. lactis* at the mucosal level. This very interesting approach will be discussed in the next pages (Guimarães et al., 2006; Chatel et al., 2008; Pontes et al., 2011).










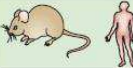




Product	Model	Application
DNA 		Vaccines
Peptide 		HIV-1, fusion inhibitors; colitis, trefoil peptides
ScFvs 		Anti-infectives
Antigens 		Vaccines
Cytokines 		Inflammatory bowel disease and colitis, vaccine adjuvants
Enzymes 		Therapy for colitis and enzyme deficiencies
Allergen 		Therapy and prevention of allergy

Figure 17. Current applications of *L. lactis* delivery. Illustration of the various molecules that have been produced in *L. lactis* and the animal models that were used. Adapted from Wells and Mercenier, 2008.

3.4 *Lactococcus lactis*: From protein to DNA delivery

3.4.1 Native *L. lactis* as DNA delivery vectors

Recently, the use of LAB as DNA vaccine delivery vehicles has been evaluated as an alternative strategy for vaccination (Guimaraes et al., 2005; Chatel et al., 2008; Tao et al., 2011). Guimaraes and co-workers (2006) were one of the first to explore the potential use of noninvasive *L. lactis* strains as a DNA delivery vehicle. It was constructed two *E. coli-L. lactis* shuttle plasmids, pLIG:BLG1 and pLIG:BLG2, containing a eukaryotic expression cassette with the cDNA of bovine β -lactoglobulin (BLG), the major allergen of cow's milk. After *in vitro* assays, pLIG:BLG1 plasmid showed better transfection capacity and was then used to transform *L. lactis* MG1363, which proved to be incapable of expressing BLG. After 3h of co-incubation with Caco-2 human colon carcinoma cells, BLG expression was only found in cells incubated with MG1363 (LL-pLIG:BLG1) strain and secretion started 48h after the beginning of the experiment. Native *L. lactis* was then considered to be capable of transferring BLG cDNA into mammalian epithelial cells, and this work demonstrated their potential to deliver *in vivo* a DNA vaccine (Guimaraes et al., 2006). 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. LL-

pLIG:BLG1 strain were able to translocate the epithelial cells of the intestinal membrane as BLG cDNA was detected in the epithelial membrane of the small intestine of 40% of the mice immunized and BLG was produced in 53% of the animals. Moreover, it was also observed a low and transitory Th1-type immune response after immunization trials which conferred protection to mice after sensitization with the allergen (Chatel et al., 2008). This study successfully demonstrated that *L. lactis* can be used as vector for genetic immunization.

3.4.2 Recombinant invasive *L. lactis* as plasmid DNA delivery vehicles

In order to increase the capacity of lactococci to deliver cDNA, some strains of *L. lactis* expressing invasins have been reported (Guimarães et al., 2005; Sleator and Hill, 2006). One very interesting strategy performed by Guimarães and collaborators was to engineer *L. lactis* to express InIA (LL-InIA+) from *L. monocytogenes*. InIA 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 InIA. Moreover, invasion rates of LL-InIA+ 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-InIA+ was able to deliver a functional eukaryotic *gfp* gene (green fluorescent protein) into epithelial Caco-2 cells (Guimarães et al., 2005).

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*. Therefore, in order to circumvent this difficulty, a new plasmid (3742 bp), termed pValac (Vaccination using Lactic acid bacteria) 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 after transfection in PK15 cells. The applicability of pValac was demonstrated by invasiveness assays of *L. lactis* inIA+ 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* inIA+ strain were able to invade Caco-2 cells and deliver pValac:*gfp* into epithelial cells (Guimaraes et al. 2009). 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-InIA+ strain presented some bottlenecks: InIA 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-InIA+ strain in guinea pigs or transgenic mice, which may be laborious and/or expensive (Wollert et al., 2007). For this reason, a new recombinant *L. lactis* strain expressing Fibronectin Binding Protein A (FnBPA) (LL-FnBPA+) of *Staphylococcus aureus* (Que et al., 2001), was constructed and therefore evaluated with the goal of improving the delivery of DNA to mammalian cells (Innocentin et al., 2009). FnBPA naturally mediates adhesion of *S. aureus* to the host tissue and its entry into non-phagocytic cells (Sinha et al., 1999). Thus, FnBPA utilization could increase the interactions of *L. lactis* with IECs leading to a more efficient delivery of DNA vaccines. *L. lactis* FnBPA+, and *L. lactis* InIA+ showed comparable internalization rates in Caco-2 cells and conventional or confocal fluorescence microscopy demonstrated big clusters of *L. lactis* FnBPA+ and *L. lactis* InIA+ 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 (Innocentin et al., 2009). 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. 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 (Pontes et al., 2012). Since FnBPA requires an adequate local concentration of fibronectin to bind to integrins (Ozeri et al., 1998; Dziewanowska et al., 2000), the interaction between FnBPA and epithelial cells may be a complex process *in vivo*.

Tao and colleagues developed a different strategy attempt to improve DNA delivery by *L. lactis* and *S. gordonii* *in vitro* without using virulent genes. They applied different chemical treatments in order to weaken the bacterial cell wall, facilitating transfection of plasmids into cells Caco-2. *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 lizozime was showed to be more effective in *S. gordonii* (Tao et al., 2011). Even

though the mechanisms by which treated bacteria could be efficiently internalized into Caco-2 cells have not yet been elucidated, this strategy is interesting because virulence genes are not involved (Pontes et al., 2011). However, the potential of these strains in delivering DNA vaccines were not tested *in vivo*.

All of the *L. lactis* strains that have been developed to be used as vaccine DNA delivery vectors are promising for mucosal immunization. Nevertheless, they all present some problems, as mentioned above. Therefore, the use of other invasin gene that can easily bind to murine epithelial cells from conventional mice as well as the use of other LAB specie that can stay longer than *L. lactis* in the gut, are being considered viable strategies that will allow the use of LAB as efficient DNA-vaccine delivery vehicles in near future (Bermúdez-Humarán et al., 2011; Pontes et al., 2011).

CHAPTER 2
AIM OF THE STUDY

MAIN AIM OF THE STUDY

Understand the mechanisms by which the bacterium *Lactococcus lactis* is able to deliver DNA vaccines to intestinal epithelial cells (IECs) both *in vitro* and *in vivo*, and to antigen presenting cells, such as dendritic cells, by using a genetically modified strain of *L. lactis* expressing *Listeria monocytogenes* mutated internalin A invasin. We also intended to investigate the immunomodulatory properties of invasive or noninvasive *L. lactis* strains after immunization trials.

SPECIFIC AIMS

- Construct a novel invasive *L. lactis* strain expressing *L. monocytogenes* mutated Internalin A (mInA),
- Detect the expression of the recombinant mInA at *L. lactis* surface using flow cytometry analysis,
- Evaluate the capacity of *L. lactis*-mInA to invade more efficiently than the wild type strain the human intestinal epithelial cell line, Caco-2, non-differentiated or differentiated in tissue culture inserts,
- Investigate the ability of *L. lactis*-mInA to deliver a cDNA of the major allergen in cows' milk, β -lactoglobulin (BLG), *in vitro* to Caco-2 cells or *in vivo* to conventional mice intestinal epithelial cells,
- Evaluation of the immune response elicited by noninvasive or invasive *L. lactis* after immunization trials,,
- Recover bone marrow dendritic cells (BMDCs) to investigate if non-invasive or invasive *L. lactis* strains are capable to deliver BLG cDNA directly to dendritic cells (DCs) after co-incubation assays,
- Evaluate if non-invasive or invasive *L. lactis* strains are capable to deliver BLG cDNA to DCs through a monolayer of differentiated Caco-2 cells after co-incubation assays.

CHAPTER 3

***In vitro* and *in vivo* characterization of DNA delivery using recombinant *Lactococcus lactis* expressing a mutated form of *L. monocytogenes* Internalin A**

CHAPTER 3: *In vitro* and *in vivo* characterization of DNA delivery using recombinant *Lactococcus lactis* expressing a mutated form of *L. monocytogenes* Internalin A

2.1 Introduction

“Naked” DNA vaccination has gained a lot of attention due to its ability to induce long-lasting humoral and cellular immune responses against an encoded antigen (Donnelly et al., 2000; Liu, 2010). This vaccine platform demonstrated to be highly efficient in rodents and mice, but not in larger animals and humans as the DNA showed to be easily degraded in those hosts (Ledwith et al., 2000). Therefore, the optimization of DNA vaccine vectors appeared to be very important to confer protection to the genetic material and thus enhance gene transfer efficiency (Manam et al., 2000). Several strategies have been explored to protect plasmids from degradation such as encapsulation into synthetic particles (cationic liposomes or polymers) or the use of viral vectors (Manam et al., 2000). Despite their potential some limitations and safety issues still remain; for example liposomes are very complex to produce, can present some toxicity to mammalian cells and have limited packaging capacity (Liu, 2011). Viral vectors, on the other hand, can have the capacity to randomly integrate their genetic material into the host genome leading to tumour formation (Kang et al., 2003; Thomas et al., 2003).

The use of bacteria as delivery vehicles for DNA vaccination has emerged as an interesting alternative to overcome many of the problems associated with viral or liposomal delivery. Bacterial carriers may be administered at mucosal surfaces eliciting secretory IgA responses, can increase and expand the magnitude of immune responses against the vector-encoded antigen due to the natural presence of Microbe-Associated Molecular Patterns (MAMPs) activating immune cells (Courvalin et al., 1995; Becker et al., 2008). Attenuated pathogens such as *Salmonella*, *Shigella*, *Listeria* and *Yersinia* have been used as experimental live delivery systems. However, a potential concern is the risk of increased virulence in young or immunocompromised individuals (Loessner et al., 2008; Wells and Mercenier, 2008).

The use of food-grade lactic acid bacteria (LAB) as DNA delivery vehicle represents an alternative and attractive strategy (Wells and Mercenier, 2008). Actually, it has been extensively demonstrated that the model LAB, *Lactococcus lactis*, is able to deliver a range of vaccine and therapeutic molecules for applications in allergic, infectious or gastrointestinal diseases (Wells, 2011). A relatively new development, however, is their use as a vehicle for genetic immunization (Pontes et al., 2011). Previous data have demonstrated that native *L. lactis* is capable to deliver DNA vaccines both *in vitro* and *in vivo* to intestinal epithelial cells (IECs). However, its DNA transfer efficiency seemed to be relative low (Chatel et al., 2008; Pontes et al., 2011). With the intent to increase the persistence of *L. lactis* in the gastrointestinal tract which could, in theory, improve

plasmid transfer to IECs, our research group decided to construct recombinant invasive *L. lactis* strains (Guimaraes et al., 2005; Innocentin et al., 2009; Pontes et al., 2012). Two strains were designed, one expressing Internalin A (InIA) from *L. monocytogenes* (LL-InIA) and another producing *S. aureus* Fibronectin Binding Protein A (FnBPA) (LL-FnBPA) (Guimaraes et al., 2005; Innocentin et al., 2009). Both of them were capable to successfully express the recombinant proteins and deliver DNA plasmids to IECs either *in vitro* or *in vivo*. Nevertheless, these two strains presented some bottlenecks: (i) *L. lactis* expressing InIA cannot interact to its receptor in mice, murine E-cadherin, being able to be administered only in transgenic mice and guinea pigs, and (ii) FnBPA requires an adequate local concentration of fibronectin to bind to its receptors, integrins, which may hamper the interpretation of *in vivo* studies (Ozeri et al., 1998; Dziewanowska et al., 2000). Hence, in this work we have decided to construct a third recombinant invasive *L. lactis* strain expressing mInIA as it is capable to bind to E-cadherin from conventional mice thus facilitating *in vivo* studies. Furthermore, besides describing the construction and characterization of this novel *L. lactis* strain, we test it as a DNA delivery vector, using cow's milk β -lactoglobulin (BLG) allergen to measure DNA transfer to IECs both *in vitro* and *in vivo*.

2.2 Materials and Methods

In order to verify if the new strain was capable to deliver more efficiently a eukaryotic expression vector than the noninvasive strain, wild type (wt) *L. lactis* (*L. lactis* NZ9000) was transformed by electroporation with a plasmid harboring mInIA gene. FACS analysis was used to confirm the expression of mInIA at *L. Lactis* surface (LL-mInIA+). The gentamicin survival assay into non-confluent intestinal epithelial cells of the cell line Caco-2 cells was used to evaluate the invasivity capacity of LL-mInIA strain which was confirmed by confocal microscopies studies. Afterwards, LL-mInIA and LL-wt were transformed by electroporation with a eukaryotic expression vector coding for the cDNA of β -lactoglobulin (pValac:BLG), the major cow's milk allergen. Co-incubation assays of LL-mInIA+ strain harboring pValac:BLG with non-confluent Caco-2 cells were performed in order to measure DNA transfer. Culture supernatant and Caco-2 extracts were collected to be assayed for the presence of BLG by ELISA. We then moved to *in vivo* experiments in which BALB/c mice, 3 to 6 weeks of age, received 1×10^9 (CFU) of invasive or non-invasive lactococci during three consecutive days. In the fourth day, mice intestinal epithelial cells were purified and their extracts were used for ELISA assays in order to quantify BLG production. Statistical significance between the groups was calculated using the One Way ANOVA (and nonparametric) test, followed by the "Bonferroni" post-test.

2.3 Results and Discussion

In this work we showed the ability of either noninvasive or recombinant invasive *L. lactis* expressing *Listeria monocytogenes* mInA as both *in vitro* and *in vivo* DNA delivery vehicle. Flow cytometry analysis demonstrated with success that mInA was expressed and properly directed to the surface of *L. lactis*. Previous studies have also shown that other invasins also derived Gram-positive bacteria, such as *Staphylococcus aureus* FnBPA and InIA, were successfully expressed in *L. lactis* confirming that the signal peptide for secretion and the anchoring signal are well recognized by the *L. lactis* machinery (Guimaraes et al., 2006; Pontes et al., 2012). Afterwards, we moved to *in vitro* assays in which we tested if the new invasive *L. lactis* (LL-mInA+) were actually capable to internalize intestinal epithelial cells (Caco-2 cells) using the gentamicin survival assay. We demonstrated that LL-mInA+ strain were almost 1000 times more invasive than the wild type strain. Moreover, a confocal image taken after gentamicin assay showed clearly that LL-mInA+ is capable of adhering to and entering in non-differentiated Caco-2 cells. Recombinant bacteria were found to be distributed at the periphery of the Caco-2 cell where the mInA receptor, E-cadherin, was accessible. Then, LL-mInA+ and LL strains were then transformed with pValac:BLG plasmid, co-incubated with Caco-2 cells and BLG expression was followed 72 h later by ELISA in order to measure the capacity of the strains in transfer DNA plasmids to mammalian cells. Both LL-mInA-BLG and LL-BLG were capable to deliver pValac:BLG to Caco-2 cells, confirming prior observations (Guimarães et al., 2009), and the invasive status improved this plasmid transfer. Cells were also capable of secreting the allergen, which may facilitate antigen uptake and presentation by professional APCs through cross-priming pathways (Donnelly et al., 2000). Subsequently, BALB/c mice received orally noninvasive and invasive *L. lactis* carrying or not pValac:BLG for 3 consecutive days and on the day 4 enterocytes from the small intestine were isolated and BLG production was measured by enzyme immunoassay (EIA). We confirmed that noninvasive lactococci are able to transfer a functional plasmid *in vivo* in mice and LL-mInA+BLG strain slightly enhanced the number of mice expressing BLG. As no significant advantages were observed by using LL-mInA-BLG compared to LL-BLG, we hypothesize that interactions of recombinant mInA with their receptors were impeded somehow in mouse intestinal epithelium hampering optimal plasmid transfer. Lack of invasion *in vivo* was also observed by another group working with *E. coli* strain expressing invasin from *Yersinia pseudotuberculosis* (Critchley-Thorne et al., 2006). It is thus possible that LL-mInA+BLG strain, which is not able to reach its receptor deeply buried in the crypt, are sampled by Microfold (M) cells in Peyer's patches. These cells are able to take up particles/bacteria from the lumen (Azizi et al., 2010). Another possibility is the interaction of the strains with other cells from the epithelial membrane such as dendritic cells, which are able to reach the lumen to sample its content (Rescigno et al., 2001). Most likely, plasmid transfer *in vivo* using either noninvasive or invasive lactococci is a combination of bacteria and released plasmid captures.

Taken together, we think that the use of another LAB, such as lactobacilli which persist longer in the gut than lactococci could be a better option for DNA delivery.

2.4 Conclusions

The use of *L. lactis* as a DNA delivery vehicle has been demonstrated to be a very interesting approach. Native lactococci have proved to be capable to transfer DNA plasmids to eukaryotic cells to Caco-2 cells and to mice IECs. However, the manner by which this DNA transfer occurs remains to be discovered. Therefore, we decided to express the mutated Internalin A (mInIA) invasin from *L. monocytogenes* in *L. lactis* to elucidate a part of the mechanism of DNA transfer and to increase the transfection ratio as in theory an invasive strain could stay longer in the host gastrointestinal tract. Flow cytometry analysis demonstrated that mInIA was successfully expressed at the surface of *L. lactis* NZ9000 strain. The final strain, named LL-mInIA+, revealed to be almost 1000 times more invasive when compared to wild type (wt) *L. lactis* strain after gentamicin survival assays into non-confluent Caco-2 cells. This invasiveness capacity was confirmed by confocal microscopy experiments wherein LL-mInIA+ strain were found to be very attached to the periphery of Caco-2 cells where the receptor for mInIA, E-cadherin, was exposed. Moreover, recombinant bacteria were also located to be inside the cells meaning that they were actually capable to invade the cells. Nor adherence neither invasion was observed for the wt strain. Afterwards, LL-mInIA+ was transformed with a second plasmid, pValac:BLG, harboring the cDNA of the allergen BLG. Strains were incubated again with Caco-2 cells and, after three hours, cell extract and culture supernatant were recovered to be assayed for the presence of BLG by ELISA. More BLG was detected for cells which were incubated with the invasive strain LLmInIA+. Therefore, we could conclude that the invasive status improved plasmid transfer *in vitro*. We then moved to *in vivo* analysis to check whether intestinal epithelial cells are the preferable cell type that can mediate plasmid transfer by using noninvasive and invasive lactococci. After immunization trials, it was observed that the number of mice expressing BLG was higher (n = 11) in the group immunized with invasive bacteria than with noninvasive bacteria (n = 8). We could clearly see a higher tendency of mice expressing the allergen in the group immunized with LLmInIA+. However, this difference observed was not statistically significant. Perhaps, IECs are not the preferable cell type that can mediate DNA transfer *in vivo* as we did not see a drastic increase in BLG expression by using recombinant invasive lactococci. Taken together, this data reinforce the use of *L. lactis*, especially invasive LLmInIA+ as an alternative tool to deliver therapeutic plasmids either *in vitro* or *in vivo*. We also intent to test if another lactic acid bacteria strain, such as the probiotic *Lactobacillus casei* BL23, that can stay longer in the gastrointestinal tract, could represent an advantage over the use of recombinant invasive *L. lactis*.

RESEARCH ARTICLE

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In vitro and *in vivo* characterization of DNA delivery using recombinant *Lactococcus lactis* expressing a mutated form of *L. monocytogenes* Internalin A

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Abstract

Background: The use of food-grade Lactic Acid Bacteria (LAB) as DNA delivery vehicles represents an attractive strategy to deliver DNA vaccines at the mucosal surfaces as they are generally regarded as safe (GRAS). We previously showed that either native *Lactococcus lactis* (LL) or recombinant invasive LL expressing Fibronectin Binding Protein A of *Staphylococcus aureus* (LL-FnBPA+) or Internalin A of *Listeria monocytogenes* (LL-InIA+), were able to deliver and trigger DNA expression by epithelial cells, either *in vitro* or *in vivo*. InIA does not bind to its receptor, the murine E-cadherin, thus limiting the use of LL-InIA+ *in vivo* murine models. Moreover, FnBPA binds to its receptors, integrins, via fibronectin introducing another limiting factor. In order to avoid the limitations of LL-InIA+ and LL-FnBPA+, a new *L. lactis* strain was engineered to produce a previously described mutated form of InIA (LL-mInIA+) allowing the binding of mInIA on murine E-cadherin.

Results: After showing the expression of mInIA at the surface of LL-mInIA+ strain, *in vitro* gentamycin survival assay in Caco-2 cells showed that LL-mInIA+ is 1000 times more invasive than LL. LL-mInIA+ invasivity was also validated by fluorescence microscopy. LL and LL-mInIA+ were transformed with pValacBLG, a plasmid containing the cDNA of bovine β -Lactoglobulin (BLG), resulting in strains LL-BLG and LL-mInIA+BLG. The plasmid transfer *in vitro* using LL-mInIA+BLG was increased 10 times compared to LL-BLG. Moreover, the number of mice producing BLG in isolated enterocytes after oral administration of LL-mInIA+BLG *in vivo* was slightly higher than after oral administration of LL-BLG.

Conclusions: We confirmed in this study that the production of mInIA at the surface of *L. lactis* is a promising strategy for plasmid transfer *in vitro* and *in vivo*.

Keywords: *Lactococcus lactis*, *Listeria monocytogenes*, Mutated internalin A, Internalization, DNA delivery

Background

DNA vaccination has gained a lot of attention since its ability to induce long-lasting humoral and cellular immune responses against an encoded antigen was discovered [1]. In addition, DNA vaccination poses no danger of integration into host cellular DNA thereby raising its safety profile [2-4]. DNA vaccines can be easily isolated

to high purity, encode multiple antigens, and possess inherent adjuvant activity due to the presence of unmethylated CpG motifs that are recognized in mammals by TLR9 [5]. So called purified "Naked" DNA vaccination was shown to be highly efficient in rodents and mice, but not in larger animals and humans [6]. Consequently, it is very important to optimize DNA vaccine vectors and develop a delivery system to facilitate cellular uptake and enhance gene transfer efficiency and expression *in situ* [7].

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Several strategies have been explored to protect plasmids from degradation, facilitating DNA uptake by phagocytic Antigen Presenting Cells (APCs) and thereby enhancing their immunological properties. This includes delivery technologies based on encapsulation into synthetic particles (cationic liposomes or polymers) or the use of viral vectors [7,8]. Despite their potential, some limitations and safety issues still remain which can restrict the application of gene therapy - e.g. the complexity of producing liposomes and their limited packaging capacity [9]. Additionally, it was shown that some viral vectors have the capacity to randomly integrate their genetic material into the host genome causing insertional mutagenesis of a cellular oncogene, leading to tumour formation [10].

The use of bacteria as delivery vehicles for DNA vaccination has emerged as an interesting alternative to overcome many of the problems associated with viral or liposomal delivery [11]. W. Schaffner was the first to observe genetic material transfer from bacteria to mammalian cells [12]. Since then, bacteria have been extensively exploited as vaccine delivery vehicles for vaccination against bacterial and viral pathogens as well as cancer immunotherapy [13-15]. The use of bacteria for mucosal delivery of DNA vaccines may be advantageous due to their potential to elicit secretory IgA responses as well as systemic immunity, when compared to conventional parenteral immunization [16]. Furthermore, bacterial carriers can increase and expand the magnitude of immune responses against the vector-encoded antigen due to the natural presence of Pathogen-Associated Molecular Patterns (PAMPs) that bind to Toll-like receptors (TLRs) and activate immune cells [5].

Presently, attenuated pathogens such as *Salmonella*, *Shigella*, *Listeria*, *Yersinia*, as well as, non-pathogenic *Escherichia coli* have been used as experimental live delivery systems [17,18]. An advantage of using attenuated pathogens as DNA vaccine vehicles is that they possess mechanisms to adhere or invade host cells with a negligible risk of reversion to a virulent strain via gene transfer or mutation. However, a potential concern is the risk of increased virulence in young or immunocompromised individuals.

The use of food-grade lactic acid bacteria (LAB) as DNA delivery vehicle represents an alternative and attractive strategy to deliver DNA vaccines at the mucosal surfaces (ref review by 19 and 20). The dietary group of LAB, including *Lactococcus lactis* and many species of *Lactobacillus*, is generally regarded as safe (GRAS) organisms of which some are intestinal commensals of humans. Indeed, it has been extensively demonstrated that these bacteria are able to deliver a range of vaccine and therapeutic molecules for applications in allergic, infectious or gastrointestinal diseases [19,21,22]. A

relatively new development, however, is their use as a vehicle for genetic immunization [23]. Previous experiments performed by our group showed that either native *L. lactis* (LL) or recombinant invasive LL expressing Fibronectin Binding Protein A (LL-FnBPA+) of *Staphylococcus aureus* or Internalin A (InIA) of *Listeria monocytogenes* (LL-InIA+) [24,25], were able to deliver DNA in epithelial cells both *in vitro* and *in vivo*, demonstrating potential as gene transfer vehicles [24-27]. However InIA does not bind to its murine receptor, E-cadherin, thus limiting the use of LL-InIA+ in *in vivo* murine model. On the other hand, FnBPA requires an adequate local concentration of fibronectin to bind to its receptors, integrins [28,29].

In order to avoid the limitations of InIA and FnBPA and improve our knowledge on the key steps by which the DNA is transferred to mammalian cells using *L. lactis*, LL was engineered to express a mutated form of Internalin A (mInIA; Ser192Asn and Tyr369Ser) that increased binding affinity to murine and human E-cadherin [30,31] thus allowing for *in vivo* experiments in conventional mice. Herein, we describe the construction and characterization of this novel *L. lactis* strain as a DNA delivery vector, using cow's milk β -lactoglobulin (BLG) allergen, to measure DNA transfer to intestinal epithelial cells (IECs) *in vitro* and *in vivo*.

Overall, the production of mInIA+ at the surface of *Lactococcus lactis* increased the invasiveness of bacterium and amount of plasmid transfer by 1000 and 10 fold, respectively. *In vivo*, BLG production was detected in isolated enterocytes after oral administration of LL-mInIA+BLG and was slightly higher than oral administration of LL-BLG.

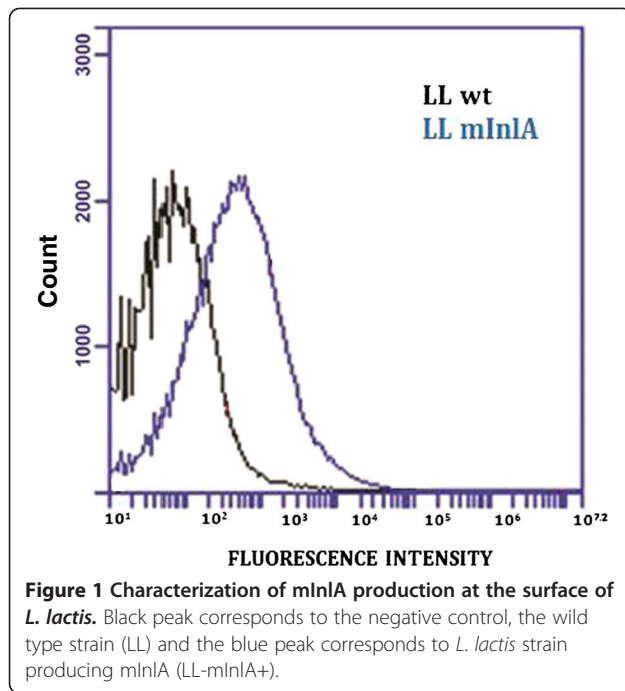
Results

Mutated internalin A is produced on the surface of recombinant *L. lactis* strain

To investigate surface expression and production of mInIA, *L. lactis* NZ9000 and LL-mInIA+ strains were incubated with specific anti-mInIA monoclonal antibody and then with FITC-conjugated anti-Mouse IgG. Stained cells were analyzed by flow cytometry. As shown in Figure 1, LL-mInIA+ strain (blue peak) showed a significant shift in the fluorescence intensity comparing to the NZ9000 strain (black peak). No shift was observed when strains were incubated with FITC-labeled anti-Mouse IgG alone (data not shown). This experiment confirmed expression of mInIA on the surface of *L. lactis*.

L. lactis producing mInIA is efficiently internalized by Caco-2 cells

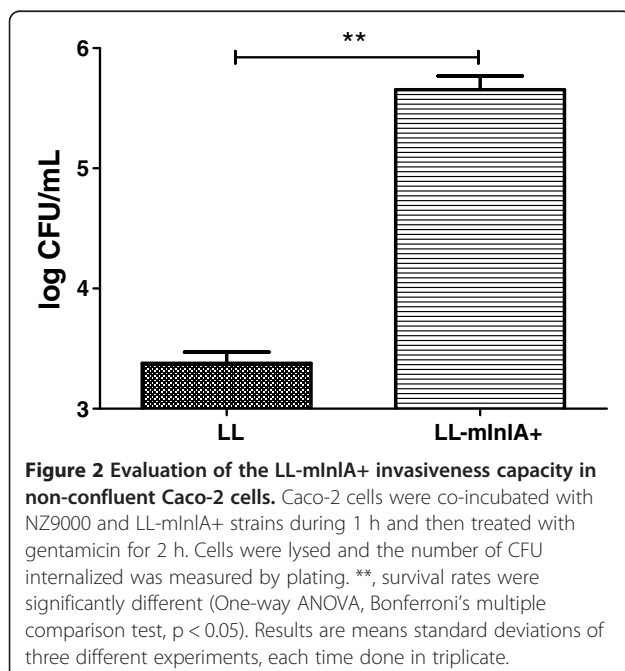
Non-confluent Caco-2 cells were incubated for 1 h with either NZ9000 or with LL-mInIA+. Non internalized bacteria were killed by gentamicin and intracellular



bacteria enumerated after lysis of the eukaryotic cells. The LL-*mInIA*+ strain exhibited 1000-fold greater invasion rate than NZ9000 strain (Figure 2).

LL-*mInIA*+ internalization analyzed by confocal microscopy

LL-*mInIA*+ and NZ9000 strains were labeled with CFSE dye and then incubated with Caco-2 cells for 1 h. Cells were fixed and confocal images were obtained. Very few



cell-associated bacteria could be detected after co-incubation with NZ9000 (Figure 3A). In contrast, the LL-*mInIA*+ strain strongly bound to the membrane of cell clusters which is compatible with the known binding of *InIA* to E-cadherin, a cell-cell adhesion molecule. In addition, LL-*mInIA*+ was located intracellularly in some cells (Figure 3C and B).

LL-*mInIA*+ can efficiently deliver *in vitro* a DNA vaccine containing β -lactoglobulin cDNA

To test the ability of LL-*mInIA*+ to deliver a DNA vaccine plasmid *in vitro* to IECs, we transformed LL-*mInIA*+ strain with pValac:BLG [32], a plasmid derived from pValac [23] containing the cDNA for BLG, under the control of an eukaryotic promoter to generate strain LL-*mInIA*+BLG (Table 1).

In order to monitor plasmid transfer and production of BLG in Caco-2 cells extracts, non-confluent Caco-2 cells were incubated with noninvasive *L. lactis* strains, LL and LL-BLG (see Table 1), or with LL-*mInIA*+BLG for three hours. After incubation with these bacteria, cell supernatant and proteins extracts from Caco-2 cells were tested for BLG expression using an EIA. BLG production was measured in Caco-2 cells protein extracts incubated with either LL-BLG or LL-*mInIA*+BLG. However, incubation with the LL-*mInIA*+BLG strain resulted in 10 fold higher levels of BLG compared to LL-BLG strain demonstrating that surface expression of *mInIA* enhanced intracellular delivery of the DNA vaccine DNA (Figure 4A).

Secreted levels of BLG were increased 2 fold after co-incubation with LL-*mInIA*+BLG compared to LL-BLG (Figure 4B). These data shows that LL and LL-*mInIA*+, can mediate gene transfer of a DNA vaccine to Caco-2 cells *in vitro* and that invasiveness significantly increases the efficiency of DNA delivery.

DNA delivery efficiency *in vivo* is slightly improved by the production of *mInIA*

Mice were intragastrically administrated with LL, LL-BLG or LL-*mInIA*+BLG for three consecutive days, and the small intestine removed for isolation of IECs. BLG production was detected in protein extracts from IECs of mice administrated with LL-BLG and LL-*mInIA*+BLG but not with control mice (Figure 5). In both of the LL-BLG and LL-*mInIA*+BLG treated groups, some mice did not show production of BLG suggesting that DNA delivery may be a stochastic event depending on environmental factors. Even if this trend was not statistically significant, the number of mice producing BLG (in each of the three individual experiments) was systematically higher (11 mice) in the group administrated with invasive bacteria than with noninvasive bacteria (8 mice producing BLG) suggesting that the LL-*mInIA*+strain is a

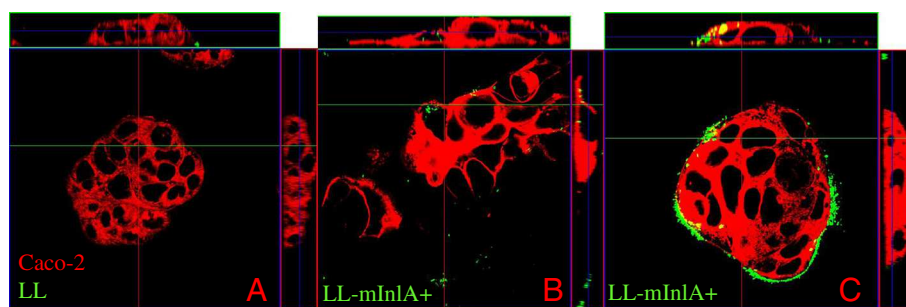


Figure 3 LL-mInIA+ internalization in Caco-2 cells analyzed by confocal microscopy. NZ9000 and *L. lactis* producing mutated internalin A (LL-mInIA+) were stained with CFSE dye (in green) and co-incubated with Caco-2 cells. Cell membranes were stained with Dil cell-labeling solution (in red) and the fluorescent samples were analyzed by confocal microscopy as described in the methods. **3A.** Non-internalization of NZ9000 strain in Caco-2 cells. **3B.** Intracellular localization of LL-mInIA+ in some cells. **3C.** LL-mInIA+ bind to the membrane of cell clusters where mInIA receptor, E-cadherin, is exposed.

slightly better DNA delivery vehicle than non-invasive strain.

Discussion

There is a large body of research demonstrating that the use of *L. lactis* is able to elicit humoral and cellular immune responses to an antigen produced in rodents (for reviews see [19-22]).

Recently, we showed the ability of either native or recombinant invasive *L. lactis* as both *in vitro* and *in vivo* DNA delivery vehicle [24-27]. Recombinant invasive *L. lactis* strains were obtained by producing heterologous invasins which are proteins expressed at the surface of pathogens responsible for their invasivity. We first built lactococci expressing Internalin A (InIA) from *Listeria monocytogenes* (LL-InIA+) and showed that LL-InIA+ were able to 1) deliver a plasmid *in vitro* and 2) be invasive *in vitro* and *in vivo* in guinea pigs [24].

Nevertheless, the use of LL-InIA+ is restricted because InIA does not bind efficiently to its murine receptor, the E-cadherin [33]. Subsequently, we produced another invasin, the Fibronectin Binding Protein A (FnBPA) from *Staphylococcus aureus* and demonstrated that LL-FnBPA + were invasive and able to transfer a plasmid *in vitro* more efficiently than non-invasive *L. lactis* [25]. However, FnBPA requires an adequate local concentration of fibronectin in order to bind to its receptors, integrins [28,29], and this limitation could be a problem *in vivo*. So, in this study we produced a mutated Internalin A (mInIA) at the surface of *L. lactis*. The two mutations introduced were demonstrated to allow the binding of mInIA to murine E-cadherin thus permitting *in vivo* experiments with conventional mice [30,31].

We first checked that mInIA was expressed and properly directed to the surface of *L. lactis*. The shift of fluorescence peak obtained for LL-mInIA+ in FACS

Table 1 Bacterial strains and plasmids used in this work

Strain/ plasmid	Relevant characteristics	Source/ reference
Bacterial strains		
NZ9000	A derivative of <i>L. lactis</i> MG1363 wild type strain generated by the integration of the <i>NisRK</i> genes	45
LL	<i>L. lactis</i> MG1363 containing pOri23 plasmid	40
LL-mInIA+	<i>L. lactis</i> NZ9000 strain containing pOri253:mInIA	This work
LL-BLG	<i>L. lactis</i> MG1363 strain containing pOri23 and pValac: BLG plasmid	32
LLmInIA+BLG	<i>L. lactis</i> NZ9000 strain expressing mInIA gene and carrying pValac: BLG plasmid	This work
Plasmids		
pPL2:mInIA	<i>E. coli</i> vector containing mInIA gene	30
pOri253Link	<i>L. lactis</i> - <i>E. coli</i> shuttle vector, Eryr	This work
pOri23	<i>L. lactis</i> - <i>E. coli</i> shuttle vector, Eryr	40
pValac: BLG	<i>L. lactis</i> - <i>E. coli</i> shuttle vector carrying the BLG gene under the control of the eukaryotic promoter IE CMV, Cmr	32
pOri253: mInIA	<i>L. lactis</i> - <i>E. coli</i> shuttle vector carrying the mInIA gene under the control of the constitutive PrfA promoter protein and harboring the native cell wall anchoring signal	This work

Eryr Erythromycin resistant; Cmr Chloramphenicol resistant.

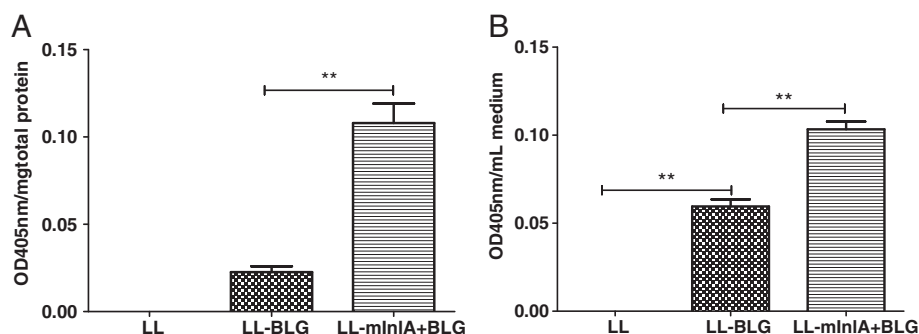


Figure 4 BLG production in Caco-2 cells after co-incubation with LL-mInIA+BLG or LL-BLG. Caco-2 cells were co-incubated with LL, LL-BLG or LL-mInIA+BLG during 3 h. BLG was assayed 72 h after co-incubation in cellular protein extracts (A) or medium (B). The results are expressed as mean \pm SE values. Statistical significance between the groups was calculated using the One Way ANOVA followed by the "Bonferroni" post-test. Values of $p < 0.05$ were considered significant.

analysis was significantly higher as compared to NZ9000 strain thus confirming successful surface expression of mInIA on *L. lactis*. Other invasins, from Gram-positive bacteria, such as InIA or FnBPA, have already been successfully expressed in *L. lactis* confirming that the signal peptide for secretion and the anchoring signal are well recognized by the *L. lactis* machinery. Production of invasins from Gram-negative bacteria, such as *Yersinia pseudotuberculosis* invasin at the surface of *L. lactis* has never been successful (Denis Mariat, personal communication).

The invasivity was assessed by gentamicin assay in non-differentiated E-cadherin expressing human epithelial cell line Caco-2 cells. This experiment showed that LL-mInIA+ strain is 1000-fold more invasive than NZ9000 strain. Wollert and collaborators (2007) observed a 2-fold increase in the adhesion and invasion efficiency of *L.*

monocytogenes strain producing mInIA compared to wild-type listeria expressing native InIA by using gentamicin-protection-invasion assays in Caco-2 cells [30]. A confocal image taken after gentamicin assay showed clearly that LL-mInIA+ is capable of adhering to and entering in non-differentiated Caco-2 cells. The preferential distribution of recombinant bacteria at the periphery of the Caco-2 cell islets can be explained by the fact that E-cadherin is accessible only at the periphery. A similar type of bacterial distribution, around the Caco-2 cell islets, was previously observed when Caco-2 cells were co-incubated with LL-FnBPA+[25].

LL-mInIA+ and LL strains were then transformed with pValac: BLG plasmid, co-incubated with Caco-2 cells and BLG expression was followed 72 h later by ELISA. BLG was detected in the cytoplasmic fraction of Caco-2 cells which were co-incubated with noninvasive and invasive strains carrying pValac: BLG. This data confirms prior observations that even noninvasive *L. lactis* can transfer functional plasmids to Caco-2 cells [23]. Cells were also capable of secreting the allergen, which is an interesting characteristic facilitating antigen uptake and presentation by professional APCs through cross-priming pathways [1]. The use of LL-mInIA+ improved BLG expression around ten times compared to noninvasive strain. Our hypothesis is that invasive lactococci can enter in higher numbers inside epithelial cells and thus deliver more plasmids.

Noninvasive and invasive *L. lactis*, carrying pValac: BLG or not, were orally administered for 3 consecutive days in BALB/c mice. On the fourth day, enterocytes from the small intestine were isolated and BLG production was measured by enzyme immunoassay (EIA). Isolated enterocytes from mice administered with invasive LL-mInIA+BLG produced the same amount of BLG as compared to mice immunized with noninvasive LL-BLG. Thus, we confirmed that noninvasive lactococci are able to transfer a functional plasmid *in vivo* in mice [27]. The

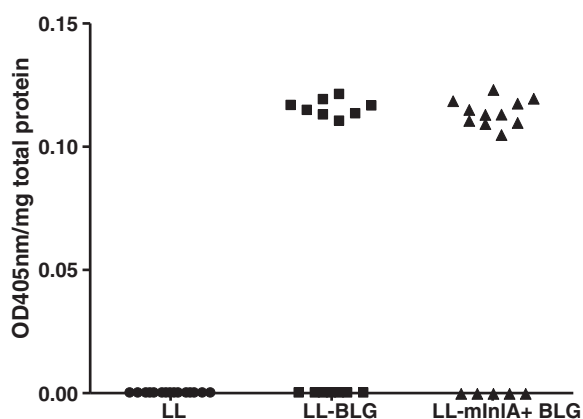


Figure 5 β -Lactoglobulin detection in mice isolated enterocytes after oral administration of noninvasive and invasive lactococci strains. Mice were orally administered 3 consecutive days with LL, LL-BLG or LL-mInIA+BLG. Seventy two hours after the last gavage, mice were sacrificed and BLG was assayed in protein extracts from isolated small intestine enterocytes. Results showed the sum of two independent experiments.

use of LL-mInIA+BLG enhanced slightly the number of mice positive for plasmid transfer. Surprisingly, BLG production was not increased.

These results partly confirmed what we published recently with LL-FnBPA+BLG *in vitro* and *in vivo* [32]. Oral administration in mice of LL-FnBPA+BLG or LL-FnBPA+GFP elicited a GFP or BLG production in enterocytes. As with LL-mInIA+ the BLG production was not increased with LL-FnBPA+. However the number of mice producing BLG was significantly higher after oral administration of LL-FnBPA+BLG compared to non invasive LL-BLG. Considering these results it seems that LL-FnBPA+strain is a better DNA delivery vehicle than LL-mInIA+.

As no significant advantages were observed by using LL-mInIA+BLG compared to LL-BLG, we hypothesize that interactions of recombinant mInIA with their receptors were impeded in mouse intestinal epithelium. This lack of invasion *in vivo* was also observed by another group working with *E. coli* strain expressing invasin from *Yersinia pseudotuberculosis* as an oral vaccine for cancer immunotherapy. They showed that invasive *E. coli* was unable to enter gut epithelial cells due to a basolateral localization of the receptor, B1-integrin [34]. They demonstrated that invasive *E. coli* expressing *Y. pseudotuberculosis* invasin were selectively uptaken from the intestinal lumen into Peyer's patches using an *ex vivo* model. Similarly, E-cadherin, the mInIA receptor, is also expressed on the basolateral membrane of IECs which are strongly linked to each other in the gut making E-cadherin less available. It has been shown recently that *L. monocytogenes* could enter the epithelial membrane through extruding epithelial cells at the top of the villi but mainly through goblet cells which are located deeper in the crypt [35]. It is thus possible that LL-mInIA+BLG strain is not able to reach its receptor deeply buried in the crypt. The pathway whereby bacteria could penetrate gut epithelial monolayers could be through Microfold (M) cells in Peyer's patches. These cells are able to take up particles/bacteria from the lumen [36]. Nevertheless, we cannot exclude any possibility that lactococci can also interact with other cells from the epithelial membrane such as dendritic cells. Some subset of dendritic cells is now well known to produce dendrites, able to reach the lumen in order to sample its content [37].

The other hypothesis is that the plasmid would be released in the lumen by lysed lactococci and then captured by the enterocytes. It has been shown that lactococci do not persist in the gut and are very sensitive to its physico-chemical condition [38]. Most likely, plasmid transfer *in vivo* is a combination of both mechanisms, bacteria and released plasmid captures. Considering these data, the use of lactobacilli which persist longer in

the gut than lactococci could be a better option for DNA delivery.

Conclusions

Mutated Internalin A protein was successfully expressed at the surface of *L. lactis* NZ9000, as demonstrated by FACS analysis. LL-mInIA+ strain was demonstrated to be 1000 times more invasive as compared to NZ9000 strain. This invasiveness capacity was confirmed by confocal microscopy experiments wherein LL-mInIA+ was found to be attached to Caco-2 cells and intracellularly located. Assays of BLG detection after BLG expression by eukaryotic cells revealed that the invasive status improved plasmid transfer *in vitro*. *In vivo*, the number of mice expressing BLG was higher (n = 11) in the group immunized with invasive bacteria than with noninvasive bacteria (n = 8). Even though this difference was not statistically significant, these study suggests that LL-mInIA+ strain can be used as a DNA delivery vehicle for *in vitro* or *in vivo* experiments. The use of other LAB species which can persist longer in the gastrointestinal tract, such as lactobacilli, to mediate DNA transfer is currently being evaluated.

Methods

DNA manipulation and plasmids construction

Procedures for DNA manipulation were carried out as described by Sambrook et al. (1989) [39], with a few modifications. Plasmids were purified by the alkaline lysis method after bacterial incubation for 30 min at 37°C in TES solution (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl pH 8) containing lysozyme (10 mg/ml). The quality of the DNA, including its concentration and purity, was estimated by measuring the absorbance at 260 nm and 280 nm in spectrophotometer (Spectra-Fluor Plus, Tecan). Restriction and modification endonucleases were used according to recommendations of the suppliers. Details concerning the plasmids used in this study are found in Table 1.

In order to construct pOri253Link:mInIA, mInIA gene was excised from pPL2:mInIA vector (9438 bp) [30] with BamHI and NotI restriction enzymes and gel purified generating a 3000 bp DNA fragment. pOri253Link plasmid (5857 bp) was derived from pOri253 [40] by modifying the multiple cloning site. Two complementary oligos CCGGGGGATCCTCGA GACGCGTCCATGGCGGCCGCTGCA and CCCTAG GAGCTCTGCGCAGGTACCGCCGGCG introducing the following restriction sites, BamHI, XhoI, MluI, NcoI and NotI were annealed and ligated into pOri253 previously digested with XmaI and PstI (underlined). BamHI/NotI-digested and purified pOri253Link and mInIA fragments were ligated using T4 DNA ligase (Invitrogen) to obtain pOri253:mInIA vector (9175 bp) (Table 1).

Finally, pOri253:mInIA was transformed in *E. coli* DH5 α and in *L. lactis* NZ9000 strain as described in the next section.

Bacterial strains, media and growth conditions

Bacterial strains are listed in Table 1. Briefly, *L. lactis* NZ9000 strain were grown in M17 medium containing 0.5% glucose (GM17) at 30°C without agitation and 10 μ g/ml of erythromycin (Ery) or 5 μ g/ml of chloramphenicol (Cm) were added, when required. Electroporation of *L. lactis* NZ9000 with pOri253:mInIA and/or pValac: BLG [32] plasmids was performed as described by Langella et al. (1993) [41]. Transformants were plated on GM17 agar plates containing Ery or Cm at the same concentration mentioned above and incubated at 30°C for two days before subsequent freezing or colony forming unit (CFU) counting. Positive clones were confirmed by colony PCR using specific oligos.

Mice handling

Specific pathogen-free BALB/c mice (females, 6 weeks of age; Janvier, France) were maintained under normal husbandry conditions in the animal facilities of the National Institute of Agricultural Research (UEAR, INRA, Jouy-en-Josas, France). All animal experiments began after allowing the animals 1 week for acclimation and were performed according to European Community rules of animal care and with authorization 78-149 of the French Veterinary Services.

Detection of mInIA expression by *L. lactis* using flow cytometry analysis

L. lactis NZ9000 and recombinant *L. lactis* expressing mInIA were centrifuged (5000 rpm), washed with phosphate buffered saline (PBS) and then resuspended at a concentration of approximately 1×10^9 CFU/ml in 500 μ l of PBS containing 0.5% of bovine serum albumin (BSA) and 10 μ g/mL of monoclonal antibody anti-InIA kindly provided by Dr. Pascale Cossart (Cell Biology and Infection Department/Unité des Interactions Bactéries-Cellules, Pasteur Institute, Paris). After one hour incubation at 4°C, the bacteria were pelleted by centrifugation washed with PBS and then resuspended in 500 μ l of PBS plus 0.5% of BSA containing fluorescein isothiocyanate (FITC)-conjugated AffiniPure Fab fragment Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research). After 1 h incubation at 4°C, bacteria were washed once more with PBS and fixed in 2% paraformaldehyde for 30 min at 4°C. FITC labeled antibody binding to InIA was assessed by flow cytometry (Accuri C6 Flow Cytometer[®]) using excitation at 494 nm and emission in the range of 510-530 nm (FL1-A channel). Data analysis was performed using CFlow Software (Accuri Cytometers, Inc.). The result was

expressed as the average of three independent experiments performed in triplicate.

Invasion assay of bacteria into intestinal epithelial cells

The human intestinal epithelial cell line Caco-2 (ATCC number HTB37) derived from a colon carcinoma was used to measure invasion capacity of each strain. Caco-2 cells were cultured in RPMI medium containing 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium) and 10% fetal calf serum in p-24 plates (Corning Glass Works) until they reached 70-80% confluence. In the assays on non-confluent Caco-2 cells, approximately 4×10^5 cells were present in each p-24 well. Bacterial strains were grown to an OD₆₀₀ of 0.9-1.0, pelleted and washed in PBS, then added to the Caco-2 cell cultures at a multiplicity of infection (MOI) of approximately 1000 bacteria per eukaryotic cell. The gentamicin survival assay was used to evaluate bacteria survival. In summary, recombinant or wild type *L. lactis* were applied in the apical side of eukaryotic cells and co-incubated during one hour at 37°C, in 5% CO₂. After this period, cells were washed in order to remove bacteria in excess and then 150 μ g/mL of gentamicin was added for 2 h to kill the extracellular bacteria. Cells were then lysed with 0.2% triton-X 100 diluted in water. Finally, serial dilutions of the cell lysate were plated for bacterial counting. CFU of intracellular bacteria were expressed as the average of three independent gentamicin assays performed in triplicate. Invasion rate was calculated as the ratio of CFU counts.

Confocal laser scanning

Bacteria were stained as described by Lee et al. (2004) [42]. Stationary phase culture of recombinant or wild type *L. lactis*, were washed twice in PBS and stained with 50 μ M of green fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) at 37°C for 20 min under constant shaking in the dark. CFSE labeled bacteria were used to perform the invasion assay as described above in non-differentiated Caco-2 cells grown on filter inserts. After 1 h of infection, cells were washed three times with PBS and fixed using 4% paraformaldehyde. Cell membranes were stained with 1 μ M Vybrant[®] CM-DiI cell-labeling solution (Invitrogen) for 1 h at room temperature. Cells were mounted in Vectashield solution (Vector Labs, Burlingame, USA) to minimize photobleaching. Confocal images were obtained using a Zeiss LSM 510 system consisting of a Zeiss Axioskop with a Zeiss Plan Neofluar 63x NA 1.3 oil objectives. Stacks of images were reconstructed using Zeiss LSM software.

β-Lactoglobulin (BLG) expression by human intestinal epithelial cells after incubation with bacteria

In order to measure BLG expression and secretion by human epithelial cells the gentamicin survival assay was performed with Caco-2 cells as described above, however, bacteria and Caco-2 cells were incubated for three hours. After gentamicin treatment, plates were maintained for 72 h at 37°C, in 5% CO₂. Supernatant was collected by centrifugation at 78.2 g (800 rpm) for 10 min and stored at -80°C. One mL of PBS supplemented with a cocktail of protease inhibitors (Roche) was then homogenized by sonication (3 times 10 s). Samples were kept at -80°C and used to measure BLG production using an Enzyme ImmunoAssay (EIA) described in the next section.

Enzyme immunometric assay (EIA) for quantification of bovine β lactoglobulin in human epithelial cells

The method used for BLG quantification is described elsewhere [43]. In summary, 96 microtitre plates were coated with 3.5 µg/ml of anti-BLG monoclonal antibody, diluted in 50 mM phosphate buffer (PB) pH 7.4, and incubated overnight at room temperature. After washing, plates were blocked with EIA buffer (0.1 M PB pH 7.4; 1 g/l BSA; 0.15 M NaCl; 0.001 M Na₂EDTA; 0.1 g/l sodium azide) and stored sealed at 4°C until use. Standard (recombinant BLG) and samples diluted in EIA buffer were added and kept at 4°C for 18 h. After this time, plates were extensively washed and then acetylcholinesterase conjugated monoclonal anti-BLG antibody (1 Ellman Unit/ml) was added for 18 h at 4°C. After washing, Ellman reagent was added and enzymatic reaction was measured at 405 nm in a spectrophotometer (SpectraFluor Plus, Tecan).

Oral administration of mice

Conventional BALB/c mice, 3 to 6 weeks of age were purchased from INRA animal care facilities (Jouy-en-Josas, France), acclimatized for 1 week before immunization under standard animal husbandry conditions in the animal facility (Unité d'Expérimentation Animale, Jouy-en-Josas, France). Mice (n = 8) were intragastrically administered with 1x10⁹ (CFU) of strains, LL, LL-BLG or LLmInLA-BLG on 3 consecutive days using a gavage tube feeding. On the fourth day, the small intestine was collected for subsequent BLG quantification in isolated IECs.

Intestinal epithelial cells isolation

Mice were euthanized, and their small intestines were removed, rinsed with complete DMEM medium (containing 2 mM L-glutamine and 10% fetal calf serum). The length of intestine was opened and submerged in buffer A (in mM: 120 NaCl, 4.7 KCl, 2.4 KCl, 1.2

KH₂PO₄, 1.2 Na₂HPO₄, 25 NaHCO₃, 10 HEPES, 5 EDTA, 0.5 DTT, 0.25% BSA; at pH 7.4 warmed to 37°C) for 20 min with agitation at 240 rpm [44]. Cells were collected by centrifugation (415.73 g – 2000 rpm – for 5 min) at room temperature, washed once with PBS and lysed by sonication (3 times, 10 s). The cell lysate was centrifuged for 10 min at 3143.98 g (5500 rpm), then the supernatant was recovered and stored at -80°C. The EIA to detect BLG was performed as described above.

Statistical analyses

The results are expressed as mean ± standard error (SE) values. Statistical significance between the groups was calculated using the One Way ANOVA (and nonparametric) test, followed by the “Bonferroni” post-test. Values of p < 0.05 were considered significant.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work presented here was carried out in collaboration between all authors. MA performed the main laboratory experiments and wrote the paper. JK helped with the confocal microscopy experiment and data analysis. FL constructed, provided pOri253:mInLA plasmid and initiated the project. PL, AM, and VA defined the research theme, helped to orient the work and revised the manuscript. JMC designed of the project, coordinated it, wrote and revised the manuscript. All authors have contributed to the writing of the paper and approved the final manuscript.

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CHAPTER 4

Immune response elicited by DNA vaccination using *Lactococcus lactis* is modified by the production of surface exposed pathogenic protein

Chapter 4: Immune response elicited by DNA vaccination using *Lactococcus lactis* is modified by the production of surface exposed pathogenic protein

3.1 Introduction

Previously, it was demonstrated both *in vitro* and *in vivo* that *L. lactis* could transfer a plasmid to mice intestinal epithelial cells (IECs) eliciting therefore a T helper cell type 1 (TH1) immune response (Guimaraes et al., 2006; Chatel et al., 2008). In order to understand the mechanism of plasmid transfer using lactococci and thus to improve it, we developed invasive *L. lactis* strains producing invasins at their surface. The first strain were engineered to express *Staphylococcus aureus* Fibronectin Biding Protein (LL-FnBPA+) which demonstrated to be a good vehicle to deliver therapeutic plasmids to mammalian cells, such as the eukaryotic expression vector pValac containing the cDNA of BLG (pValac:BLG) (Innocentin et al., 2009; Pontes et al., 2012). The other invasive lactococci was designed to express *Listeria monocytogenes* Internalin A (InIA) (LL-InIA+) and we showed that it could be used to transfer plasmid *in vivo* to guinea pigs (Guimaraes and others 2005). Nevertheless, as InIA cannot efficiently bind to its receptor, E-cadherin, in mice we decided to construct another invasive *L. lactis* strain expressing a mutated form of the InIA (mInIA). The use of mInIA appeared to be very interesting as it can bind to murine E-cadherin thus facilitating *in vivo* studies in conventional mice. Furthermore, we LL-mInIA+ could successfully deliver pValac:BLG to IECs *in vitro* and *in vivo* it tended to increase plasmid transfer (De Azevedo et al., 2012).

This work had the intent to compare if the immune response elicited by *L. lactis*-mediated DNA immunization could be modified by the surface-exposed production of invasins. Thus, conventional BALB/c mice were administered with noninvasive and invasive strains, both carrying pValac:BLG plasmid, and our results showed that either oral or nasal immunization with LL-FnBPA+ showed a tendency to induce a Th2 polarization while mice that received noninvasive lactococci showed a tendency to polarize the immune response to a Th1 type. We then checked weather the expression of an invasin at *L. lactis* surface could be the reason of this observed immune response. Therefore, LL-mInIA+ was used to answer to this question. Mice that received noninvasive *L. lactis* expressed a higher amount of INF- γ , a cytokine characteristic for a Th1 immune profile. On the other hand mice immunized with invasive strains (LL-FnBPA+ and LL-mInIA+) did not show any expression of INF- γ confirming what was observed before. The suppression of the Th1 type immune response was then attributed to the expression of invasins at *L. lactis* cell wall as LL-FnBPA+ and LL-mInIA+ elicited almost the same immune response.

3.2 Materials and methods

Specific pathogen-free BALB/c mice were used for immunization assays. For measurement of immune response mice received orally (10^{10} CFU) or intranasally (10^9 CFU) noninvasive (LL-BLG) or invasive (LL-FnBPA+ and LL-mInIA+) *L. lactis* strains during three consecutive days. Two weeks after the last administration mice received the same amount of bacteria again for 3 days consecutively. Subsequent to the last administration mice were either killed and bled after one week or sensitized by intraperitoneal injection of 5 μ g BLG after two weeks (Adel-Patient et al., 2003). Then, two weeks after BLG injection, sensitized mice were killed and bled. Serum of non-sensitized or sensitized mice was collected to be assayed for the presence of BLG-specific IgE, IgG1 and IgG2a by ELISA. Spleens were also collected from non-sensitized or sensitized mice; harvested and isolated splenocytes were reactivated with BLG for cytokines secretion (IFN- γ , IL-4 and IL-5) which was measured by ELISA as well. All statistical tests were done using GraphPad Prism version 5.00 and data were analyzed using ANOVA and Tukey's multiple comparison test. A p-value less than 0.05 were considered significant.

3.3 Results

It has been shown that DNA immunization using invasive (LL-FnBPA+) or noninvasive (LL-BLG) *L. lactis* strains elicits different BLG-specific primary immune responses. No BLG specific IgG1, IgG2a or IgE could be detected in the serum of orally or intranasally immunized animals. Splenocytes of mice administered with LL-FnBPA+ BLG strain exhibited a low TH2 BLG-specific immune response (secretion of IL-4 and IL-5) whereas a classical TH1 BLG-specific immune response was observed in mice treated with LL-BLG (secretion of IFN- γ). This TH1 profile which was induced by noninvasive *L. lactis* could protect the animals from further BLG sensitization with a significant decrease of 45% of BLG-specific IgE level, in contrast to mice pre-administered with LL-FnBPA+ BLG. In order to evaluate if the same cytokine profile could be induced in sensitized animals which were previously immunized with strains by oral or intranasal route, splenocytes were collected and assayed for the presence of IFN- γ , IL-4 and IL-5 cytokines. This analysis confirmed the Th2 orientation of immune response elicited by the administration of LL-FnBPA+ BLG (secretion of IL-4 and IL-5). We then moved to *in vivo* experiments with another invasive *L. lactis* (LL-mInIA+) to understand if this immune polarization could be due to the expression of invasins at the surface of the bacterium. Splenocytes of mice intranasally pre-treated with LL-BLG secreted only IFN- γ whereas no cytokines were detected in splenocytes of mice immunized with both invasive LL-FnBPA+ BLG or LL-mInIA+ BLG strains. This data confirmed that invasive lactococci does not elicit a Th1 BLG-specific primary immune response. As peptidoglycan (PG) of the cell wall plays an important role in bacterial adjuvanticity, we decided to investigate whether its composition were modified due to the expression of invasins at the surface of *L. lactis*. No differences could be detected.

3.4 Discussion

We demonstrated that *L. lactis* is able to transfer *in vivo* a fully functional plasmid to IECs (Guimaraes et al., 2006; Chatel et al., 2008). In order to understand and improve the DNA delivery we developed *L. lactis* strains producing invasins (LL-FnBPA+, LL-InIA+, LL-mInIA+) and we showed that, at least *in vitro*, they are better delivery vehicles compared to the noninvasive *L. Lactis* (Innocentin et al., 2009; De Azevedo et al., 2012; Pontes et al., 2012). *In vivo*, only LL-FnBPA+ strain could significantly increase the number of mice expressing BLG.

We previously published that a Th1 immune response is elicited by DNA vaccination using non-invasive lactococci (Chatel et al., 2008). In this work we observed the same immune polarization (TH1), characterized by the secretion of INF- γ by mice splenocytes. Differently, administration of LL-FnBPA+ BLG strain elicited a Th2 immune response characterized by secretion of IL-4 or IL-5 from splenocytes of mice pretreated with this strain. We investigated if this phenomenon was FnBPA-specific or if it was linked to the invasivity properties of LL-FnBPA+ strain. Another invasive *L. lactis*, LL-mInIA, was used to answer to this question and thus, together with the other ones, were administered in mice for measurement of immune response. Neither IL-4 nor IL-5 was detected but the level of IFN- γ secreted in splenocytes from mice administered with LL-BLG was significantly higher, suggesting that the expressing of both FnBPA and mInIA invasins direct the immune response more toward a weak Th2 response. It has been recently described that production of invasins at the surface of lactobacilli can modify their immunomodulatory properties (Fredriksen et al., 2012).

The majority of the works describes the use of attenuated pathogens as DNA delivery vector and, in general, they elicit a TH1 type immune response (Pamer 2004; Schoen et al., 2004; Vassaux et al., 2006; Abdul-Wahid and Faubert 2007). However, we have to point out that TH2 cytokines were not assayed in those works. DNA vaccination using non-invasive lactococci elicited this "classical" Th1 immune response while the use of LL-FnBPA+ or LL-mInIA+ invasive strains led to a low Th2 immune response. This difference in the immune response could be explained by the altered pathway that the strains will present after its inoculation in mice. For example, it was demonstrated that *E. coli* expressing an invasin from *Y. pseudotuberculosis* was mainly in the Peyer's patches of the animals while noninvasive *E. coli* can gain also entry but with less efficiently (Critchley-Thorne et al., 2006). Invasive *L. lactis* expressing InIA was able to enter in the epithelial membrane mainly through goblet cells, as it was demonstrated for *L. monocytogenes* (Nikitas et al., 2011), while the noninvasive strain which is not able to express neither InIA nor mInIA could cross the monolayer through a different pathway. The different immune response observed for invasive and noninvasive lactococci could also be explained by the composition of their cell wall. Some recent studies have revealed the crucial role of cell wall components in immune response against bacteria (Grangette et al., 2005; Mazmanian et al., 2005; Mazmanian et al., 2008). Therefore, we decided to check if the expression of invasins at the

surface of *L. lactis* could modify the peptidoglycan composition of our recombinant strain, but no differences were detected. The mechanisms by which invasive lactococci induces an immune response polarized to a TH2 profile remains to be discovered.

3.5 Conclusions

In this work we demonstrated that DNA immunization of conventional BALB/c mice with invasive (LL-FnBPA+, LL-mInIA+) or noninvasive (LL-BLG) *L. lactis* strains induces different BLG-specific primary immune responses. Mice administered with LL-FnBPA+ BLG strain revealed a low TH2 BLG-specific immune response, characterized by the secretion of IL-4 and IL-5, while a TH1 BLG-specific immune response was observed in mice treated with LL-BLG, characterized by the secretion of INF- γ . Furthermore, the induction of INF- γ protected the animals from further BLG sensitization. This effect was not observed in mice pre-treated with the invasive strain. The same immune profile was obtained even in sensitized animals which were earlier immunized with strains by oral or intranasal route, confirming previous results. Experiments using another invasive *L. lactis* (LL-mInIA+) had the intent to clarify if the immune polarization could be due to the expression of invasins at the surface of *L. lactis*. Mice intranasally immunized with LL-BLG secreted INF- γ whereas no cytokines were detected in administered with LL-FnBPA+ BLG or LL-mInIA+ BLG strains. This data confirmed that invasive lactococci does not elicit a Th1 BLG-specific primary immune response. Modification on the peptidoglycan (PG) caused by the expression of invasins at bacterial cell wall was implicated as the cause of this different immunogenicity observed between invasive and noninvasive *L. lactis*. No differences on PG composition could be identified. Taken together our data clearly demonstrates that the production of the invasins at *L. lactis* surface can modify their immunomodulatory properties, although no mechanism has been elucidated in this work.

1 Immune response elicited by DNA vaccination using *Lactococcus lactis* is modified by
2 the production of surface exposed pathogenic protein
3

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1 Abstract

2 Here, we compared immune responses elicited by DNA immunization using *Lactococcus*
3 *lactis* or *L. lactis* expressing the *Staphylococcus aureus* invasin Fibronectin Binding Protein
4 A (FnBPA) at its surface. Both strains carried pValac:BLG, a plasmid containing the cDNA
5 of Beta-Lactoglobulin (BLG), and were called respectively LL-BLG and LL-FnBPA+ BLG.
6 A low T_{H2} immune response characterized by the secretion of IL-4 and IL-5 in medium of
7 BLG reactivated splenocytes was detected after either oral or intranasal administration of LL-
8 FnBPA+. In contrast, intranasal administration of LL-BLG elicited a strong T_{H1} immune
9 response. After BLG sensitization, mice previously intranasally administered with LL-BLG
10 showed a significantly lower concentration of BLG-specific IgE than the mice non-
11 administered. Whereas administration LL-FnBPA+ BLG didn't modify the BLG-specific IgE
12 concentration obtained after sensitization confirming thus the T_{H2} orientation of the immune
13 response. To determine if the T_{H2}-skewed immune response obtained with LL-FnBpA+ BLG
14 was FnBPA-specific or not, mice received intranasally another *L. lactis* strain producing a
15 mutated form of the *Listeria monocytogenes* invasin Internalin A and containing pValac:BLG
16 (LL-mInIA+ BLG). As with LL-FnBPA+ BLG, LL-mInIA+ BLG was not able to elicit a T_{H1}
17 immune response. Furthermore, we observed that these difference were not due to the
18 peptidoglycan composition of the cell wall as LL-FnBPA+ BLG, LL-mInIA+ BLG and LL-
19 BLG strains share the same one. Thus, DNA vaccination using LL-BLG elicited a pro-
20 inflammatory T_{H1} immune response while using LL-FnBPA+ BLG or LL-mInIA+ BLG
21 elicited a low anti-inflammatory T_{H2} immune response.

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23

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1 Introduction

2

3 We previously developed an innovative strategy using *Lactococcus lactis*, a food grade
4 bacterium, to deliver plasmids *in vitro* [1] and *in vivo* [2]. We demonstrated first that a non
5 invasive, transiting bacterium could transfer a plasmid to the epithelial membrane of the small
6 intestine in mice and such elicit a T helper cell type 1 (T_{H1}) immune response [2]. T_{H1}
7 immune is typical for DNA vaccination [3] even if various factors as antigen nature or mouse
8 strain could influence the immune response [4]. To understand the mechanism of plasmid
9 transfer and thus to improve it, we developed invasive *L. lactis* strains by expressing invasins
10 at their surface.

11 We constructed LL-FnBPA+, a recombinant *L. lactis* strain producing at its surface the
12 *Staphylococcus aureus* Fibronectin Binding protein A (FnBPA). We demonstrated that LL-
13 FnBPA+ can be used to deliver plasmid DNA *in vitro* [5] and more recently that the use of
14 invasive LL-FnBPA+ increased plasmid transfer rate/efficacy *in vivo* [6]. We constructed also
15 a recombinant *L. lactis* strain producing *Listeria monocytogenes* Internalin A (LL-InlA) and
16 showed that LL-InlA could be used to transfer plasmid *in vivo* to guinea pigs [7]. As InlA
17 binds only poorly to murine E-cadherin, the use of this strain is thereby limited to either
18 transgenic mice expressing human E-cadherin [8] or guinea pigs. InlA has been mutated to
19 increase its binding to murine E-cadherin [9]. We produced the mutated mInlA at the surface
20 of *L. lactis*, LL-mInlA+, and showed that LL-mInlA+ did not increase significantly the
21 plasmid transfer in mice [10].

22 In this paper, we evaluated if the immune response elicited by *L. lactis*-mediated DNA
23 immunization could be modified by the surface-exposed production of invasins. We first
24 demonstrated that intranasal or oral DNA administration using invasive LL-FnBPA carrying
25 pValacBLG, LL-FnBPA+ BLG, elicits a low T_{H2} primary immune response while the non
26 invasive strain LL-BLG elicited a classical T_{H1} immune response. These results were
27 confirmed using another *L. lactis* strain producing a mutated InlA and carrying pValacBLG,
28 LL-mInlA+ BLG. It was also shown that the differences between immune responses observed
29 after mucosal administration of invasive and non invasive strains were not due to a difference
30 in peptidoglycan composition. Our data thus evidenced that production of two unrelated
31 different invasins from pathogens led to the similar modification of the intrinsic
32 immunomodulatory properties of *L. lactis*.

33

34

1 Material and Methods.

2 Ethics statement

3 All procedures were carried out in accordance with European and French guidelines for the
4 care and use of laboratory animals. Permission 78-123 is a permit number dedicated to
5 P.Langella. MICALIS (Microbiologie de l'Alimentation au Service de laSante') review board
6 specifically approved this study.

7

8 Bacterial strains, plasmids, media and growth conditions. Bacterial strains and plasmids
9 used in this study are listed in Table 1. *L. lactis* subsp. *cremoris* strains were grown in M17
10 medium containing 0.5% glucose (GM17) at 30°C. *E. coli* strains were grown in Luria–
11 Bertani medium and incubated at 37°C with vigorous shaking. Antibiotics were added at the
12 indicated concentrations as necessary: erythromycin, 500 µg/ml for *E. coli*, and 5 µg/ml for *L.*
13 *lactis*; chloramphenicol, 10 µg/ml for *E. coli* and *L. lactis*.

14

15 Mice handling.

16 Specific pathogen-free BALB/c mice (females, 6 weeks of age; Janvier, France) were
17 maintained under normal husbandry conditions in the animal facilities of the National
18 Institute of Agricultural Research (UEAR, INRA, Jouy-en-Josas, France). All animal
19 experiments were started after the animals were allowed 2 weeks of acclimation and were
20 performed according to European Community rules of animal care and with authorization 78-
21 149 of the French Veterinary Services.

22 Apparatus and reagents. All enzymatic immunoassays were performed in 96-well microtitre
23 plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek
24 microtitration equipment from Labsystems (Helsinki, Finland). Unless otherwise stated, all
25 reagents were of analytical grade from Sigma (St Louis, MO, USA). BLG was purified from
26 cow's milk as previously described [11].

27 Quantification of BLG-specific IgE, IgG1 and IgG2a. Blood samples were obtained from
28 the retro-orbital venous plexus, centrifuged, 0.1% sodium azide was added as preservative,
29 and the sera were stored at -20 °C until further assay. Naïve mice were bled on the same days
30 to assess non-specific binding. Each immunization group was composed of 8 mice. BLG-
31 specific IgE, IgG1 and IgG2a were measured using immunoassays, as previously described,
32 allowing quantification of antibodies recognizing both native and denatured BLG [12].

1 Quantification of specific IgE is preceded by removal of serum IgG using protein G
2 immobilized on porous glass (PROSEP, Bioprocessing, Consett, UK) avoiding thus IgG
3 interference in IgE detection [13]. The minimum detectable concentrations are 8 pg/ml for
4 IgE, 7 pg/ml for IgG1 and 12 pg/ml for IgG2a.

5 Cytokines production. Mice were humanely killed and spleens were harvested under sterile
6 conditions, and pooled per immunization group. After lysis of red blood cells (180 mM
7 NH_4Cl , 17 mM Na_2EDTA) and several washes, splenocytes were resuspended in RPMI-10
8 (RPMI supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U penicillin, 100
9 mg/mL streptomycin). Cells were incubated for 60 h at 37°C (5% CO_2) in 96-well culture
10 plates (10^6 cells/well) in the presence of BLG (20 $\mu\text{g/mL}$) or concanavalin A (1 $\mu\text{g/mL}$,
11 positive control). Incubations with PBS or ovalbumin (20 $\mu\text{g/mL}$) were done as negative
12 controls. Supernatants were then removed and stored at -80°C until further assay. IFN- γ , IL-4
13 and IL-5 was assayed using CytoSets™ kits (BioSource International Europe, Nivelles,
14 Belgium). Limits of detection are respectively of 5 pg/ml, 3 pg/ml and 1 pg/ml for IL-4, IL-5
15 and IFN- γ .

16 Primary immune response elicited by oral or intranasal administration of bacteria
17 strains. Strains were grown to saturation (overnight (ON) cultures) as described above.
18 Before administration strains were centrifuged 10 min, 5,000 g at 4°C, and then resuspended
19 in PBS to wash the bacteria. Washed bacteria were centrifuged again and the pellet was
20 resuspended in PBS containing Fetal Calf Serum 10% to provide fibronectin during 2 hours at
21 4°C [14]. Then the strains were pelleted and resuspended in PBS. Group of mice (n=8) were
22 fed orally with 10^{10} CFU/mouse or received intranasally 10^9 CFU in 10 μl per nostril during 3
23 days. Two weeks after the last administration mice received again 3 days consecutively 10^{10}
24 CFU/mouse by oral administration or 10^9 CFU by intranasal administration. One week after
25 the last administration mice were killed and bled. Naïve mice were killed and bled the same
26 day to assess non-specific immune response.

27 Immune response after sensitization in mice intranasally or orally administered with
28 bacteria strains. Strains were grown to saturation (overnight (ON) cultures) as described
29 above. Before administration strains were centrifuged 10 min, 5,000 g at 4°C, and then
30 resuspended in PBS to wash the bacteria. Washed bacteria were centrifuged again and the

1 pellet was resuspended in PBS containing Fetal Calf Serum 10%, to provide fibronectin,
2 during 2 hours at 4°C [14]. Then the strains were pelleted and resuspended in PBS. Group of
3 mice (n=8) were fed orally with 10¹⁰ CFU/mouse or received intranasally 10⁹ CFU in 10 µl
4 per nostril during 3 days. Two weeks after the last administration mice received again 3 days
5 consecutively 10¹⁰ CFU/mouse by oral administration or 10⁹ CFU by intranasal
6 administration. Two weeks after all mice were sensitized by i.p. injection of 5 µg BLG
7 adsorbed on alum (1 mg/mouse; Alhydrogel 3%, Superfos Biosector als, Denmark) [15].
8 Injected volume was 0.2 ml per mouse. Two weeks after mice were humanely killed and bled.
9 Serum was collected to assessed BLG-specific IgE, IgG1 and IgG2a concentrations as
10 described above. Naïve mice (n=8) were bled the same day to assess non-specific immune
11 response. Naive sensitized mice received PBS orally or intranasally instead of bacteria.
12 Spleen were harvested and isolated splenocytes were reactivated for cytokines secretion as
13 described above.

14 **Statistical analyses.** All statistical calculations were done using GraphPad Prism version 5.00
15 for Windows (GraphPad Software, San Diego, CA). Data were analyzed using analysis of
16 variance (ANOVA) and Tukey's multiple comparison test. A p-value less than 0.05 was
17 considered significant.

18

19 **Peptidoglycan structural analysis.** Peptidoglycan was extracted from exponential phase
20 cultures of the different *L. lactis* strains as described previously [16]. Peptidoglycan was then
21 hydrolyzed with mutanolysin and the reduced soluble muropeptides were then separated by
22 RP-HPLC with an Agilent UHPLC1290 system using ammonium phosphate buffer and
23 methanol linear gradient as described previously [17].

24

1

2 Results

3 DNA immunization using LL-FnBPA+ BLG or LL BLG strains elicits different BLG-
4 specific primary immune responses.

5 In order to know if the production of FnBPA at the surface of *L. lactis* could influence its
6 immunomodulatory properties, mice were orally or intranasally administered with LL-wt
7 (control), LL-BLG or LL-FnBPA+ BLG strains and the BLG specific primary immune
8 response was monitored. No BLG specific IgG1, IgG2a or IgE could be detected in mice sera
9 whatever the administration route (data not shown). Splenocytes from orally and intranasally
10 administered mice were reactivated with BLG and secreted cytokines, IFN- γ , IL-4 and IL-5
11 were assayed in the medium. Splenocytes of mice intranasally (Fig. 1) or orally immunized
12 (Fig. 2) with LL- BLG strain secreted only IFN- γ . IL-4 and IL-5 were detected in medium of
13 BLG-reactivated splenocytes from mice receiving intranasally LL-FnBPA+ BLG (Fig. 1).
14 Moreover, only IL-5 cytokine secretion by BLG-reactivated splenocytes from mice orally
15 immunized with LL-FnBPA+ BLG was observed (Fig. 2). No IL-4 secretion was detected in
16 mice orally administered with LL-FnBPA+ BLG (data not shown). Thereby, mice treated
17 with LL-FnBPA+ BLG exhibit a low T_H2 BLG-specific immune response whereas a classical
18 T_H1 BLG-specific immune response was observed in mice treated with LL-BLG.

19

20 After intranasal administration with LL-BLG mice were protected from further BLG
21 sensitization in contrast to mice pre-administered with LL-FnBPA+ BLG.

22 After intranasal or oral administration of LL-wt, LL-BLG or LL-FnBPA+ BLG strains, mice
23 were sensitized with BLG in alum in order to elicit a Th2 immune response. The
24 concentrations of BLG-specific IgG1, IgG2a and IgE were monitored in sera of sensitized and
25 intranasally pre-treated mice (Fig. 3). Naïve sensitized mice (mice not receiving bacteria)
26 exhibited a T_H2 immune response characterized by a high level of BLG-specific IgG1 and
27 IgE. Pre-treatment with LL-wt control strain had no effect on IgG1, IgG2a or IgE level.
28 Despite a decrease of 25% in average, there was no significant difference in IgE level
29 between naive sensitized mice and mice pre-treated with LL-FnBPA+ BLG (Fig. 3). In
30 contrast, mice pre-treated with LL-BLG showed a statistically significant decrease of 45% of
31 BLG-specific IgE level (Fig. 3).

32 No differences in the levels of IgG1, IgG2a or IgE could be detected between mice orally
33 immunized with LL-wt, LL-BLG or LL-FnBPA+ BLG strains and naive sensitized mice (data
34 not shown).

1
2 Cytokines Secreted by BLG reactivated splenocytes after BLG sensitization confirmed
3 the Th2 orientation of immune response elicited by the administration of LL-FnBPA+
4 BLG.
5 Splenocytes of mice sensitized after intranasal or oral pre-treatment with either LL-FnBPA+
6 BLG or LL-BLG strains were reactivated by BLG and IFN- γ , IL-4 and IL-5 cytokines were
7 assayed in medium. In mice pre-treated with LL-wt control strain, the IFN- γ (Fig. 4A, Fig.
8 5A) and IL-5 (Fig. 4C, Fig. 5C) levels were lower compared to the naïve-sensitized group.
9 Therefore, a slight increase of IL-4 levels (Fig. 5B) was observed only for mice orally pre-
10 treated with LL-wt strain. Intranasal and oral pre-treatments with LL-BLG did not modify the
11 levels of any cytokines. However, spleen cells from all sensitized mice pre-treated with LL-
12 FnBPA+ BLG secreted higher amounts of IL-4 (Fig. 4B, Fig. 5B) and IL-5 (Fig. 4C, Fig. 5C)
13 than those of the naïve-sensitized group.
14
15 Intranasal administration of invasive LL-mInLA+ BLG does not elicit a Th1 BLG-
16 specific primary immune response.
17 To study if the Th2 orientation of the immune response was or not FnBPA-specific, another
18 invasive strain, LL-mInLA+ BLG, was administered intranasally to mice. Splenocytes from
19 mice pre-treated with LL-BLG, LL-FnBPA+ BLG or LL-mInLA+ BLG were reactivated with
20 BLG and IFN- γ , IL-4 and IL-5 were assayed in the medium. Splenocytes of mice pre-treated
21 with non invasive LL-BLG secreted only IFN- γ (Fig. 6) whereas no cytokines were detected
22 in media of mice pre-treated with both invasive LL-FnBPA+ BLG or LL-mInLA+ BLG
23 strains.
24
25 Peptidoglycan composition of cell wall from LL-BLG, LL-FnBPA+ BLG and LL-
26 mInLA+ BLG is highly similar.
27 Peptidoglycan (PG) of cell wall is well known for its adjuvanticity role. We studied if the
28 production of heterologous proteins as invasins at the surface of *L. lactis* would modify its
29 composition. The PG structure of the different strains LL-BLG, LL-FnBPA+ BLG and LL-
30 mInLA+ BLG was compared. For this purpose, PG was extracted from each strain, digested
31 with mutanolysin and the resulting muropeptides were separated by RP-HPLC. Comparison
32 of the obtained muropeptide profiles (Fig. 7) did not reveal any differences at the level of PG
33 structure between the strains.

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Discussion

In this paper, we compared the immune response induced in mice by administration of either non invasive or recombinant *L. lactis* strain producing FnBPA or mInLA. We previously demonstrated *in vitro* and *in vivo* that *L.lactis* is able to transfer a fully functional plasmid to eukaryotic cells of the murine epithelial membrane [1,2]. In order to understand and improve the DNA delivery we developed recombinant strains producing invasins from various pathogens, as FnBPA, InlA or a mutated form of InLA. *In vitro*, we showed that all our invasive strains were better DNA delivery vectors than non invasive strains [7,10,14]. *In vivo*, the use of the invasive LL-FnBPA+ BLG strain increased the number of mice producing BLG but not the quantity of BLG produced [14] whereas the use of LL-mInlA+ BLG did not change significantly none of both parameters [10]. Here, we would like to determine the incidence of the invasin expression on the nature of the immune response elicited by administration of such strains compared to on invasive ones.

Although since 1996 it was clearly shown [3] that Th1 was the main immune response elicited by DNA immunization, several previously approaches had demonstrated that the nature of the antigen [4,18,19], the route of administration [20,21,22] or the genetic background of the host [4,23] could influence the immune response.

We previously published that a Th1 immune response is elicited by DNA vaccination using non-invasive lactococci [2]. Here, we confirmed this result and we reported for the first time intranasal DNA immunization using our recombinant invasive lactococci strains. After intranasal administration, the wt LL-BLG strain can induce a Th1 immune response. We compared the immune responses observed with LL-BLG using different routes of administration and showed that intranasal immunization was more effective than oral immunization. Similar results have already been described for protein vaccination using LAB [24,25].

Surprisingly, intranasal and oral administration of LL-FnBPA+ BLG strain elicited a Th2 immune response characterized by secretion of IL-4 or IL-5 by BLG reactivated splenocytes in mice orally or intranasally pretreated with LL-FnBPA+ BLG. After BLG sensitization, no decrease of IgE concentration was observed in mice. The results clearly showed that DNA immunization using invasive LL-FnBPA+ BLG strain orientated the immune response toward a low Th2-type in contrast to the Th1-type response elicited by DNA vaccination using non invasive LL-BLG strain. We explored if this phenomenon was FnBPA-specific or if it was linked to the invasivity properties of our strain. In order to answer

1 to this question, we performed the same type of experiments using a new recombinant
2 invasive strain producing a mutated form of Internalin A from *Listeria monocytogenes*, LL-
3 mInIA+ BLG. Invasivity of LL-FnBPA+ BLG and LL-mInIA+ BLG were tested *in vitro* and
4 were comparable [10]. *In vivo* BLG expression detected in enterocytes was comparable
5 between mice orally administered with LL-BLG, LL-FnBPA+ BLG or LL-mInIA+ BLG (data
6 not shown). We were not able to detect any IFN γ in medium of BLG reactivated splenocytes
7 from mice treated with LL-mInIA+ BLG. In this experiment, neither IL-4 nor IL-5 was
8 detected but the level of IFN γ secreted by BLG reactivated splenocytes from mice
9 administered with LL-BLG was 10-times higher than in the previous experiments described in
10 this paper. This suggests that the production of both FnBPA and mInIA invasins at the surface
11 of *L. lactis* direct the immune response not toward a Th1 immune response but more toward a
12 weak Th2 response.

13 It has been recently described that production of invasins at the surface of lactobacilli
14 can modify their immunomodulatory properties. The production of invasin from *Yersinia*
15 *pseudotuberculosis* at the surface of *Lactobacillus plantarum* changed its profile from neutral
16 to pro-inflammatory [26]. They determined the immunomodulatory properties of their strain
17 only *in vitro* using monocytes stably transfected with NF- κ B reporter system.

18 Usually, most of the bacteria used as DNA delivery vector are attenuated pathogens
19 like *L. monocytogenes* or recombinant invasive *E. coli* [27,28]. *L. monocytogenes* infection is
20 accompanied by a strong innate immune response followed by a T-cell activation of Th1 type
21 [29]. Attenuated *Salmonella typhimurium* delivering a DNA vaccine coding for cyst wall
22 protein-2 (CWP-2) from *Giardia lamblia* was able to elicit a mixed CWP-2 specific cellular
23 Th1/Th2 immune response after oral administration [30]. Humoral immune response was
24 characterized by the presence of CWP-2 specific IgG2a in sera. The use of invasive *E. coli*
25 expressing the invasin from *Y. pseudotuberculosis* and Listeriolysin-O (LLO) from *L.*
26 *monocytogenes* could elicit a Th1 immune response characterized by secretion of IFN γ from
27 reactivated splenocytes. Same results were obtained when non invasive *E. coli* was used as
28 DNA delivery vector but the induction of immune response is less effective. It has to be noted
29 that no Th2 cytokines were assayed. Protection against challenge could be induced with a
30 better efficiency than naked DNA[31]. Using the same type of bacterial vector to deliver GFP,
31 Harms et al. observed a mixed Th1/Th2 immune response for both invasive and non invasive
32 *E. coli*. The secondary immune response provides a more defining Th1 cytokine profile [32].
33 Our results with DNA vaccination using non-invasive lactococci elicited this "classical" Th1

1 immune response while the use of LL-FnBPA+ or LL-mInlA+ invasive strains led to a low
2 Th2 immune response. It has to be noticed that the immune response elicited by the use of
3 invasive *L. lactis* is a low level response. It is possible that the main immune response was a
4 tolerization characterized by a high number of Treg.

5 We know that *L. lactis* invasive or non invasive strains can transfer plasmids *in vivo*,
6 to enterocytes [14] but, other subset of epithelial cells could be targeted by the invasive LAB
7 thus modifying the immune response. Tropism of non invasive *E. coli* and invasive *E. coli*
8 expressing at its surface the invasins from *Y. pseudotuberculosis*, LLO from *L. monocytogenes*,
9 both also expressing GFP protein, were observed *ex vivo* in murine small intestine tissue.
10 Invasive *E. coli* was mainly detected in the Peyer's patches. Non invasive *E. coli* can gain also
11 entry to the Peyer's patches but with less efficiency [33]. Invasive bacteria were also assayed
12 in Peyer's patch antigen-presenting cells. 3% of the DCs and 0.5% of the leukocytes were
13 GFP positive [33]. E-cadherin, mInlA receptor, is expressed on the basolateral membrane of
14 epithelial cells which are strongly linked to each other in the gut turning E-cadherin less
15 available. It has been shown recently that *L. monocytogenes* could enter in the epithelial
16 membrane through extruding epithelial cells at the top of the villi but mainly through goblet
17 cells which are located deeper in the crypt [34].

18 The difference of immune response between invasive and non invasive lactococci
19 could also be explained by a difference in the composition of the cell wall. Indeed some
20 recent studies have revealed the crucial role of cell wall components in immune response
21 against bacteria like *Bacteroides fragilis* [35,36] or probiotics [37,38]. Thus, we decided to
22 check if the expression of invasins at the surface of *L. lactis* could modify the peptidoglycan
23 composition of our recombinant strain, but no differences were detected.

24 To better understand why *L. lactis* recombinant invasive strains produced a low Th2
25 response, we need to go further in the characterization of the immune response, for example
26 the subset of T cell involved, the nature of cells taken up our bacteria or plasmid. It would be
27 interesting to know if the same bias is observed when we use our invasive bacteria to deliver
28 proteins instead of plasmid. The data described here will help us to choose the vehicle
29 depending on the nature of the desired immune response, pro-inflammatory or anti-
30 inflammatory, to be induced by DNA delivery.

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1
2 Figure legends.

3
4 Figure 1. BLG-specific cytokines secreted by BLG-reactivated splenocytes from mice
5 intranasally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG.

6 Mice were intranasally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG then
7 splenocytes were purified and reactivated with BLG. Secreted cytokines, IFN- γ (A), IL-4 (B)
8 and IL-5 (C) were assayed in medium. Sum of two independent experiments, 8 mice/group.

9
10 Figure 2. BLG-specific cytokines secreted by BLG-reactivated splenocytes from mice
11 orally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG.

12 Mice were orally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG then splenocytes
13 were purified and reactivated with BLG. Secreted cytokines, IFN γ (A) and IL-5 (B) were
14 assayed in medium. 8 mice/group.

15
16 Figure 3. BLG-specific IgG1, IgG2a and IgE in sera of mice intranasally administered
17 with LL-wt, LL-BLG or LL-FnBPA+ BLG and then sensitized with BLG. BLG specific
18 IgG1 (A), IgG2a (B) and IgE (C) were assayed in sera of mice intranasally administered or
19 not (naive-sensitized, NS) with LL-wt, LL-BLG or LL-FnBPA+ BLG then sensitized with
20 BLG in Alum in order to elicit a Th2 immune response. Sum of two independent experiments,
21 8 mice/group.* indicates $P < 0.05$, ANOVA and Tukey's post test.

22
23 Figure 4. BLG-specific cytokines secreted by BLG-reactivated splenocytes from mice
24 intranasally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG then BLG
25 sensitized. Mice were intranasally administered or not (NS) with LL-wt, LL-BLG or LL-
26 FnBPA+ BLG then sensitized with BLG in Alum. Splenocytes were purified and reactivated
27 with BLG. Secreted cytokines, IFN- γ (A), IL-4 (B) and IL-5 (C) were assayed in medium.
28 The results presented here are from one experiment representative of two performed
29 independently, 8 mice/group.

30
31 Figure 5. BLG-specific cytokines secreted by BLG-reactivated splenocytes from mice
32 orally administered with LL-wt, LL-BLG or LL-FnBPA+ BLG then BLG sensitized.
33 Mice were orally administered or not (NS) with LL-wt, LL-BLG or LL-FnBPA+ BLG then
34 sensitized with BLG in Alum. Splenocytes were purified and reactivated with BLG. Secreted

1 cytokines, IFN- γ (A), IL-4 (B) and IL-5 (C) were assayed in medium. The results presented
2 here are from one experiment representative of two performed independently, 8 mice/group.

3

4 Figure 6. BLG-specific cytokines secreted by BLG-reactivated splenocytes from mice
5 intranasally administered with LL-wt, LL-BLG, LL-FnBPA+ BLG or LL-mInIA+ BLG.
6 Mice were intranasally administered with LL-wt, LL-BLG, LL-FnBPA+ BLG or LL-mInIA+
7 BLG then splenocytes were purified and reactivated with BLG. Secreted IFN- γ was assayed
8 in medium.

9

10 Figure 7. Peptidoglycan composition of cell wall from lactococci recombinant strains

11 The PG structure of the different strains LL-BLG (A), LL-FnBPA+ BLG (B) and LL-mInIA+
12 BLG (C) was compared. PG was extracted from each strain, digested with mutanolysin and
13 the resulting muropeptides were separated by RP-HPLC. Absorbance was monitored at 220
14 nm.

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2 Table 1 Plasmids and strains used in this study

Strains	Properties	Reference
LL-wt	MG1363 <i>L. lactis</i> strain carrying pIL253 plasmid, Ery ^r	[39]
LL- BLG	MG1363 <i>L. lactis</i> strain carrying pIL253 and pValac:BLG plasmid, Ery ^r , Cm ^r	[14]
LL-FnBPA+ BLG	MG1363 <i>L. lactis</i> strain expressing FnBPA and carrying pValac:BLG, Ery ^r , Cm ^r	[14]
LL-mInIA+ BLG	NZ9000 <i>L. lactis</i> strain expressing mInIA and carrying pValac:BLG, Ery ^r , Cm ^r	[40]

3 Ery^r, Erythromycin; Cm^r, Chloramphenicol

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FIGURE 1

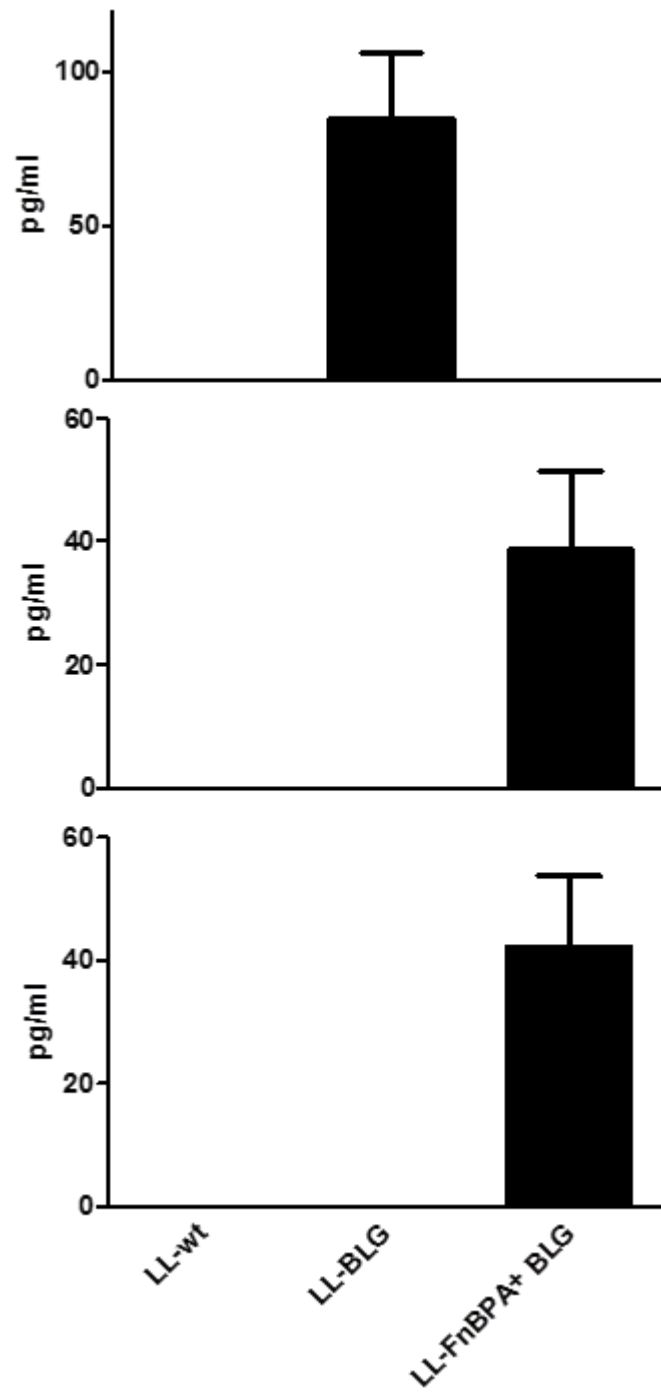


FIGURE 2

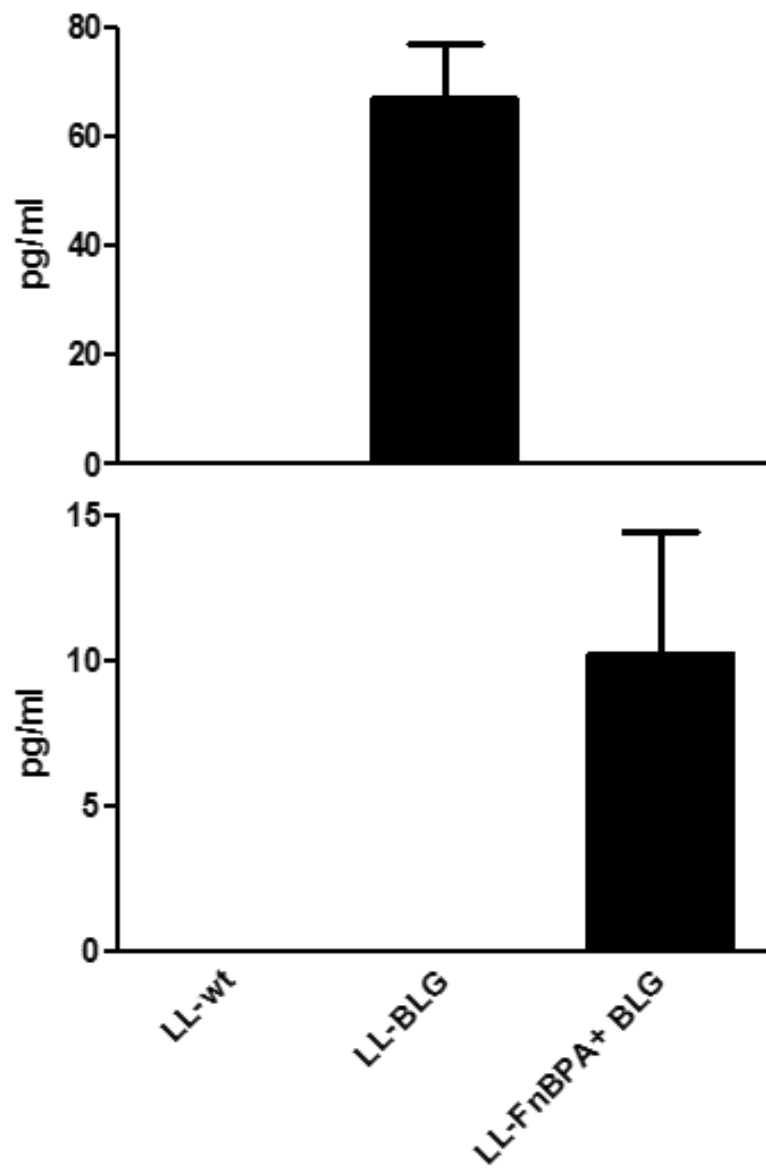


FIGURE 3

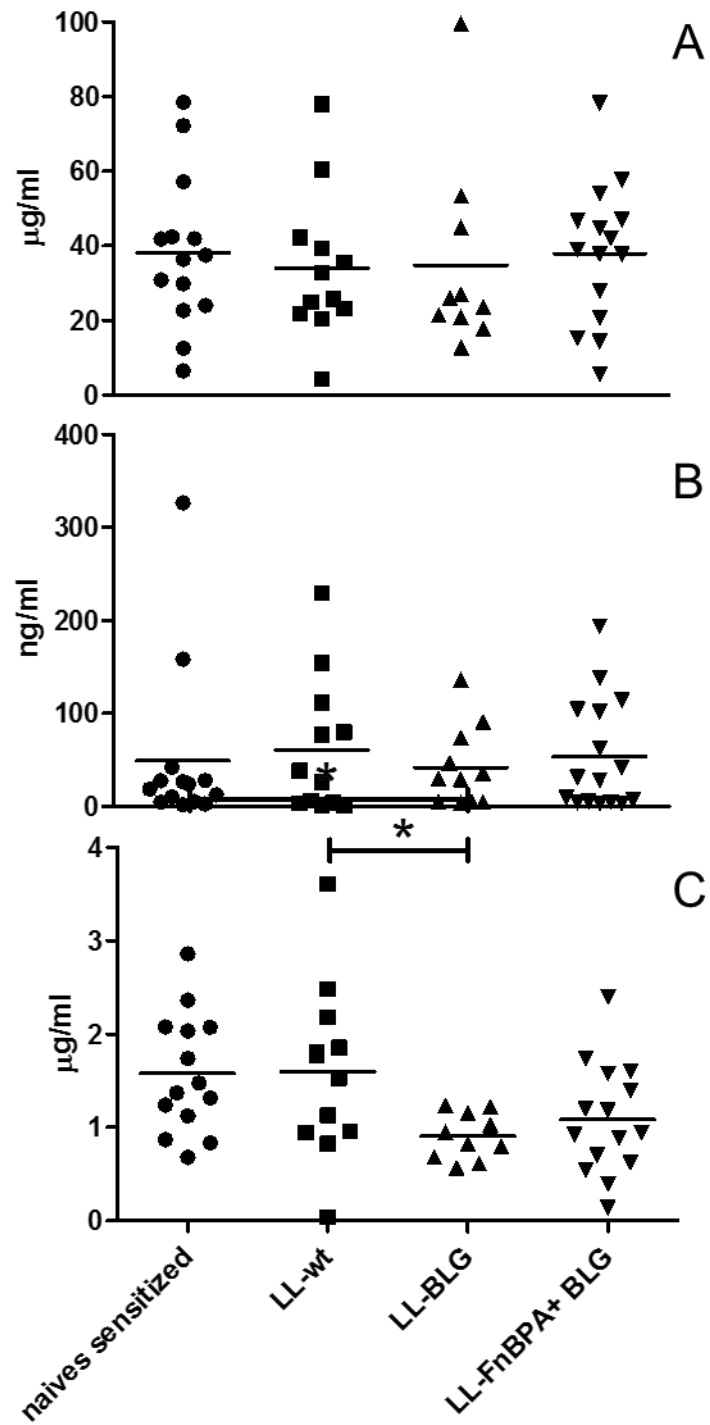


FIGURE 4

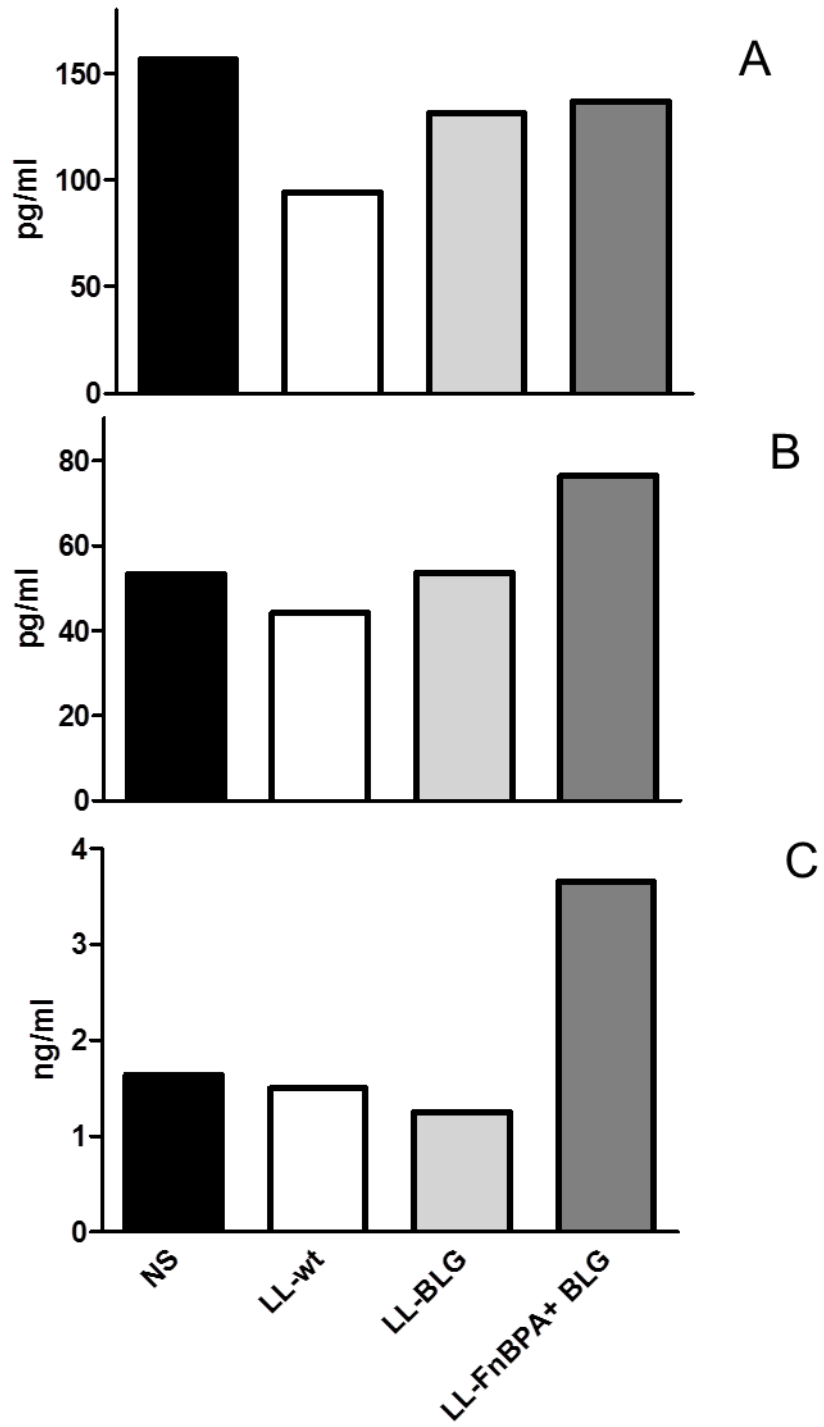


FIGURE 5

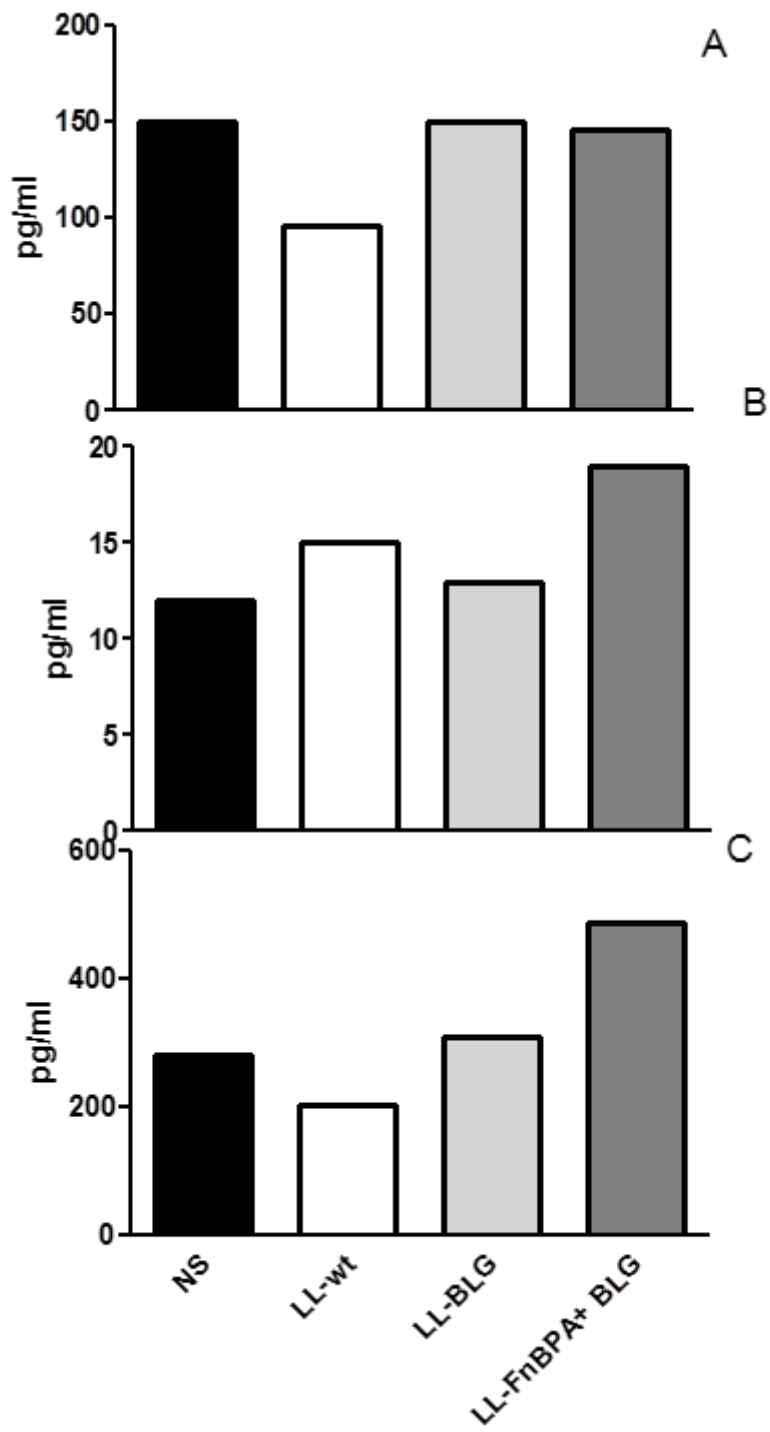


FIGURE 6

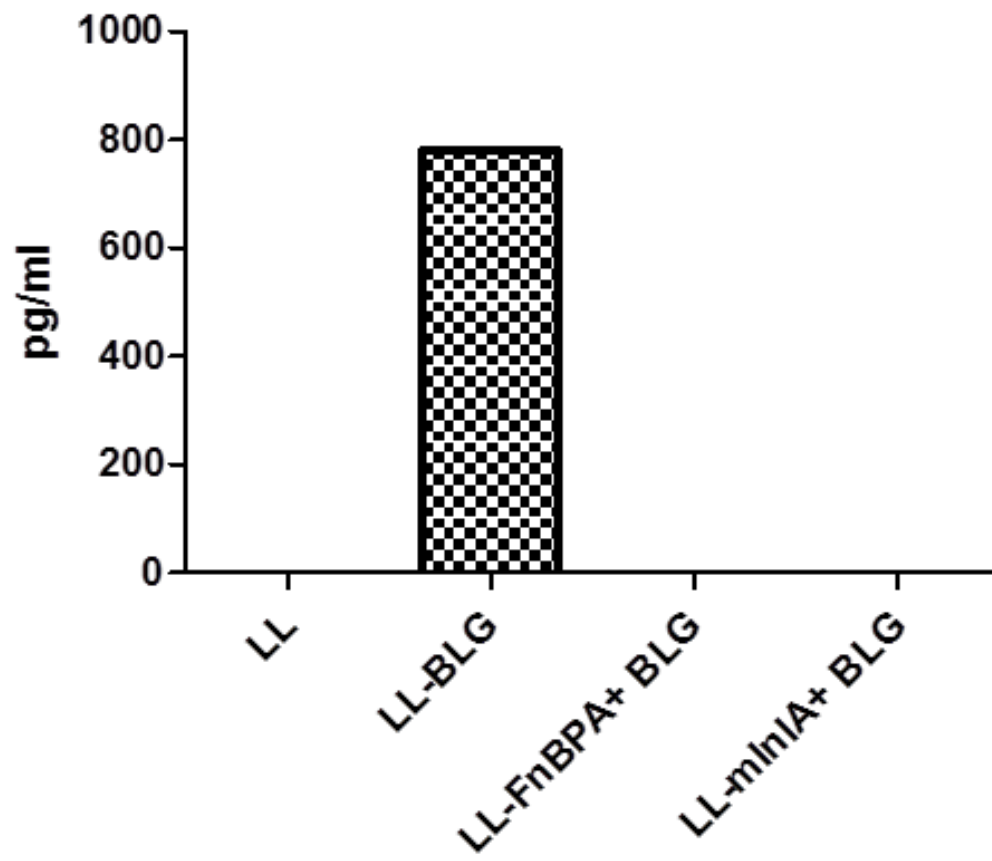
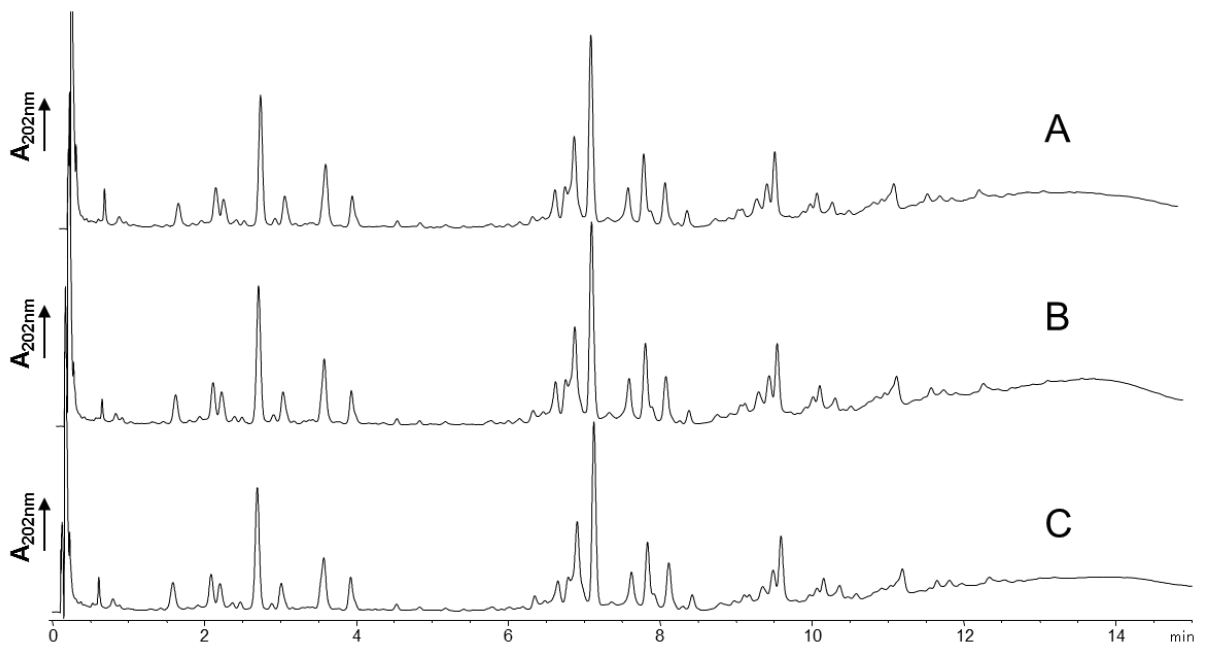


FIGURE 7



CHAPTER 5

Recombinant invasive *Lactococcus lactis* can transfer DNA vaccines either directly to dendritic cells or across an epithelial cell monolayer

Chapter 5: Recombinant invasive *Lactococcus lactis* can transfer DNA vaccines either directly to dendritic cells or across an epithelial cell monolayer

5.1 Introduction

DNA vaccination has been of great interest since its discovery owing to its ability to stimulate both cell-mediated and humoral immune responses (Ingolotti et al., 2010). However, one of the concerns about naked DNA immunization is its low immunogenicity as the antigen is produced in very small amounts in larger animals (Srivastava and Liu, 2003). Besides this, it is well known that DNA-based vaccines target poorly professional antigen-presenting cells (APC), such as dendritic cells (DCs) (Daftarian et al., 2011). Hence, several strategies have been designed to increase the potency of DNA immunization such as the use of bacteria as a vector for DNA delivery (Schoen et al., 2004). One attractive feature of bacterial carriers is their potential for oral administration which may stimulate both mucosal and systemic immune responses (Srivastava and Liu, 2003). Another advantage consists on the fact that they can protect the DNA against degradation by endonucleases (Seow and Wood, 2009). Enteropathogenic species have been used as experimental delivery systems, however, reversion to the virulent phenotype remain as a major concern (Pontes et al., 2011).

Recently, the potential use of *Lactococcus lactis* (LL), the model Lactic Acid Bacteria (LAB), for plasmid DNA delivery to eukaryotic cells is being explored (Bermúdez-Humarán et al., 2011; Pontes et al. 2011; Wells, 2011). *L. lactis* is considered an advantageous vector because it has an established safety profile generated through its long use in the dairy industry as starters for food fermentations (Pontes et al. 2011). Guimarães and collaborators demonstrated that *L. lactis* was capable to transfer a β -lactoglobulin (BLG) eukaryotic expression plasmid to human intestinal epithelial cell line Caco-2 (Guimarães et al., 2006). Later, Chatel et al. demonstrated that this bacterium was also able to transfer BLG cDNA *in vivo* after oral administration in mice (Chatel et al., 2008). Nevertheless, plasmid transfer ratio observed was low. With the intent to increase the capacity of *L. lactis* to transfer DNA plasmids, recombinant strains expressing invasins derived from pathogenic species were constructed. Both *L. lactis* expressing either *Listeria monocytogenes* Internalin A (InIA) or *Staphylococcus aureus* Fibronectin Biding Protein A (FnBPA) improved DNA delivery *in vitro* (Guimarães et al., 2005; Innocentin et al., 2009). However, even though interesting, these two strategies presented some bottlenecks as InIA invasin does not recognize its receptor in mice, murine E-cadherin (Wollert et al., 2007), and FnBPA requires an adequate local concentration of fibronectin to bind to integrins thus limiting the *in vivo* studies in conventional mice (De Azevedo et al., 2012). Therefore, another recombinant invasive *L. lactis* strain expressing a mutated form of InIA (LLmInIA) was recently

described. *In vitro*, it was shown that the invasive status improved both invasivity and DNA delivery capacity to Caco-2 cells and, *in vivo*, it was demonstrated a tendency of LL-mInIA to increase the phenomenon of plasmid transfer to mice epithelial cells (De azevedo et al., 2012). Considering that current knowledge about DNA vaccination using *L. lactis* is mostly based on data obtained in experiments performed with intestinal epithelial cells (IECs), we had the intent to evaluate DNA transfer capacity of noninvasive or invasive (LL-FnBPA and LL-mInIA) *L. lactis* to DCs as well which are considered to be the major APCs of the immune system. We also measured the capacity of these strains to deliver DNA in a Transwell co-culture model and we evaluated DCs immune response after direct co-incubation with bacteria.

5.2 Materials and methods

Bone-marrow derived dendritic cells (BMDC) from conventional BALB/c were isolated and differentiated with granulocyte-macrophage colony-stimulating-factor (GM-CSF) before being directly co-incubated with invasive (LL-FnBPA-BLG, LL-mInIA-BLG) and noninvasive (LL-BLG), all strains carrying the eukaryotic expression vector pValac:BLG. We then used flow cytometry analysis in order to quantify the amount of differentiated DCs. After bacterial co-incubation, expression of BLG by BMDCs was quantified by ELISA as well as secretion of the cytokines IL-12 and IL-10. In order to evaluate the capacity of noninvasive or invasive *L. lactis* to transfer pValac:BLG to DCs grown under a monolayer of differentiated IECs, we first grown epithelial cells of the cell line Caco-2 at the apical side and DCs at the basolateral side of porous inserts. After bacterial co-incubation with the system, we measured the capacity of the strains to invade the monolayer of epithelial cells using the gentamicin survival assay. Finally, we quantified BLG production at both apical and basolateral supernatants as well as in BMDCs and Caco-2 extracts by ELISA. Statistical significance between the groups was calculated using the One Way ANOVA (and nonparametric) test, followed by the “Bonferroni” post-test. Values of $p < 0.05$ were considered significant.

5.3 Results

BMDCs directly co-incubated with LL-FnBPA+ were able to express 1.5 times higher amounts of BLG compared to the noninvasive (LL-BLG) and wild type (wt) strains while BMDCs incubated with LLmInIA-BLG strain could express almost 2.5 times greater quantities of BLG. Furthermore, all strains induced the expression of the pro-inflammatory cytokine IL-12 and nor the expression of BLG neither the invasive status of strains did not affect IL-12 expression. We then moved to other *in vitro* assays to study if the strains could transfer pValac:BLG across a monolayer of differentiated IECs. The first step was to evaluate weather LL-FnBPA or LL-mInIA

invasive lactococci were able to invade the monolayer and afterwards establish a closer contact with the BMDCs grown at the basolateral side of the insert. After co-incubation with bacteria and treatment with gentamicin, we observed that recombinant LL-mInIA and LL-FnBPA strains are around 100 times more invasive than the wild type strains (NZ9000 and MG1363). Afterwards, we measured BLG expression in DCs co-cultured with the monolayer of Caco-2 cells. Apical and basolateral supernatants were also assayed for the presence of BLG. DCs co-cultured with the IEC monolayer previously co-incubated with LLmInIA-BLG strain could express significant higher amounts of BLG when compared to LL strain lacking pValac:BLG vaccine. Furthermore, no BLG expression by DCs was observed with cells cultured with LL, LL-BLG or LLFnBPA-BLG strains and no BLG was detected neither in apical or basolateral culture supernatants.

5.4 Discussion

We have shown that *L. lactis* is capable to deliver cDNA plasmids either *in vitro* or *in vivo* to intestinal epithelial cells (IECs) (Guimarães et al., 2005; Chatel et al., 2008; Innocentin et al., 2009; De Azevedo et al., 2012). Recent data demonstrated that the use of recombinant invasive strains expressing invasins from pathogenic species increased DNA transfer *in vitro* and showed a tendency to improve DNA delivery *in vivo* to mice IECs (Pontes et al., 2012; De Azevedo et al., 2012). All experiments performed so far aimed to evaluate the transfer of pValac:BLG plasmid using noninvasive or invasive *L. lactis* only to IECs. In this work we decided to measure the capacity of these strains to deliver a plasmid DNA to DCs as well. These cells are in direct contact with the IEC monolayer being capable to uptake antigens diluted in the intestinal lumen, and stimulate both mucosal and systemic immune responses (Steinman et al., 2002, Rimoldi et al, 2006). For example, stimulated DCs can release interleukins such as IL-12 that activates natural killer cells polarizing T cells to the protective Th1 phenotype (Macatonia et al., 1995).

BMDCs co-incubated with LL-FnBPA and LL-mInIA were shown to express great amounts of BLG, demonstrating that both strains were capable to successfully transfer pValac:BLG plasmid. The fact that LLmInIA-BLG strain could transfer more efficiently the cDNA of BLG to BMDCs may be due to the expression of E-cadherin, receptor of mInIA, in these cells (Borkowski et al., 1994). The improved plasmid transfer observed for LL-FnBPA strain could be also due to the presence of receptors expressed at the surface of DCs that are involved in the endocytosis of *S. aureus* through FnBPA invasin. Nevertheless, definitive information about how FnBPA interacts with receptors expressed by DCs is lacking.

To evaluate DCs immune response against noninvasive and invasive lactococci, isolated BMDCs were co-incubated with strains. After this period, IL-12, a pro-inflammatory interleukin involved in the differentiation of naïve T cells into Th1 cells (Hsieh et al., 1993) and IL-10, that exhibits pleiotropic effects in immune-regulation and inflammation down-regulating the expression of Th1 cytokines (Pestka et al., 2004), were measured in culture supernatants. BMDCs co-cultivated with either noninvasive or invasive lactococci were able to release elevated levels of IL-12 (300 pg/mL) while no IL-10 expression was observed.

This data partially confirms what was found by Yam and collaborators in 2008 where they showed that *L. lactis* NZ9000 strain incubated with isolated BMDCs induced the secretion of IL-1 β (a pro-inflammatory cytokine) and IL-12. Nonetheless, differently from what was obtained in this work, they found very strong induced mRNA levels of IL-10 (Yam et al., 2008). Others have shown that the type of immune response elicited by LAB is strain dependent as they may favour either a Th1 response, a Th2 humoral or tolerogenic, or only an inflammatory response (Gonella et al., 1998).

It was vastly demonstrated that the heterologous expression of certain antigens from pathogenic species on *L. lactis* surface can induce a desired type of immune response (Th1 or Th2) (Pontes et al., 2011). In this work the expression of *L. monocytogenes* mInIA or *S. aureus* FnBPA did not changed IL-12 production by DCs. The expression of BLG by the BMDCs also did not modified IL-12 levels in culture supernatants.

To study the dialog between strains, differentiated IECs and BMDCs, we choose to work with a model that mimics the *in vivo* situation in which Caco-2 cells were cultured at the apical side of the insert and BMDCs were grown at the basolateral side. At first we checked weather recombinant invasive *L. lactis* strains, LL-FnBPA+ and LL-mInIA+, are able to internalize a monolayer of IECs more efficiently than the wt strains. The invasion rate obtained for the invasive strains were similar and about 100-fold higher than the invasion rate obtained for wt strains. De Azevedo and collaborators showed that LL-mInIA strain is 1000 times more invasive than the wt in experiments performed with non-confluent Caco-2 cells. The lower invasiveness score obtained using the IEC monolayer was probably because cells were very attached to each other turning E-cadherin receptor less accessible. Afterwards, bacteria were incubated with the co-culture system (BMDCs and Caco-2 cells) in order to understand the mechanisms by which *L. lactis* can transfer DNA vaccines *in vivo*.

5.5 Conclusion

We demonstrated that recombinant invasive *L. lactis* expressing either *S. aureus* FnBPA or *L. monocytogenes* mInIA are capable to transfer DNA directly to BMDCs. Furthermore, this

direct contact lead to the secretion of IL-12 by the cells, independent of the invasive status. No IL-10 production was observed. We also checked that invasive lactococci are able to invade a monolayer of differentiated Caco-2 cells 100 times more efficiently than the wt strains. Using a co-culture model we demonstrated that LL-mInIA strain can transfer pValac:BLG to DCs across a monolayer of epithelial cells. Taken together, this study gives new insights on the mechanism of lactococci uptake for delivery of therapeutics.

Recombinant invasive *Lactococcus lactis* can transfer DNA vaccines either directly to dendritic cells or across an epithelial cell monolayer

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Abstract | The use of bacterial carriers for oral delivery of DNA vaccines constitutes a promising vaccination strategy. *Lactococcus lactis* (LL), a generally regarded as safe (GRAS) bacterium (LAB) widely used in the dairy industry, has recently been explored as mucosal DNA delivery vector. Due to its safe status, LL represents an attractive alternative to attenuated pathogens which are the most commonly bacterial vectors used for DNA immunization. Previous studies showed that either native or recombinant invasive LL are able to deliver and trigger DNA expression by mammalian cells both *in vitro* and *in vivo*. However, current knowledge about DNA vaccination using LL is mostly based on data obtained in studies with intestinal epithelial cells (IECs). Considering that dendritic cells (DCs) serve as potent inducers of specific cell-mediated immune responses acting as professional antigen presenting cells (APCs), in this work we evaluated DNA transfer capacity of LL to DCs. We have shown for the first time that invasive *L. lactis* strains expressing either *Staphylococcus aureus* Fibronectin Binding Protein A (LL-FnBPA+), or *Listeria monocytogenes* mutated Internalin A (LL-mInIA+) can transfect bone marrow-derived DCs (BMDCs) and deliver a plasmid DNA vaccine (pValac) encoding for the cow milk allergen β -lactoglobulin (BLG). BMDCs co-cultured with non-invasive or invasive Lactococci were able to secrete elevated levels of the pro-inflammatory cytokine IL-12. In order to understand the mechanism by which lactococci could transfer pValac:BLG *in vivo*, differentiated Caco-2 cells were used to measure the capacity of strains to invade a polarized monolayer of epithelial cells, mimicking the *in vivo* situation. Gentamycin survival assay in these cells showed that LL-mInIA+ is 100 times more invasive than LL. We also investigated the cross-talk between differentiated IECs, BMDCs and bacteria using an *in vitro* Transwell co-culture model. Co-incubation of strains with the co-culture model has shown that DCs maintained with LLmInIA-BLG strain could express significant higher levels of BLG. This result suggest that *in vivo* DCs could sample bacteria containing the DNA vaccine across the epithelial barrier; express the antigen and/or present it to naïve T cells stimulating adaptive immune responses.

1. Introduction

DNA vaccination has been of great interest since its discovery in the 1990s owing to its ability to stimulate both cell-mediated and humoral immune responses (Ingolotti et al., 2010). It is now well proven that intramuscular injection of a plasmid DNA can induce antibodies, helper T cell responses, cytotoxic T cell (CTL) responses, and protective immunity against different viral, bacterial, and parasitic infections in various animal models (Donnelly et al., 2000). Although US Food and Drug Administration (FDA) still did not approved DNA vaccines for use in humans, phase I clinical studies have been reported for the prevention and/or

treatment of influenza, HIV, malaria, hepatitis B, SARS and many other infectious agents (Klinman et al., 2011). Recently, four DNA vaccines have been licensed for administration in horses, salmon, dogs and swine turning this vaccine platform very promising (Findik and Çiftci, 2012).

One of the concerns about naked DNA immunization is its low immunogenicity as the antigen is produced in very small amounts *in vivo* due to its non-replicative nature (Srivastava and Liu, 2003). Besides this, it is well known that DNA-based vaccines poorly target professional antigen-presenting cells (APC) (Daftarian et al., 2011). Therefore, several strategies have been designed to increase the potency of DNA immunization (Chen et al., 2012). The use of bacteria as a vector for DNA delivery into eukaryotic cells has emerged as a potential approach to enhance the immunogenicity of DNA vaccines (Schoen et al., 2004). One attractive feature of bacterial carriers is their potential for oral administration which may stimulate both mucosal and systemic immune responses (Srivastava and Liu, 2003). Some attenuated pathogenic species contains an innate tropism for specific tissues of the host directing systemic responses towards the mucosa (Schoen et al., 2004). Furthermore, besides protecting the plasmid against degradation by endonucleases (Hoebe et al., 2004), bacteria can act as adjuvants due to the presence of pathogen-associated molecular patterns (PAMPs) in their surface (Schoen et al., 2004). Another advantage consists on the fact that bacterial vectors can accommodate large-sized plasmids, which allows the insertion of multiple genes or chemokines of interest (Seow and Wood, 2009). Finally, this vaccine platform is considered to be low cost as it does not require further steps of plasmid purification, reducing cost and labor (Hoebe et al., 2004; Schoen et al., 2004).

Shigella, *Salmonella*, and *Listeria monocytogenes* have been used as experimental delivery systems. Even though the bacteria used for such proposed vaccines are attenuated strains, preexisting immunity, reactogenicity and reversion to virulence remain major concerns, especially considering their administration in children and immunosuppressed patients (Pontes et al., 2011). Recently, the potential use of *Lactococcus lactis* (LL), the model Lactic Acid Bacteria (LAB), for the production of biologically useful proteins and for plasmid DNA delivery to eukaryotic cells is being explored (for a review see Bermúdez-Humarán et al., 2011; Pontes et al. 2011; Wells, 2011). *L. lactis* is considered an advantageous vector because it has an established safety profile generated through its long use in the dairy industry as starters for food fermentations (Pontes et al. 2011). That is the reason why the FDA has been granted this bacterium with the GRAS (Generally Recognized As Safe) status. Moreover, *L. lactis* is easy to handle, does not produce lipopolysaccharides (LPS) in its outer membrane, contains a number of genetic tools developed in the last couple of decades and does not induce strong host

immune responses (Bermúdez-Humarán et al., 2011; Pontes et al. 2011). Guimarães and collaborators showed that *L. lactis* was capable to transfer a β -lactoglobulin (BLG) eukaryotic expression plasmid to human intestinal epithelial cell line Caco-2. Expression of BLG by Caco-2 cells was detected after co-culture with *L. lactis* carrying the expression plasmid (Guimarães et al., 2006). Later, Chatel et al. demonstrated that this bacterium was also able to transfer BLG cDNA *in vivo* after oral administration in mice (Chatel et al., 2008). With the intent to increase the capacity of *L. lactis* to transfer DNA plasmids, recombinant strains expressing invasins derived from pathogenic species were constructed. One was designed to express *Listeria monocytogenes* Internalin A (InIA) (LLInIA) (Guimarães et al., 2005) and the other to produce *Staphylococcus aureus* Fibronectin Binding Protein A (FnBPA) (LLFnBPA) (Innocentin et al., 2009). *In vitro*, they showed a higher ability to invade mammalian cells compared to the wild type (*wt*) Lactococci and, as a result, more GFP expression plasmid was delivered to Caco-2 cells. However, even though interesting, these two strategies presented some bottlenecks: InIA invasin does not recognize its receptor in mice, murine E-cadherin (Wollert et al., 2007), and FnBPA requires an adequate local concentration of fibronectin to bind to integrins (De Azevedo et al., 2012) thus limiting the *in vivo* studies in conventional mice. Another recombinant invasive *L. lactis* strain expressing a mutated form of Internalin A (mInIA) (LLmInIA) was recently described (De azevedo et al., 2012). It was shown that the invasive status improved both invasivity and DNA deliver capacity *in vitro* to Caco-2 cells. Moreover, it was demonstrated a tendency of the invasive bacteria to increase the phenomenon of plasmid transfer to mice epithelial cells.

Current knowledge about DNA vaccination using *L. lactis* is mostly based on data obtained in experiments performed with intestinal epithelial cells (IECs). Considering that dendritic cells (DCs) serve as potent inducers of specific cell-mediated immune responses, it is very interesting to evaluate the capacity of *L. lactis* to transfer DNA vaccines to these cells as well. Besides being in direct contact with the IEC monolayer, DCs are capable to uptake antigens, other cells or bacteria carrying a DNA vaccine through the epithelial tight junctions (Rescigno et al., 2001). They can also generate great amounts of MHC-peptide complexes, migrate to the primary lymphoid organs and present antigens to naïve T cells, acting as professional antigen presenting cells (APCs) (Joffre et al., 2012). Moreover, DCs are able to secrete interleukins, such as IL-12, that recruits other DCs and T cells polarizing them to the protective Th1 phenotype (Steinman et al., 2002).

The purpose of this work was to measure the ability of noninvasive or invasive *L. lactis* expressing either *S. aureus* FnBPA – LL-FnBPA+ – or *L. monocytogenes* mutated InIA – LL-mInIA+ – to deliver a plasmid DNA vaccine (pValac:BLG) encoding for the cow's milk allergen β -

lactoglobulin (BLG) (Guimarães et al., 2009) directly to bone marrow-derived DCs (BMDCs). The capacity of these strains to deliver pValac:BLG in a Transwell co-culture model (where the BMDCs were grown on the basolateral side of the IEC monolayer) was also investigated. Finally, DCs immune response was measured after direct co-incubation with bacteria.

2. Materials and Methods

2.1 Bacterial strains, plasmids, media and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Noninvasive *L. lactis* NZ9000 (Kleeberzem et al., 1997), *L. lactis* MG1363 (Gasson, 1983), *L. lactis* carrying pOri23 plasmid (LL) (Que et al., 2000), *L. lactis* harboring both pOri23 and pValac:BLG plasmids (LL-BLG) (Pontes et al., 2012) and invasive *L. lactis* strains expressing *S. aureus* FnBPA (LL-FnBPA+) (Que et al., 2001) or *L. monocytogenes* mInIA (LL-mInIA+) (De Azevedo et al., 2012) were grown in M17 medium supplemented with 0.5% (wt/vol) glucose (GM17) at 30°C without agitation. Erythromycin (Ery) (10 µg/ml) and/or chloramphenicol (Cm) (5 µg/ml) were added to the medium when needed. DNA manipulation was performed following standard procedures (Sambrook et al., 1989), with a few modifications. Plasmids were purified by lyses alkaline method after bacterial incubation for 30 min at 37 °C in TES solution (25% sucrose, 1 mM EDTA, 50 mM Tris–HCl pH 8) containing lysozyme (10 mg/ml). The quality of the products obtained, including its concentration and purity, were estimated measuring the absorbance at 260 nm and 280 nm in spectrophotometer. Restriction and modification endonucleases were used in accordance with the manufacturer's recommendations (Invitrogen).

2.2 Polarized intestinal epithelial cell cultures on Transwell filters. The human intestinal epithelial cell line Caco-2 (ATCC number HTB37) was maintained in DMEM medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Omnilab, Switzerland) and 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium). The culture medium was switched every 2 days, and cell viability was determined by trypan blue staining. Trypsin-treated cells were seeded on permeable supports with microporous membranes (Corning Glass Works, 0.4 µm pore size PET membrane 24 well formats) for 14 days into p-24 plates (Corning Glass Works), to ensure cell polarization with a distinguished apical and basal side (Jumarie and Malo., 1991). Cells were grown at 37°C in the presence of 5% CO₂ and 95% air atmosphere.

2.3 Invasion assay of bacteria into differentiated human intestinal epithelial cells.

Differentiated Caco-2 cells grown on Transwell filters were used to perform bacterial internalization into human epithelial cells. Recombinant or *wt L. lactis* was grown to an OD₆₀₀ of 0.9–1.0, pellet was recovered, washed two times with PBS 1X and diluted in order to obtain a multiplicity of infection (MOI) of around 1000 bacteria per eukaryotic cell. Before infection, polarized monolayers of Caco-2 cells were treated with 10mM EDTA buffer (Sigma-Aldrich, St. Louis, MO) to disrupt tight junctions and the transepithelial electrical resistance (TER) was monitored across the monolayers as described by Karczewski et al. (2010). The gentamicin survival assay was used to evaluate bacteria survival (Isberg and Falkow, 1985). Briefly, recombinant or *wt L. lactis* were applied in the apical side of eukaryotic cells and co-incubated during one hour at 37°C, in 5% CO₂. After this period, cells were washed in order to remove bacteria in excess and then 150 µg/mL of gentamicin were added. Plates were maintained for more 2 hours before cells were washed and lysed with 0.2% of triton. The quantity of live intracellular bacteria was determined by plating serial dilutions of cell lysates and counting colony-forming units (CFU).

2.4 Animals. Female BALB/c mice were used at six-to-eight weeks and housed in a light and temperature controlled facility of Wageningen University to generate bone marrow-derived dendritic cells (BMDCs). The study was approved by the Wageningen University Ethics Committee (Research, Wageningen UR, Wageningen University).

2.5 Murine bone-marrow derived dendritic cells (BMDC) isolation. To obtain BMDC cells, 6-10-week BALB/c mice were euthanized; femurs were isolated, washed and gently crushed in 10 ml of RPMI-1640 medium (zonder HEPES) supplemented with 1% penicillin/Streptomycin (Sigma-Aldrich). Cells were filtered using a Steriflip® Filter Unit (Millipore) and stained with a trypan blue staining solution to determine the number of live and dead cells. Around 2×10^7 live cells were seeded in a sterile petri dish in complete media (RPMI-1640 medium containing 10% heat-inactivated fetal calf serum [FCS, Sigma-Aldrich], 1% penicillin/Streptomycin [Sigma-Aldrich], 20 ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating-factor (GM-CSF) [R&D systems] and 0.05mM of β-mercaptoethanol [Invitrogen]). Cells were incubated at 37°C in CO₂ atmosphere and medium was changed every three days. At day six BMDCs were removed from petri dishes plates to determine the number and size of cells using fluorescence activated cell-sorting (FACS) analysis. 24-Well Cell Culture Plates (Corning) containing 5×10^5 BMDC per well were then prepared and after used for both DNA transfer and BMDCs stimulation experiments.

2.6 Flow cytometry analysis. BMDCs cultured during six days were harvested from the plate, centrifuged (300 ×g, 10 min, 4 °C), resuspended in appropriate medium and analyzed using a

BD Biosciences FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) to measure CD marker expression. Cells were stained with FITC-conjugated anti-mouse CD11c, PE/Cy7-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD40 antibodies (all from BD Biosciences) and suitable isotype control monoclonal antibodies. Data was analyzed with either BD FACSDiva or Flowjo software and the level of expression was expressed as the geometric mean of fluorescence intensity (MFI).

2.7 Bacterial incubation with BMDCs for β -lactoglobulin (BLG) detection. In order to evaluate the capacity that invasive or noninvasive *L. lactis* contains in transferring DNA vaccines to APCs, BLG expression and secretion by isolated BMDCs were quantified after performing the gentamicin survival assay. Briefly, noninvasive *L. lactis* (LL and LL-BLG) and invasive *L. lactis* (LL-FnBPA+ and LL-mInIA+) (table 1) were incubated with BMDCs for one hour, 150 μ g/mL of gentamicin was added to kill extracellular bacteria, cells were washed after 2 hours and gentamicin-treated cells were incubated during 72 hours at 37 °C, in 5% CO₂. Plates were centrifuged at 78.2 g for 10 minutes; supernatant was collected and stored at -80°C. 500 μ l of PBS containing a cocktail of protease inhibitors (Roche) was added to the cells and then homogenized using an ultrasonic treatment (3 times 10 seconds). Samples were stored at -80°C and thus used to measure BLG expression by ELISA.

2.8 *In vitro* transwell co-culture system of differentiated Caco-2 cells and BMDCs. The method to prepare epithelial cell monolayers in contact with BMDCs is described elsewhere (Rescigno et al., 2001; Zoumpopoulou et al., 2009). In summary, intestinal epithelial cell line Caco-2 was cultured on the upper face of 3- μ m pore Transwell filters (costar, polycarbonate Membrane) for 10–15 days in a 24-well plate (Corning Glass Works) using DMEM medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Omnilab, Switzerland) and 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium). 4×10^5 of isolated BMDCs were seeded on the basolateral side of differentiated Caco-2 and maintained in RPMI-1640 (Zonder Hepes), supplemented with 10% heat-inactivated FCS (Sigma-Aldrich) and 20 ng/ml of GM-CSF (R&D systems) during 18 h at 37 °C in a 5% CO₂/95% air atmosphere. After this period the medium from all compartments was changed and the system was used for assessing BLG expression by epithelial and dendritic cells after bacterial co-incubation.

2.9 BLG detection in BMDCs and Caco-2 monolayers co-cultured on transwell inserts after bacterial incubation. A co-culture system model was used to evaluate DNA transfer capacity of invasive or noninvasive *L. lactis* strains to dendritic cells across an epithelial cell monolayer. Inserts containing fully differentiated Caco-2 cells monolayers in the apical side and BMDCs cultured in the basolateral side were prepared, as described in section 2.8. No EDTA treatment was applied to disrupt tight junctions before co-incubation with strains. MOI of 1000 bacteria

per cell was incubated from the apical side of the epithelium at 37°C in 5% CO₂ for 3 hours. All compartments were washed and gentamicin (150 µg/mL) treatment was applied for 2 hours to avoid bacteria overgrowth. Following washing, plate was incubated for 72 hours in 5% CO₂ at 37°C; 250 µl of PBS supplemented with a cocktail of protease inhibitors (Roche) were added to the wells; cells were scraped from the inserts and harvested using an ultrasonic treatment (3 times 10 seconds). Samples as well as apical and basolateral supernatant containing protease inhibitors were stored at -80°C and assayed for BLG production by ELISA.

2.10 Detection of bovine β- lactoglobulin in either BMDCs or differentiated Caco-2 cells using an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated by adding 100 µl/well of sample. Plates were maintained at 4°C overnight and then washed three times with washing buffer (PBS 1X; 0.05% Tween 20) and saturated with blocking buffer (PBS 1X; 1% BSA – Sigma, Aldrich), sealed and kept at room temperature for one hour. 3.5 µg/ml of anti BLG-antibody (Genway Biotech, Inc. Protein and Antibody solutions) diluted in PBS 1X containing 0.1% BSA (Sigma-Aldrich) were added to the plates and incubated at room temperature during 1 hour. After this period plates were washed again by filling the wells with washing buffer. Rabbit anti-sheep IgG conjugated with Horse Radish Peroxidase antibody (Bethyl) diluted 1:1000 in PBS 1X 0.1% BSA were added to the wells. Plates were incubated for 1 hour at 37°C in the dark and washed again. ABTS Peroxidase Substrate (Roche Applied Science) was added in each well; plate was kept in the dark and, after sufficient color development, reaction was stopped by adding 1M of H₂SO₄. Optical density was measured at 405nm in a spectrophotometer (SpectraFluor Plus, Tecan). Standard curves were fitted.

2.11 BMDCs bacterial stimulation and cytokine quantification in culture supernatants. 4x10⁵ of isolated BMDCs were plated in 24-well plates (Corning Glass Works) as indicated in section 2.5 and bacteria were applied (or not) to the cells (MOI 40 bacteria/cell). Plate was maintained at 37°C in 5% CO₂ during 24 hours and supernatant was used to quantify mouse interleukine-10 (IL-10) and IL-12 using commercially available ELISA kits (Mabtech, Stockholm, Sweden) following the manufacturer's instructions.

2.12 Statistical analyses. The results are expressed as mean ± SE values. Statistical significance between the groups was calculated using the One Way ANOVA (and nonparametric) test, followed by the “Bonferroni” post-test. Values of p< 0.05 were considered significant.

3. Results

3.1 Invasive *L. lactis* strains can transfer a β -lactoglobulin cDNA directly to BMDCs. We previously demonstrated that invasive *L. lactis* LL-FnBPA⁺ and LL-mInIA⁺ strains carrying pValac:BLG (Pontes et al., 2012; De Azevedo et al., 2012), a plasmid derived from pValac containing the cDNA for BLG under the control of an eukaryotic promoter, could transfer BLG cDNA to IECs (De Azevedo et al., 2012). To measure the capacity of strains to deliver DNA to DCs, BMDCs maturation was confirmed by FACS analysis (Figure 1). Next, matured DCs were incubated with non-invasive (LL, LL-BLG) and invasive *L. lactis* carrying pValac:BLG vaccine (LLFnBPA-BLG, LLmInIA-BLG). After three days, BLG was quantified in both culture supernatants and BMDCs extracts. DCs co-incubated with LLFnBPA-BLG were able to express 1.5 times higher amounts of BLG compared to LL-BLG and LL strains while cells incubated with LLmInIA-BLG strain could express almost 2.5 times greater quantities of BLG, compared to noninvasive strains (Figure 2). No BLG secretion was observed in culture supernatants (data not shown).

3.2 Lactococci induces the secretion of IL-12 in BMDCs. BMDCs were directly challenged by addition (or not) of the bacteria at MOI 40:1. Plate was kept overnight at 37°C in 5% CO₂/95% air atmosphere and supernatants were assayed for the presence of IL-10 and IL-12. BMDCs co-cultivated with either noninvasive (LL, LL-BLG) or invasive (LLmInIA-BLG, LLFnBPA-BLG) *L. lactis* were able to secrete elevated levels of IL-12 compared to cells which were not incubated with bacteria (medium). Moreover, expression of BLG by DCs as well as the invasive status of strains did not affect IL-12 expression. The induction of IL-10 measured by ELISA was not observed in culture supernatants incubated or not with bacteria (data not shown) (Figure 3).

3.3 Recombinant *L. lactis* expressing invasins are capable to internalize differentiated IECs. The capacity of invasive (LL-FnBPA⁺ and LL-mInIA⁺) or noninvasive (NZ9000 and MG1363) *L. lactis* to internalize a monolayer of Caco-2 cells was investigated. Before co-incubation with bacteria, cell monolayer was treated with 10 mM EDTA solution to disrupt tight junctions (TJ) and, therefore, expose mInIA receptor, E-cadherin, which is expressed below the TJ on the basolateral side of the cell. 30 min of treatment with EDTA buffer was sufficient to completely disrupt the transepithelial electrical resistance of the monolayer (data not shown). Differentiated Caco-2 cells were incubated with strains for one hour, non-internalized bacteria were killed by gentamicin and intracellular bacteria were enumerated after cell lysis. Recombinant LL-mInIA⁺ and LL-FnBPA⁺ strains showed 100-fold greater invasion rate compared to NZ9000 and MG1363 noninvasive strains as indicated in figure 4.

3.4 Invasive *L. lactis* are able to deliver pValac:BLG to BMDCs through the IEC monolayer.

With the intent to evaluate DNA transfer capacity of invasive or noninvasive *L. lactis* strains using another model that better mimics the *in vivo* situation, BMDCs were co-cultured in the basolateral side of the differentiated Caco-2 cells. The bacterial strains tested were added at the apical surface of the IECs, after three hours gentamicin treatment was applied. For this experiment, no EDTA treatment was applied before co-incubation with strains as we would like to observe if DCs are able to cross the monolayer going through the tight junction between adjacent epithelial cells and sample bacteria present in the apical compartment. After 72 hours, cells were lysed, collected, as well as basal and apical culture supernatants and all compartments were used to investigate BLG expression by ELISA. As demonstrated in Figure 5, DCs co-cultured with the IEC monolayer which were co-incubated with LLmInIA-BLG strain could express significant higher amounts of BLG when compared to LL strain lacking pValac:BLG vaccine. No BLG expression by DCs was observed with cells cultured with LL, LL-BLG or LLFnBPA-BLG strains. Furthermore, BLG was detected neither in apical or basal culture supernatants nor in Caco-2 extracts (data not shown).

4. Discussion

Several species of Lactic acid bacteria (LAB) have proved to be effective mucosal delivery vehicles for vaccines and therapeutic molecules (Wells, 2011; Tarahomjoo, 2012). Among all LAB species, the food-grade *L. lactis* is one of the most studied and over the last decade efficient genetic tools have been designed allowing the expression of heterologous proteins in this bacterium (Kleerebezem et al., 2002; Wells, 2011; Pontes et al., 2011). Numerous antigenic proteins from bacterial, viral, and parasitic infective agents were successfully expressed in *L. lactis*. Protection studies with this recombinant strains have been performed and results are very encouraging as protection or partial protection after challenge was observed (Pontes et al., 2011; Bermudez-Humaran et al., 2011; Tarahomjoo, 2012). However, even though interesting, this strategy presents some problems associated with post-translational modifications performed on recombinant proteins by *L. lactis* that affects the bioactivity and function of these proteins (Marreddy et al., 2011). In order to circumvent those problems, the use of *L. lactis* to deliver cDNA instead of recombinant proteins at the mucosal level is being explored (Guimarães et al., 2006; Chatel et al., 2008; Pontes et al., 2011). We have shown that *L. lactis* is capable to deliver cDNA plasmids either *in vitro* or *in vivo* to intestinal epithelial cells (IECs) (Guimarães et al., 2005; Chatel et al., 2008; Innocentin et al., 2009; De Azevedo et al., 2012). Recent data demonstrated that the use of recombinant invasive strains expressing *S. aureus* Fibronectin Binding Protein A (FnBPA) or *L. monocytogenes* mutated Internalin A (mInIA) increased DNA transfer *in vitro* and showed a tendency to

improve DNA delivery *in vivo* to mice IECs (Pontes et al., 2012; De Azevedo et al., 2012). All experiments performed so far aimed to evaluate the transfer of pValac:BLG plasmid using noninvasive or invasive *L. lactis* only to IECs. In this work we decided to measure the capacity of these strains to deliver a plasmid DNA to DCs as well as these cells are in direct contact with the IEC monolayer being capable to cross the epithelial membrane through the tight junctions (TJ) between adjacent epithelial cells and uptake antigens present in the lumen. Moreover, they can generate great amounts of MHC-peptide complexes and present them to naïve T cells stimulating both mucosal and systemic immune responses (Steinman et al., 2002, Rimoldi et al., 2006). Stimulated DCs can also release interleukins such as IL-12 that activates natural killer cells polarizing T cells to the protective Th1 phenotype (Macatonia et al., 1995).

With the intent to study the capacity of noninvasive or invasive *L. lactis* to deliver a DNA vaccine to DCs, LL, LL-FnBPA+ and LL-mInIA+ strains transformed with pValac:BLG vaccine (Pontes et al., 2012; De Azevedo et al., 2012) were incubated with isolated BMDCs and after 72h BLG was assayed in culture supernatants and cell extracts by ELISA. Despite not being able to secrete the allergen as no BLG was found in culture supernatants, DCs co-incubated with either LLFnBPA-BLG or LLmInIA-BLG were able to produce significant higher amounts of BLG compared to noninvasive (LL, LL-BLG) *L. lactis* (Figure 2). The fact that LLmInIA-BLG strain could transfer more efficiently pValac:BLG plasmid to BMDCs can be explained by the fact that these cells grown in GM-CSF-supplemented media express E-cadherin, receptor of mInIA (Borkowski et al., 1994); fact that can facilitate bacterial adherence and invasion resulting in a more efficient delivery of the DNA vaccine to the DCs. The improved plasmid transfer to BMDCs was also observed for the other invasive *L. lactis* strain, LLFnBPA-BLG. Probably, some of the surface receptors expressed by DCs that participate in receptor-mediated endocytosis could be recognizing *S. aureus* FnBPA invasin. It is already known that *S. aureus* enters host cells through FnBPA interaction with cell surface $\alpha_5\beta_1$ integrins expressed in epithelial cells, endothelial cells and keratinocytes (Sinha et al., 1999; Edwards et al., 2011). Recently, it was demonstrated that DCs readily take up *S. aureus* in response to the recognition of staphylococcal DNA resulting in the activation of TLR9 signaling (Parker and Prince, 2012). Nevertheless, definitive information about how FnBPA interacts with receptors expressed by DCs is lacking.

In this work, we have shown for the first time that recombinant invasive *L. lactis* are able to transfect dendritic cells. The majority of the works about DNA transfer in DCs is based on a technology that uses dendrimers as gene delivery vectors. It seems that this technology provides favorable DNA condensation turning it easier to get inside the cells (Fant et al., 2010; Daftarian et al., 2011; Jia et al., 2011). However, their application is limited due to its inherent

cytotoxicity to mammalian cells (Fenske and Cullis, 2008; Fant et al., 2010). Therefore, the use of GRAS bacteria, such as *L. lactis*, is an alternative strategy particularly interesting.

To evaluate DCs immune response against noninvasive and invasive lactococci, isolated BMDCs were co-incubated with strains for 24 hours. After this time, we choose to quantify the expression of IL-12, a pro-inflammatory interleukin which is involved in the differentiation of naïve T cells into Th1 cells (Hsieh et al., 1993) and IL-10, that exhibits pleiotropic effects in immune-regulation and inflammation down-regulating the expression of Th1 cytokines (Pestka et al., 2004), in culture supernatants by ELISA. BMDCs co-cultivated with either noninvasive or invasive lactococci were able to release elevated levels of IL-12 (300 pg/mL) compared to cells not infected with bacteria while no IL-10 expression was observed, as shown in figure 3. This data partially confirms what was found by Yam and collaborators in 2008 (Yam et al., 2008). They incubated *L. lactis* NZ9000 strain with isolated BMDCs and observed that this bacterium exhibited pro-inflammatory properties as it was capable of inducing significant levels of IL-1 β (a pro-inflammatory cytokine) and IL-12 mRNA from DCs. Nonetheless, differently from what was obtained in this work, they found very strong induced mRNA levels of IL-10 (Yam et al., 2008). Others have shown that the type of immune response elicited by LAB is strain dependent as they may favour either a Th1 response, a Th2 humoral or tolerogenic, or only an inflammatory response (Gonella et al., 1998). For instance, *in vivo* it was observed an increase of interleukins IL-10 and IL-4 in mice immunized with *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus casei*, while a significant induction of IL-2 and IL-12 was only detected with mice fed with *L. acidophilus* (Perdigón et al., 2002). The exact mechanisms are currently a topic of intensive research. It is known that the exposure of the DCs to microbial products can induce the activation of the transcription factor NF- κ B pathway through Toll-like receptor (TLR) signaling, leading to IL-12 induction (Murphy et al., 1995). On the other hand, microbial products can polarize DCs towards a Th2- type immune response or towards peripheral immune tolerance via the induction of regulatory T cells (Kushwah and Hu, 2011).

It was vastly demonstrated that the heterologous expression of certain antigens from pathogenic species on *L. lactis* surface can induce a desired type of immune response (Th1 or Th2) (For a review see Wells and Mercenier, 2008 and Pontes et al., 2011). In this study, the expression of *L. monocytogenes* mInIA or *S. aureus* FnBPA in *L. lactis* did not increase IL-12 production by DCs, when compared to levels obtained in cells incubated with non-invasive bacteria. The expression of BLG, an allergen that usually drives a Th2-mediated immune response, by DCs did not change IL-12 levels in culture supernatants as well.

Understanding how bacteria interact with the intestinal barrier is a fundamental topic in the development of effective bacterial vaccine vectors (Canny and McCormick, 2008).

Therefore, we decided to study the dialog between strains, differentiated IECs and BMDCs. At first we checked whether recombinant invasive *L. lactis* strains, LL-FnBPA+ and LL-mInIA+, are able to internalize a monolayer of IECs more efficiently than the *wt* strains using the gentamicin protection assay (Isberg and Falkow, 1985). We choose to work with Caco-2 cells differentiated into villus like enterocytes that resembles intestinal epithelium both functionally and structurally as this model better mimics the *in vivo* state. Before co-incubation with bacteria, 10mM of EDTA was applied to the monolayer with the intent to disrupt tight junctions (TJ) allowing E-cadherin exposure (Wollert et al., 2007). The invasion rate of LL-mInIA and LL-FnBPA were similar and about 100-fold higher than the invasion rate obtained for *wt* strains (NZ9000 and MG1363) (Figure 4). Previously data demonstrated that LL-mInIA+ were 1000 times more invasive than NZ9000 strain in experiments performed with non-confluent Caco-2 cells (De Azevedo et al., 2012). The lower invasiveness score obtained after bacterial co-incubation with the IEC monolayer was probably do to the fact that cells were very attached to each other turning both E-cadherin and $\alpha_5\beta_1$ integrins receptors less accessible.

In order to understand the mechanisms by which *L. lactis* can transfer DNA vaccines *in vivo*, bacteria was co-incubated with a transwell murine co-culture model mimicking the intestinal barrier. DCs were grown on the basolateral side of the inserts while differentiated IECs were cultivated on the apical side. It is important to mention that no EDTA treatment was performed with this system because we would like to check the capacity of DCs to extend their dendrites and penetrate the epithelial monolayer to sample bacteria present in the apical compartment, as observed by Rescigno and collaborators (2001). After incubation with strains, BLG production was assayed in apical and basolateral supernatant as well as in Caco-2 and DCs extracts. BLG production was only found in DCs extracts co-cultured with the IEC monolayer which were co-incubated with LLmInIA-BLG (Figure 5). In experiments performed with isolated BMDCs cells we also observed a higher DNA transfer using this strain. This data shows for the first time that DCs can sample an invasive *L. lactis* strain through the epithelium giving new insights on how the DNA transfer process occurs *in vivo*. No BLG secretion by either DCs or Caco-2 cells was observed, differently of what was obtained in previously work in which non-confluent Caco-2 cells co-incubated with LLFnBPA-BLG and LLmInIA-BLG could secrete the allergen (De Azevedo et al., 2012). As mentioned before, even with the use of EDTA to disrupt the epithelial tight junctions, internalization score of strains is lower in experiments performed in differentiated IECs. The fact that cells are very attached in a monolayer may hamper the contact with bacteria, preventing efficient DNA transfer, and therefore lowering BLG production and secretion. Follow-up studies will be performed in near future to evaluate whether DCs could secrete BLG *in vivo* after oral immunization trials with non-invasive or

invasive *L. lactis* strains. It is also interesting to study the fate of these bacteria after inoculation as this knowledge may help to elucidate or improve the mechanisms by which *L. lactis* can transfer DNA vaccines to mammalian cells.

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Figure Legends

Figure 1. FACS analysis of surface CD11c, CD40 and CD86 receptors in isolated BMDCs.

BMDCs cultured during six days were analyzed using a BD Biosciences FACSCanto II flow to measure CD marker expression. Cells were stained with FITC-conjugated anti-mouse CD11c, PE/Cy7-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD40 antibodies and results were analyzed with either BD FACSDiva or Flowjo software.

Figure 2. BLG detection in BMDCs extracts after co-incubation with non-invasive (LL and LL-BLG) or invasive *L. lactis* (LLmInIA-BLG and LLFnBPA-BLG) strains carrying or not pValac:BLG.

BMDCs were isolated from BALB/c mice, cultivated with GM-CSF and incubated with strains (MOI 1:1000) for one hour. Cells were washed, treated with gentamicin and BLG was assayed in both cells extract and culture supernatants after three days. Data representative from two independent experiments * $p < 0.05$.

Figure 3. IL-12 secretion by BMDCs after co-incubation with *L. lactis*.

Isolated BMDCs were co-incubated with non-invasive or invasive *L. lactis* (MOI 1:40) during 24 hours and culture supernatant was tested for the presence of IL-12 using commercially available ELISA kits. Data representative from two independent experiments.

Figure 4. Invasiveness scores of wild type or invasive recombinant Lactococci into differentiated Caco-2 cells.

Caco-2 cells were cultivated on inserts and after 14 days a monolayer of fully differentiated IECs were obtained. MG1363, NZ9000, LL-mInIA+ and LL-FnBPA+ strains were then co-incubated with cells during 1 hour and, after this period, treated with gentamicin for 2 hours. Cells were lysed and the number of CFU internalized was measured by plating. **, survival rates were significantly different (One-way ANOVA, Bonferroni's multiple comparison test, $p < 0.05$). Results are means standard deviations of two different experiments, each time done in triplicate.

Figure 5. BLG expression by BMDCs after incubation with non-invasive or invasive lactococci using a co-culture model.

IECs were apically challenged by addition of bacteria (MOI 1:1000) in the presence BMDCs in the lower compartment. After bacterial stimulation, BLG expression or secretion was measured in extract from IECs, BMDCs, apical or basolateral supernatants by ELISA. Results are expressed as the average between two experiments. * $p < 0.05$

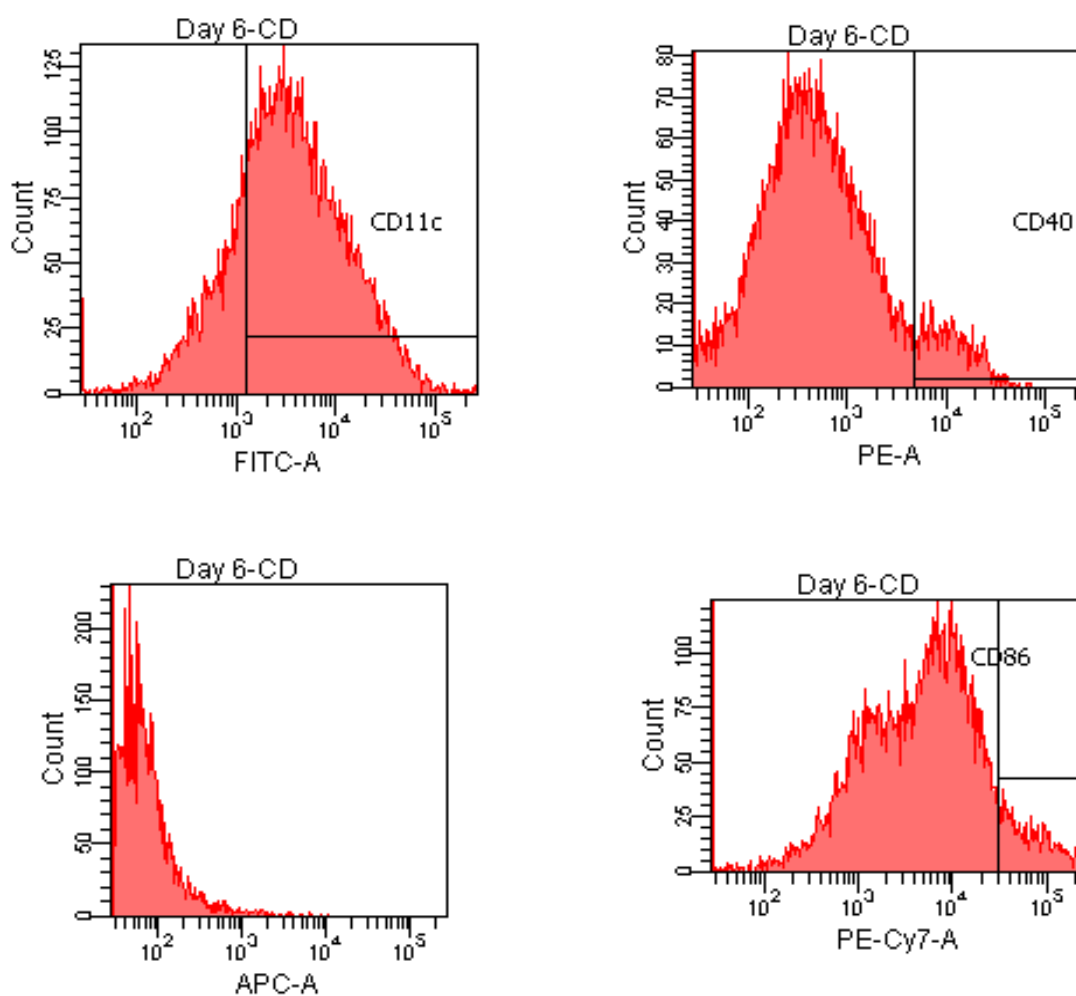
Table 1 Bacterial strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Source/reference
Bacterial strains		
MG1363	<i>L. lactis</i> MG1363 wild type strain	Gasson, 1983
NZ9000	A derivative of <i>L. lactis</i> MG1363 wild type strain generated by the integration of the <i>NisRK</i> genes	Kleeberzem et al., 1997
LL	<i>L. lactis</i> MG1363 containing pOri23 plasmid	Que et al., 2000
LL-mInIA+	<i>L. lactis</i> NZ9000 strain containing pOri253:mInIA	De Azevedo et al., 2012
LL-FnBPA+	<i>L. lactis</i> MG1363 strain harbouring pOri23:FnBPA plasmid	Que et al., 2001
LL-BLG	<i>L. lactis</i> MG1363 strain containing pOri23 and pValac:BLG plasmid	Pontes et al., 2012
LlmInIA- BLG	<i>L. lactis</i> NZ9000 strain expressing mInIA gene and carrying pValac:BLG plasmid	De Azevedo et al., 2012
LLFnBPA-BLG	<i>L. lactis</i> MG1363 strain producing FnBPA gene and carrying pValac:BLG plasmid	Pontes et al., 2012
Plasmids		
pPL2:mInIA	<i>E. coli</i> vector containing mInIA gene	Que et al., 2000
pOri253Ink	<i>L. lactis-E. coli</i> shuttle vector, Ery ^r	De Azevedo et al., 2012
pOri23	<i>L. lactis-E. coli</i> shuttle vector, Ery ^r	Que et al., 2000
pValac:BLG	<i>L. lactis-E. coli</i> shuttle vector carrying the BLG gene under the control of the eukaryotic promoter IE CMV, Cm ^r	Pontes et al., 2012

pOri253:mInIA	<i>L. lactis-E. coli</i> shuttle vector carrying the mInIA gene under the control of the constitutive PrfA promoter protein and harboring the native cell wall anchoring signal, Ery ^r	De Azevedo et al., 2012
pOri23:FnBPA	<i>L. lactis-E. coli</i> shuttle vector carrying the FnBPA gene of <i>S. aureus</i> ; Ery ^r	Que et al., 2001

Ery^r, Erythromycin resistant; Cm^r, Chloramphenicol resistant

FIGURE 1



Tube: CD			
Population	#Events	%Parent	%Total
All Events	21,568	###	100.0
Cells of interest	10,000	46.4	46.4
CD11c	7,811	78.1	36.2
CD40	589	5.9	2.7
CD86	968	9.7	4.5

FIGURE 2

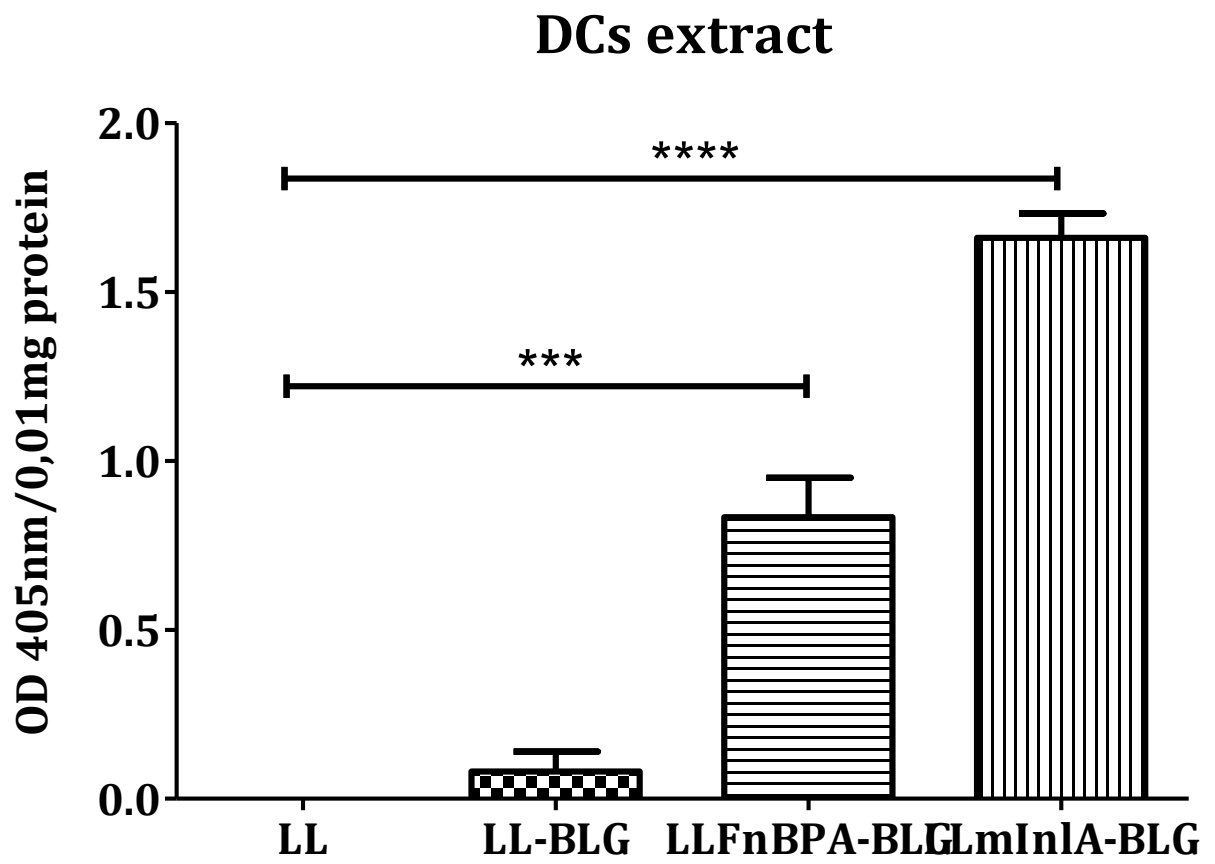
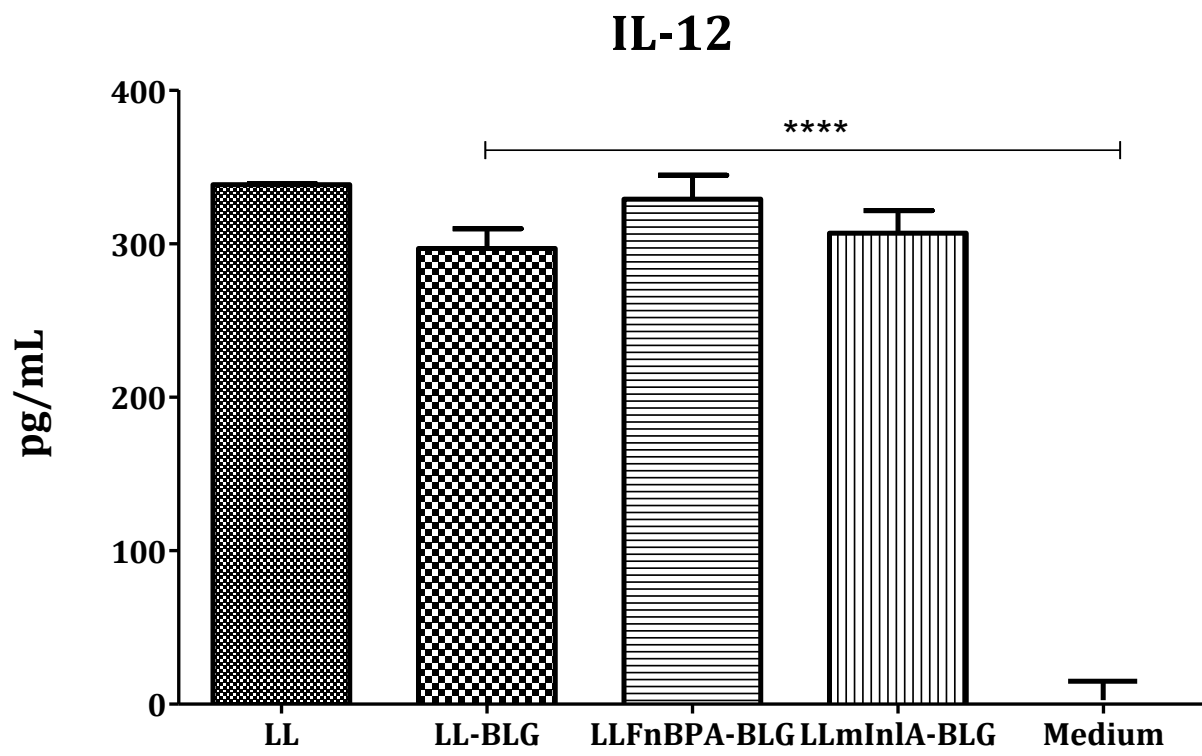


FIGURE 3



[

FIGURE 4

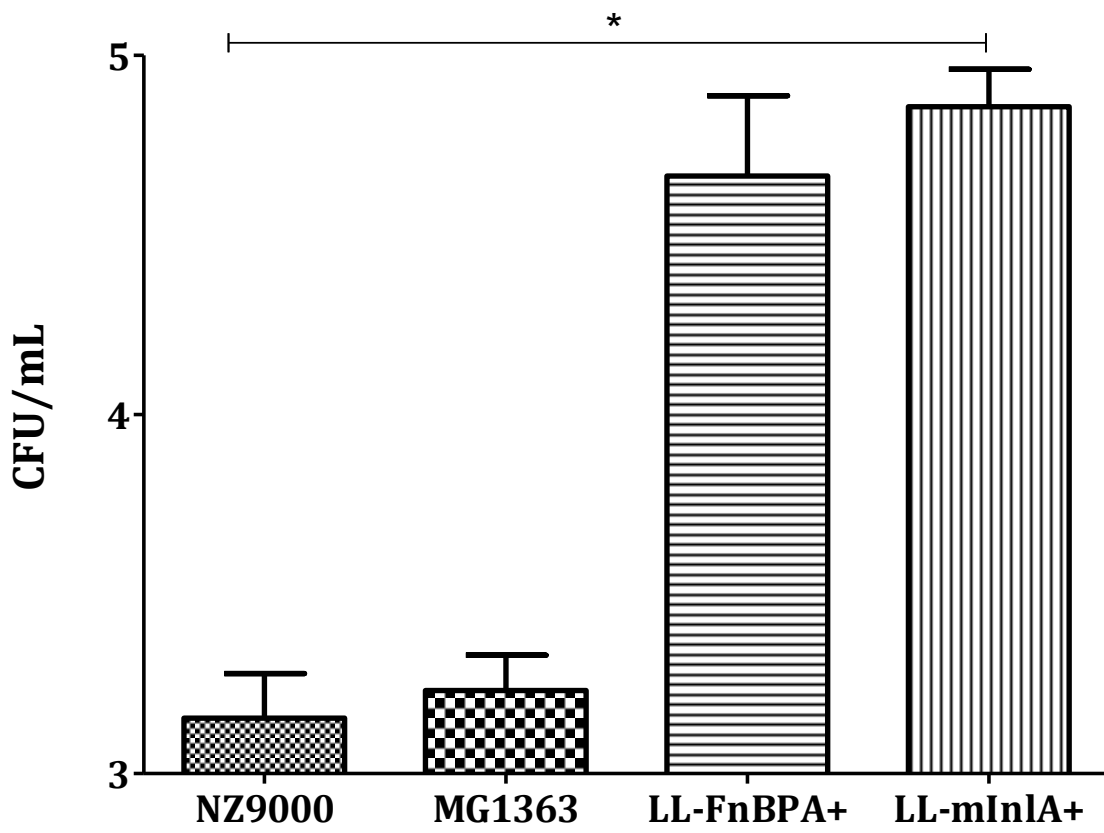
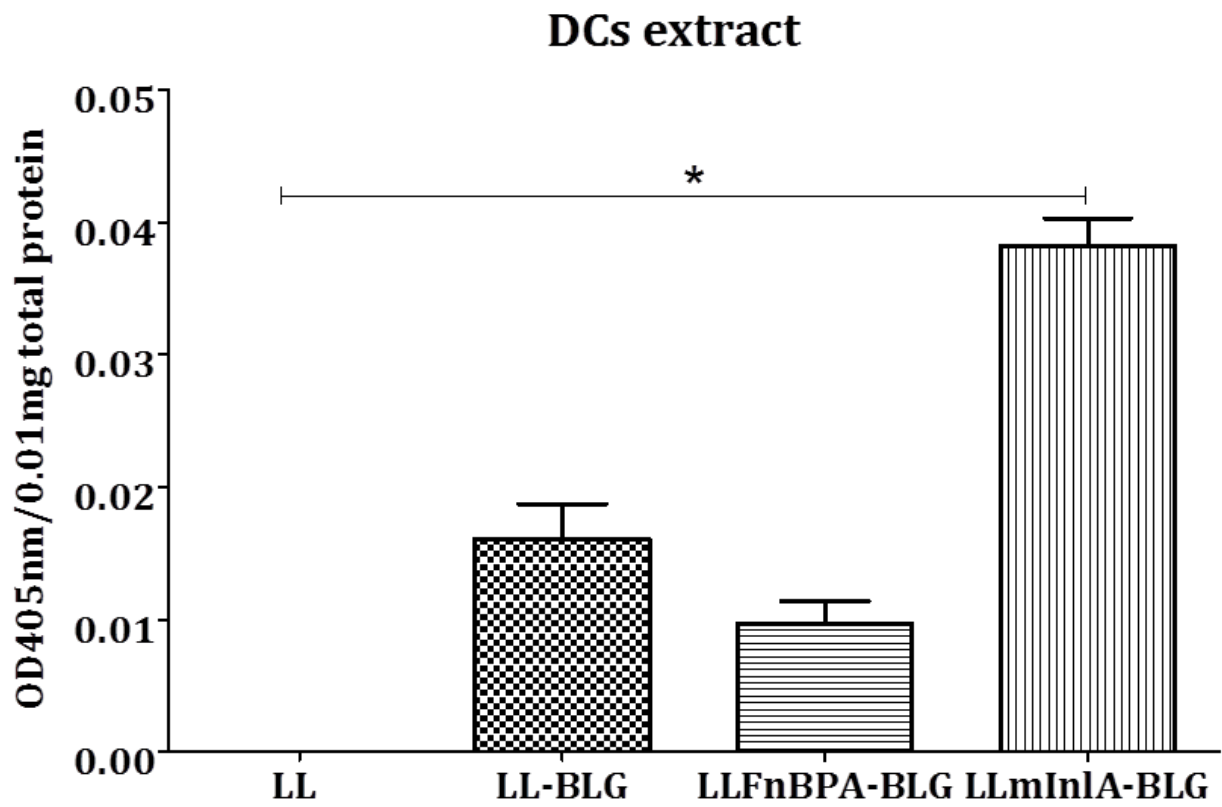


FIGURE 5



CHAPTER 6

**GENERAL DISCUSSION, MAIN CONCLUSIONS AND
DIRECTIONS FOR FUTURE WORK**

GENERAL DISCUSSION, MAIN CONCLUSIONS AND DIRECTIONS FOR FUTURE WORK

Several studies demonstrated with success that *L. lactis* is capable to express heterologous antigenic proteins eliciting mucosal, cellular, and humoral immunity against it (Wells and Mercenier, 2008; Bermudéz-Humaran et al., 2011). Ever since, recombinant *L. lactis* strains are being suggested as an alternative strategy to combat infectious diseases (Pontes et al., 2011). More recently, a new immunization system has been proposed in which *L. lactis* is used to deliver DNA vaccines instead of heterologous antigenic proteins. Guimaraes and co-workers showed that non-invasive *L. lactis* could deliver a eukaryotic expression vector *in vitro* to mammalian epithelial cells, demonstrating the potential of this new vaccination strategy (Guimaraes et al., 2006). Later Chatel and collaborators (2008) observed that *L. lactis* carrying the same eukaryotic expression plasmid were able to deliver it *in vivo* to intestinal epithelial cells of conventional mice (Chatel et al., 2008). The *in vivo* data demonstrated that *L. lactis* were actually able to successfully deliver DNA vaccines at mucosal surfaces.

As *L. lactis* is not a pathogen/invasive bacterium, this feature could be, in theory, a limiting factor for efficient DNA delivery. In order to better understand the mechanisms by which the DNA is transferred to mammalian cells and to test if a more invasive lactococci could improve gene transfer, our research group decided to construct recombinant invasive strains. In this work we constructed with success *L. lactis* expressing one invasin named mutated Internalin A (mInIA) derived *L. monocytogenes*. Although a *L. lactis* strain expressing a similar invasin, native InIA, have been already described and demonstrated to be more invasive than the wt strain and able to deliver more eukaryotic gfp gene into IECs, its use seemed to be limited in guinea pigs as InIA cannot bind to its receptor (E-cadherin) in conventional mice (Guimaraes et al., 2005; Pontes et al., 2011). Therefore, in order to facilitate *in vivo* studies we choose to work with mInIA invasin as it can bind to E-cadherin from both human and conventional mice.

We reported that *L. lactis* were able to express mInIA under the transcriptional control of the native promoter. FACS analysis revealed that recombinant lactococci efficiently displayed mInIA at its cell wall. LL-mInIA strain also demonstrated to be almost 1000 times more invasive than the wt strain (Azevedo et al., 2012) and equally invasive compared to other recombinant invasive *L. lactis* strains: one expressing *S. aureus* Fibronectin Binding Protein A (LL-FnBPA) (Innocentin et al., 2009) and *L. lactis* producing native InIA (LL-InIA) (Guimaraes et al., 2005) (data not shown). Confocal microscopy studies confirmed the invasive status of LL-mInIA strain as internalized bacteria could be detected in Caco-2 cells. Same data was previously obtained for other invasive lactococci, LL-FnBPA and LL-InIA after conventional and confocal fluorescence microscopy. Big clusters of both strains were uptaken by Caco-2 cells much more efficiently compared to the wt strain (Innocentin et al., 2009). Co-

incubation assays demonstrated that Caco-2 cells incubated with LL-mInIA strain carrying pValac:BLG eukaryotic expression vector were able to express significantly more BLG when compared to cells incubated with non-invasive strain harboring the same plasmid. The invasive status of lactococci showed to be advantageous *in vitro*, as demonstrated before with LL-FnBPA strain used to deliver a eukaryotic cGFP expression plasmid in Caco-2 cells which showed a highest percentage of fluorescence when compared with the control strain (Innocentin et al., 2009). Studies comparing plasmid transfer capacity *in vitro* between LL-mInIA, LL-InIA and LL-FnBPA strains are currently in progress. Differently from the *in vitro* data, after mice oral immunization with invasive and non-invasive lactococci we did not observe any significant increase on plasmid transfer by the use of LL-mInIA strain, even though the number of mice expressing BLG was higher in the group immunized with this strain. Pontes and collaborators in 2012 showed the same phenomenon using another invasive Lactococci, LL-FnBPA. They observed that this strain when treated with fetal calf serum could increase the number of mice producing BLG, but not the level of expressed BLG (Pontes et al., 2012). As we did not see a significant increase on plasmid transfer by the use of recombinant invasive lactococci we think that *in vivo* this is a complicated process where the complex structure of tissues (endogenous microbiota, mucus layer, immune system) could hamper optimal interaction of strains with mouse intestinal epithelium. Moreover, we believe that the main pathway whereby non-invasive or invasive *L. lactis* penetrates gut epithelial monolayers to deliver plasmids could be through Microfold (M) cells in Peyer's patches, instead of intestinal epithelial cells (IECs). Actually, another research group have shown that invasive *E. coli* expressing *Y. pseudotuberculosis* invasin is selectively uptaken from the intestinal lumen into Peyer's patches by using an *ex vivo* model (Critchley-Thorne et al., 2006).

As major conclusions our results demonstrated the successful expression of mInIA at *L. lactis* surface and that LL-mInIA+ strain is more invasive than the wt strain after co-incubation experiments with non-confluent Caco-2 cells. The invasive status was confirmed by confocal microscopy analysis which showed LL-mInIA+ strongly bound to the membrane of cell clusters and intracellular located in some cells. Furthermore, co-incubation of noninvasive (LL) and invasive (LL-mInIA+) lactococci, both carrying pValac:BLG eukaryotic expression plasmid, with non-confluent Caco-2 demonstrated that both strains were able mediate gene transfer. We also concluded that the invasive status significantly increased the efficiency of DNA delivery *in vitro* as more BLG was detected in Caco-2 cells extracts as well as in the culture supernatants which were incubated with LL-mInIA+. After oral administration in conventional BALB/c mice, we observed that *L. lactis* was able to transfer pValac:BLG to mice IECs leading to a subsequent BLG production by these cells. The use of LL-mInIA+ BLG strain showed a tendency to increase the number of mice expressing BLG suggesting that it is a slightly better DNA delivery vehicle when compared to the noninvasive Lactococci. Thus, we think that plasmid transfer

in vivo is a combination of bacteria and released plasmid captures and that DNA delivery may be a stochastic event depending on environmental factors. This research has thrown up many questions in need of further investigation. We believe that studies about bacterial co-localization after immunization trials using immunohistochemical techniques and the use of bacterial strains expressing or carrying DNA vaccines with reporter genes could give us more information about the fate of invasive or non-invasive lactococci. To study the influence of bacterium physiology on plasmid transfer by using another lactic acid bacterium strain instead of *L. lactis*, such as the probiotic *Lactobacillus casei* BL23 for example would be also very interesting.

Regarding the second chapter, we had the intent to compare the immune response elicited by DNA vaccination using invasive or noninvasive recombinant lactococci strains in a mouse model of food allergy. In this work we showed that DNA intranasal administration using invasive *L. lactis* FnBPA expressing strain tended to induce a Th2 primary immune response, characterized by the secretion of the cytokines IL-4 and IL-5, while the use of noninvasive strain tended to mount a Th1 response. Chatel and co-workers showed a decrease of the IgE concentration after sensitization in mice intranasally administered with noninvasive *L. lactis* (LL-BLG) (Chatel et al., 2008). Thus, reduction of IgE levels previously observed could be due to an increase of Th1 population, observed in this work by the increased secretion of IFN γ after mice immunization with LL-BLG strain. In order to evaluate if the expression of an invasin at *L. lactis* surface could change its immunogenicity, LLmInIA-BLG strain were administrated in conventional mice as well. It has been recently described that production of invasins at the surface of other LAB, such as lactobacilli may modify their immunomodulatory properties turning them neutral to pro-inflammatory strain (Fredriksen et al., 2012). It has been shown that immunized animals did not produce IFN γ , confirming the suppression of the pro-inflammatory immune profile which was induced by the noninvasive lactococci.

After oral or intranasal administration, we have shown that invasive LL-FnBPA+ elicited a low Th2 immune response while the intranasal administration of noninvasive *L. lactis* generated a Th1 immune response in the animals. It was also shown that sensitization of mice pre-treated with LL BLG strain demonstrated lower concentration of BLG specific-IgE. The results of this investigation demonstrated that mice orally or intranasally pre-treated with invasive *L. lactis* presented a Th2 immune polarization when compared with mice pre-treated with noninvasive *L. lactis*, confirming the Th2 orientation of the immune response elicited by the invasive strain. Experiments performed with LL-mInIA+ BLG reaffirmed that invasive lactococci do not polarize the immune response for a Th1 profile. The composition of the Peptidoglycan (PG) that could be modified by the expression of invasins at *L. lactis* cell wall was thought to be the reason of this skewed immune response. No differences could be detected in PG composition from invasive and noninvasive *L. lactis*. We could conclude that the DNA vaccination using noninvasive strain elicits a more pro-inflammatory response

after oral or intranasal administration while DNA vaccination using invasive strain elicits a more anti-inflammatory immune response.

In order to understand the reasons why invasive lactococci elicits a Th2 immune profile and non-invasive strain induces a Th1 immune response it would be interesting to assess the subsets of T cells involved in the response against these strains by flow cytometry analysis. We also intent to check if some bias is observed when recombinant proteins are delivered by invasive *L. lactis* instead of plasmids. A future study investigating which innate immune receptors (Toll like receptors – TLRs – or Nod like receptors – NLRs) expressed by epithelial cells or dendritic cells are involved in the recognition of non-invasive or invasive *L. lactis* using TLR-or NLR transfected HEK cells and bone marrow derived macrophages (BMDM) from wt and TLR/NLR knockout mice would be very interesting.

Considering the last chapter of this thesis, we had the curiosity to study DNA transfer in dendritic cells (DCs) by using non-invasive and invasive *L. lactis*. Current knowledge about DNA vaccination using *L. lactis* is mostly based on data obtained in studies with IECs. As DCs serve as potent inducers of specific cell-mediated immune responses acting as professional antigen-presenting cells (APCs), we evaluated DNA transfer capacity of this bacterium to DCs as well. The purpose of this work was to measure the ability of noninvasive or invasive *L. lactis* to deliver pValac:BLG directly to bone marrow-derived dendritic cells (BMDCs). It was also investigated the capacity of these strains to deliver pValac:BLG in a Transwell co-culture model where BMDCs were grown on the basolateral side of the IEC monolayer. Finally, immune response was also measured after infection with bacteria. After direct co-incubation of DCs with LLFnBPA-BLG and LLmInIA-BLG strains we have shown that cells were able to express significant higher amounts of BLG compared with the ones previously incubated with noninvasive (LL-BLG) or *L. lactis* lacking pValac:BLG plasmid. This data clearly demonstrates that *L. lactis* can transfect APCs besides IECs. Furthermore, differently of what was observed for Caco-2 cells, we did not see any secretion of BLG by DCs (data not shown). Other research groups have already demonstrated DNA transfer to DCs using bacteria. In 2005, Kudela and co-workers performed a similar work in which they demonstrated that DCs incubated with gram-negative bacterial ghosts loaded with eukaryotic expression plasmids encoding GFP exhibited high expression levels of GFP (up to 85%) (Kudela et al., 2005). Another one performed by Paglia and collaborators have shown that a auxotrophic mutant of *Salmonella typhimurium* carrying vectors with GFP under the control of an eukaryotic promoter were able to transfect either splenocytes of mice, macrophages or DCs as they were scored positive for GFP expression after cytofluorometric analysis (Paglia et al., 1998). However, we show here for the first time, that DCs can be directly and specifically transduced by a lactic acid bacterium, *L. lactis*, which harbors the GRAS status and is considered to be safer than attenuated pathogens and gram-negative hosts (Pontes et

al., 2011). In order to evaluate if *L. lactis* can transfer DNA vaccines across the intestinal epithelium to DCs we chose to work with an interesting *in vitro* model that mimic the *in vivo* situation. DCs were grown on the basolateral side of permeable inserts and Caco-2 cells were polarized on its apical side. This model have been used for studies attempt to understand the cross-talk between bacteria, epithelial and dendritic cells (Zoumpopoulou et al., 2009). We observed that DCs co-cultured with the IEC monolayer which were infected with LLmInIA-BLG strain could express significant higher amounts of BLG when compared with *L. lactis* strain lacking pValac:BLG vaccine. The fact that we could detect BLG only in DCs incubated with LLmInIA-BLG could be due to E-cadherin expression by DCs (Bossche et al., 2012) which may have facilitated bacterial-cell interactions. Besides this, LLmInIA-BLG were probably phagocytized by the DCs which were in contact with the monolayer of epithelial cells, then pValac:BLG plasmid were delivered to their nucleus allowing BLG expression. This data gives new insights on how the DNA transfer process occurs *in vivo*. Furthermore, the monolayer of Caco-2 cells could not secrete the allergen as no BLG was detected in apical or basal culture supernatants. Furthermore, no BLG was found in Caco-2 extracts. Differently, we previously have shown that direct co-incubation of LLmInIA-BLG strain with non-confluent Caco-2 cells made them capable of either express or secrete BLG (Azevedo et al., 2012). Thus, we think that when Caco-2 cells are grown in a polarized fashion the interaction with both noninvasive and invasive *L. lactis* may be hampered due to the presence of epithelial tight junctions. Actually, the receptor for mInIA, E-cadherin, is expressed at the basolateral side of the epithelial cell and this fact may limit bacteria-epithelial cell interaction, decrease pValac:BLG transfection to the cell nucleus and therefore diminish BLG expression. We also observed that DCs co-cultivated with noninvasive or invasive lactococci were able to secrete elevated levels of the pro-inflammatory cytokine IL-12 compared to cells which were not incubated with bacteria (medium). It has been demonstrated that wild type (wt) *L. lactis* can provide constant stimulation to immune-related cells at mucosal surfaces. For example, *L. lactis* subsp. *cremoris* FC could induce the production of cytokines such as IL-10, IL-6, TNF- α and a high production of IL-12 from murine bone marrow DCs (Kosaka et al., 2012). Another recent work has shown that the co-incubation of wt *L. lactis* MG1363 with murine induced elevated levels of TNF α and IL-10 (Smelt et al., 2012). We also detect that the expression of BLG by DCs neither the invasive status of strains did not change the level of IL-12 produced.

This study has shown that non-invasive or invasive *L. lactis* (LL-mInIA+ and LL-FnBPA+) carrying pValac:BLG can mediate gene transfer in DCs as BLG was detected in BMDCs extracts after direct co-incubation with strains. A higher amount of BLG was produced by DCs which were co-incubated with the invasive strains demonstrating that the invasive *status* was advantageous *in vitro*. Furthermore, DCs were not able to secrete the allergen. Another finding was that noninvasive or LL-mInIA+ and LL-FnBPA+ strains induced the secretion of the proinflammatory cytokine IL-12 in BMDCs.

Invasive *L. lactis* demonstrated to be 100 times more invasive than the wt strain in a monolayer of differentiated IECs. In a co-culture system where BMDCs were grown on the basolateral side of the IEC monolayer we have also shown LLmInIA+ BLG strain was the only one capable to transfer pValac:BLG to DCs across the monolayer of IECs providing additional evidence on how might be taken up *in vivo* . The results of this investigation show new insights on the mechanism of lactococci uptake for delivery of therapeutics. Further research might investigate *in vitro* activation of T lymphocytes with DCs previously stimulated with invasive and non-invasive *L. lactis* in order to compare immune response generated by both strains. Future research should also concentrate efforts to evaluate DCs immune response elicited by non-invasive and invasive lactococci using a co-culture system and to explore *in vivo* DNA transfer to DCs.

CHAPTER 7
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APPENDICE 1-

**Recombinant *Lactococcus lactis* expressing both
Listeria monocytogenes Lysteriolysin O and mutated
internalin A applied for DNA vaccination**

Perspectives on the use of recombinant *Lactococcus lactis* expressing both Listeriolysin O and mutated internalin A from *Listeria monocytogenes* as a tool for DNA vaccination

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Abstract: *Lactococcus lactis* have been engineered in this work to express mutated internalin A at its surface and to secrete great amounts of Listeriolysin O (LLO), both proteins derived from the food born pathogen *Listeria monocytogenes*, in order to be used as a tool for DNA vaccination. Western Blot experiments demonstrated that the bacterium was able to express LLO in both cytoplasmic and extracellular compartments, with a higher quantity found in culture supernatants. Hemolytic assay have shown that the recombinant strain is capable of secreting 250 ng of LLO/mg of total protein in active format. Finally, we believe that mInIA/LLO *L. lactis* producing this strain could have the potential to be used as an alternative tool in DNA vaccination against a number of infectious diseases or in cancer therapy.

Key words: Lactic Acid Bacteria, *Lactococcus lactis*, *Listeria monocytogenes*, Listeriolysin O, Mutated internalin A

Introduction

The strategy to use GRAS (Generally Regarded as Safe) bacteria, such as the most economically important Lactic Acid Bacteria (LAB), *Lactococcus lactis*, as a vector to deliver therapeutic plasmids seems a very interesting alternative approach. *L. lactis* has been proposed as a safe and effective vaccine platform for the delivery of therapeutic molecules to the immune system.¹ It has been shown that wild type (wt) *L. lactis* is capable to deliver DNA vaccines both *in vitro* and *in vivo* after oral inoculation of mice.^{2,3} Nevertheless, the ratio of DNA transferred to mammalian cells observed was low. We hypothesized that this low efficiency detected could be due to the transient nature of *L. lactis* which might avoid its optimal interaction with intestinal epithelial cells (IECs) from the gastrointestinal tract and therefore prevent DNA translocation. Thus, strains of *L. lactis* expressing recombinant virulent genes were recently constructed in attempt to improve their capacity to stay longer in the gastrointestinal tract and therefore deliver more DNA vaccines.^{4,5} Guimaraes and co-workers were one of the first to explore the potential use of recombinant *L. lactis* strains engineered to express invasins naturally produced by enteropathogenic species, such as Internalin A (InIA) from the food-borne pathogen *L. monocytogenes*.^{2,6} InIA is a sortase anchored cell wall protein which contains 800 amino acids (aa) in length that mediates bacterial entry into mammalian epithelial cells.⁷ Due to InIA, *L. monocytogenes* can invade mucosal surfaces in the small intestine and interact with Peyer's patch-based immune system, including intestinal dendritic cells, which are potent antigen presenting cells.⁸ InIA-expressing *L. lactis* (LL-InIA+) strain showed to be able to invade Caco-2 cells *in vitro* and also intestinal cells after oral immunization in guinea pigs. Furthermore, LL-InIA+ strain were able to deliver a functional eukaryotic gfp gene (green fluorescent protein) into epithelial Caco-2 cells more efficiently than the wt lactococci demonstrating to be a good tool for gene delivery.² Nevertheless, even though interesting, the use of LL-InIA+ strain presented some bottlenecks as InIA can only bind to its receptor (E-cadherin) in guinea pigs or transgenic mice, turning *in vivo* studies laborious and/or expensive.⁹ For this reason, an *L. lactis* strain producing a mutated form of InIA (LL-mInIA+), which can bind to E-cadherin from conventional mice was recently reported by our research group. Mutated internalin A (mInIA) rendered *L. lactis* an invasive status, improving its interaction with intestinal epithelial cells, leading to a higher level of DNA transfer *in vitro*.¹⁰ As *in vivo* the use of LL-mInIA+ did not statically increased the DNA delivery, we decided to construct another strain able to express both mInIA and another virulence factor from *L. monocytogenes*, the haemolysin listeriolysin O (LLO). In this pathogen LLO expression is up-regulated at low pH (~pH 5.5) inside de phagosome which then oligomerize to form a pre-pore complex, rupturing the Listeria-containing phagosome.^{11,12} Therefore, the expression of LLO in *L. lactis* would in theory facilitate the escape of the plasmid DNA from the cytoplasm to the nucleus. Thus, the result could be the cellular expression of the transfected gene in a higher efficiency. Actually, it was already demonstrated that LLO incorporated in an anionic liposome was very suitable to enhance plasmid DNA delivery.¹³ Besides increasing plasmid transfer, it was demonstrated that LLO-containing pH-sensitive liposomes encapsulated with immunostimulatory CpG oligonucleotides was able to efficiently deliver Ovalbumin (OVA) antigen to the cytosol of APCs and stimulate cytotoxic T lymphocytes (CTLs) driving Th1-type immune responses.¹⁴ Another research group demonstrated that LLO-liposome-mediating cytosolic delivery of antigens *in vivo* could enhance antigen-specific cytotoxic T lymphocyte frequency, activity conferring tumor protection.¹⁵ Thus, in the present work, food-grade *L.*

lactis NZ9000 strain was engineered to constitutively express both mInIA and LLO. This strain might then serve as a vehicle for DNA vaccination in near future.

Results

***L. lactis* is able to successfully express mutated internalin A on its surface.**

In this work we proposed two approaches thought to enhance plasmid transfer both *in vitro* and *in vivo* by expressing in *L. lactis* two major immunodominant antigens, LLO and mInIA, derived from *L. monocytogenes*. After transformation of *L. lactis* NZ9000 strain with pLL31 plasmid by electroporation, flow cytometry analyses were performed to firstly detect recombinant mInIA. Plasmid pLL31 harbours the native *hlyA* gene (encoding LLO) containing its constitutive promoter, the signal peptide and the ribosomal binding site. It also contains the mutated internalin A ORF, the constitutive PrfA promoter protein and the cell wall anchoring signal. *L. lactis* expressing native InIA, wt *L. lactis* NZ9000 and the strain constructed in this work producing both LLO and mInIA (LL-mInIA+LLO+) were incubated with monoclonal antibody anti-InIA and afterwards with (FITC)-conjugated AffiniPure Fab fragment Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research). Data was then analyzed by flow cytometry analysis. As demonstrated in Figure 1, strains producing mInIA (blue and yellow peak) or native InIA (red peak) could significantly shift the distribution curve comparing to the wt strain (black peak). Fluorescence intensity obtained for mInIA producing strain was two times higher compared to the wt strain while LL-InIA could express four times more recombinant protein. This assay confirmed the expression of mInIA on the surface of *L. lactis*.

***L. monocytogenes* Listeriolysin O is constitutively produced and secreted by *L. lactis*.**

After confirming mInIA expression on *L. lactis* surface, western blotting experiments were conducted with the intent to detect recombinant LLO in *L. lactis* cytoplasmic and extracellular cell compartments. Strains (wt and LL-mInIA+LLO+) were grown for protein extraction. Both supernatant and cytoplasmic proteins were collected and submitted to Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were then blotted against a nitrocellulose membrane, which was incubated with primary anti-LLO antibody (abcam, France) and secondary goat Anti-Rabbit IgG (γ heavy chain specific) and signals of LLO were determined. Analysis of stationary-phase cell lysates showed the presence of a band at the expected position, which is in accordance with the molecular weight of LLO (58 kDa) in both soluble and insoluble cytoplasmic protein fractions as well as in culture supernatants, with a great amount of recombinant protein found in this compartment (Figure 2). LLO was not detected in protein extracts from supernatant and cytoplasmic compartments derived from the wt strain.

Recombinant Listeriolysin O is produced as an active pore-forming toxin in *L. lactis*.

Subsequently, the haemolytic assay was carried out to assess if LLO was being produced as an active protein. Culture supernatant and cytoplasmic proteins derived from both wt and LL-mInIA+LLO+ strains were extracted and then used in the assay. A purified recombinant LLO (rLLO) was used as a standard to measure protein activity and to quantify the amount of LLO produced by *L. lactis*. Either rLLO or protein preparations from wt and *L. lactis* producing mInIA-LLO strain were incubated with Red Blood Cells. Afterwards, hemolytic activity was measured at 540 nm in a

spectrophotometer and LLO production was quantified using the same approach. As demonstrated in Figure 3, protein extracts (both soluble and insoluble) as well as culture supernatants derived from recombinant *L. lactis* revealed a strong haemolytic activity when compared to the negative control (LLwt). We also observed that LLO from the insoluble compartment has pore-forming activity similar to LLO derived from the soluble fraction. Furthermore, we also checked that the highest hemolytic activity was found in the culture supernatant. Moreover, almost 250 ng/mg of total protein were found to be constitutively secreted by *L. lactis*. This hemolytic assay clearly revealed that LLO is well secreted in *L. lactis* as a highly active pore-forming toxin.

Discussion

Attenuated strains of *L. monocytogenes* have been proposed as a vehicle to deliver therapeutic plasmids to eukaryotic cells. This bacterium has the capacity to induce its own uptake into nonphagocytic mammalian cells by the expression of InIA and to form pores in phagolysosomal membranes due to the production of LLO facilitating DNA plasmid transfer to the mammalian cell nucleus.^{8,16} Several preclinical studies have demonstrated the ability of *L. monocytogenes* for intracellular gene delivery. Furthermore, this vector has also displayed a relatively safety and efficacy in some clinical trial.¹⁶ However, even though attractive, there is always the insecurity that attenuated vectors might restore the ability to replicate and cause disease in the patient. This fact should be taken into further consideration when it comes to the administration in infants and immuno-compromised individuals. Actually, reversion to virulence, preexisting immunity, and reactogenicity will always remain major concerns when pathogenic species are considered as a vaccine platform.¹⁷ Thus, the scientific community has recently been exploring the use of non-pathogenic bacteria, such as lactic acid bacteria (LAB), as prophylactic or therapeutic tools and more recently as a DNA delivery vehicle for genetic immunization.^{5,18} In 2008, Chatel and collaborators have shown that the model LAB, *L. lactis*, was able to deliver a plasmid to the epithelial cells of the intestinal membrane of conventional mice. Besides this study has demonstrated that *L. lactis* could be used as a vector for genetic immunization, the ratio of plasmid transfer observed was low.³ The transient physiology of *L. lactis* was considered to be the cause of this limitation. Thus, recent research is focusing on the construction of recombinant invasive lactococci strains as this feature could increase plasmid DNA delivery.^{4,5,10} One very interesting strategy performed by Guimarães and collaborators was to engineer *L. lactis* to express InIA (LL-InIA+) from *L. monocytogenes*. The recombinant strain proved to be able to enter intestinal cells *in vivo*, after oral inoculation of guinea pigs. After internalization, LL-InIA+ was able to deliver a functional eukaryotic gfp (green fluorescent protein) into epithelial Caco-2 cells.² Nevertheless, the disadvantage was the limitation of the strain in *in vivo* experiments as InIA does not bind to its receptor, E-cadherin, in conventional mice. Therefore, a mutated form of InIA that can bind to murine E-cadherin, mInIA, was successfully expressed in *L. lactis* thus facilitating *in vivo* studies. De Azevedo et al.² demonstrated that the expression of mInIA at *L. lactis* surface could improve its invasive capacity in experiments performed with IECs. Moreover, the invasive characteristic increased the DNA vaccine delivery *in vitro* but not *in vivo*.¹⁰ In this work we decided to engineer *L. lactis* to express besides mInIA another virulent factor of *L. monocytogenes*, LLO, as it could facilitate plasmid escape to the nucleus increasing the production of the gene of interest. We demonstrated

with success that mInIA was correctly expressed at *L. lactis* surface using flow cytometry analysis. The antibody used in the assay was specific for native InIA what could explain the decreased detection of mInIA. After confirming mInIA expression we moved to experiments designed to identify the production of LLO by *L. lactis*. Western Blot assay exhibited a dual localization of LLO, being found in both extracellular and intracellular compartments. It is interesting to notice that half of the LLO produced was found in the soluble fraction with a great amount correctly secreted to the extracellular medium. The other half was detected in the insoluble fraction demonstrating that LLO was attached to the cell envelope. This result could be likely due to the presence of inclusion bodies. It is known that recombinant proteins expressed in *L. lactis* can be subjected to inadequate stability and/or solubility, leading to protein improper folding, inclusion body formation and/or protein degradation.¹⁹ Thus, it is possible that a post-translational process could be limiting LLO secretion, such as insoluble aggregates formation.

The hemolytic assay demonstrated that the protein is being produced as an active pore forming toxin. This is a very important aspect when considering further experiments with the strain as it ensures that phagosomal membranes certainly will be disrupted helping plasmid escape. Recently Vadia and collaborators showed that LLO can also serve as an invasion factor sufficient to induce the internalization of noninvasive *Listeria innocua*.²⁰ This might be another advantage feature for *L. lactis* producing mInIA-LLO strain which would be able to establish a more intimate contact with host cells from the gastrointestinal tract and then promote a higher plasmid transfer after oral inoculation. Other research groups have reported the use of bacteria expressing recombinant LLO for vaccination proposals. *L. lactis* have been engineered to produce this protein induced by an expensive peptide named nisin, or with the gene chromosomally integrated as a vaccine against Listeriosis.^{21,22} The fact that LLO was engineered in this work to be constitutively expressed might facilitate its commercialization and lower the cost of purification process, when compared to this other LLO-*L. lactis* producing strain in which nisin is needed to induce gene expression.⁹

Perhaps the use of *L. lactis* expressing both virulent factors from *L. monocytogenes*, mInIA and LLO, as a tool to deliver DNA plasmids may eventually represent an efficient strategy that could have significant applications in basic research, cancer therapy or in the vaccinology field. Another application could be the use of the strain as a source of pure LLO as *L. lactis* does not produce both inclusion bodies neither endotoxins (LPS), differently from recombinant *E. coli* expressing LLO that contains LPS in its outer membrane.²³ Finally, we are currently evaluating DNA transfer capacity of this novel *L. lactis* strain in experiments performed with IECs *in vitro*. Afterwards our intent is to measure DNA transfer *in vivo* after oral inoculation the strain in conventional BALB/c mice.

Material and Methods

Bacterial strains, media and growth conditions. Bacterial strains and plasmids used in this work are listed in table 1. *Lactococcus lactis* NZ9000 was used as host strain for pLL31 plasmid, which was gently provided by MORU VAZE Company. Electrocompetent *L. lactis* strains were prepared and transformed with the corresponding pLL31 expression vector and plated into M17 agar supplemented with 0.5% glucose and 10 µg/mL of Erythromycin. Colonies were screened by PCR analysis. Procedures for DNA manipulation were carried out as described by Sambrook et al.²⁴

Detection of recombinant mInIA by flow cytometry analysis. Bacterial cells were collected and resuspended in PBS containing 0.5% of bovine serum albumin (BSA) (Sigma) and 10 $\mu\text{g}/\text{mL}$ of monoclonal antibody anti-mInIA. After incubation at 4°C, suspension were centrifuged and pellets were resuspended in PBS containing 0.5% of BSA (Sigma) and FITC-conjugated AffiniPure Fab fragment Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research). Bacteria were then fixed in 2% paraformaldehyde and flow cytometry was performed on Accuri's C6 Flow Cytometer® System. FITC fluorescence was measured by using two-color excitation in the FL1-A channel. Data analysis was performed using CFlow Software (Accuri Cytometers, Inc.).

Investigation of LLO production by Western blot analysis. Protein sample preparation from *L. lactis* cultures was performed as previously described²⁵ and then used for immunodetection. Supernatant was collected as well soluble and insoluble cytoplasmic protein extracts which were assayed for the presence of LLO. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed and gels were blotted against a nitrocellulose membrane and then blocked overnight at 4°C in 5% BSA (Sigma). Primary anti-LLO antibody (abcam, France) and secondary goat Anti-Rabbit IgG (γ heavy chain specific) were used at 1/15000 and 1/20000 dilutions in 5% BSA, respectively. Blots were then scanned and the signals of rLLO were analyzed using a commercial protein standard.

Assessment of recombinant LLO haemolytic activity. In order to quantify and verify if LLO was being produced in active form, the haemolytic activity of protein preparations were performed. Firstly, total protein was quantified by Bradford assay and Assay Buffer (AB) was prepared to give a 1% Red Blood Cell (RBC) solution diluted in Low pH Assay Buffer (LpHAB) [125 mM NaCl (Synth), 35 mM dibasic sodium phosphate (J.T.Baker), 0.5 mg/mL BSA (Sigma), pH 5.5]. 1 mg of total protein derived from culture supernatants, 0.25 mg of total protein from soluble and insoluble cytoplasmic extracts and 50 ng/ml of recombinant LLO diluted in LpHAB as well as the positive control (40% μl AB solution; 50% of LpHAB; 10% of Triton) were added to a 96 well microtiter plate-V-bottom format (Edge Bio). Negative control (LpHAB buffer) was also included. Samples were serially diluted two times and plate was incubated at 37°C. After spin down, 100 μL of the supernatant were transferred to a flat-bottomed microtiter plate (Edge Bio) and absorbance was measured at 540 nm in a spectrophotometer. Data was quantitatively analyzed using a standard curve, which was fitted based on rLLO.

Statistical analyses. Statistical significance between the groups was calculated using the One Way ANOVA test, followed by the “Bonferroni” post-test. Values of $p < 0.05$ were considered significant.

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Figure and table Legends

Fig.1 Detection of mutated InIA at *L.lactis* surface by fluorometry analysis. wt lactococci (black peak, negative control) or recombinant *Lactococcus lactis* expressing *Listeria monocytogenes* Internalin A (InIA) (red peak, positive control), mutated InIA (mInIA) (blue peak) or both Listeriolysin (LLO) and mInIA (yellow peak)

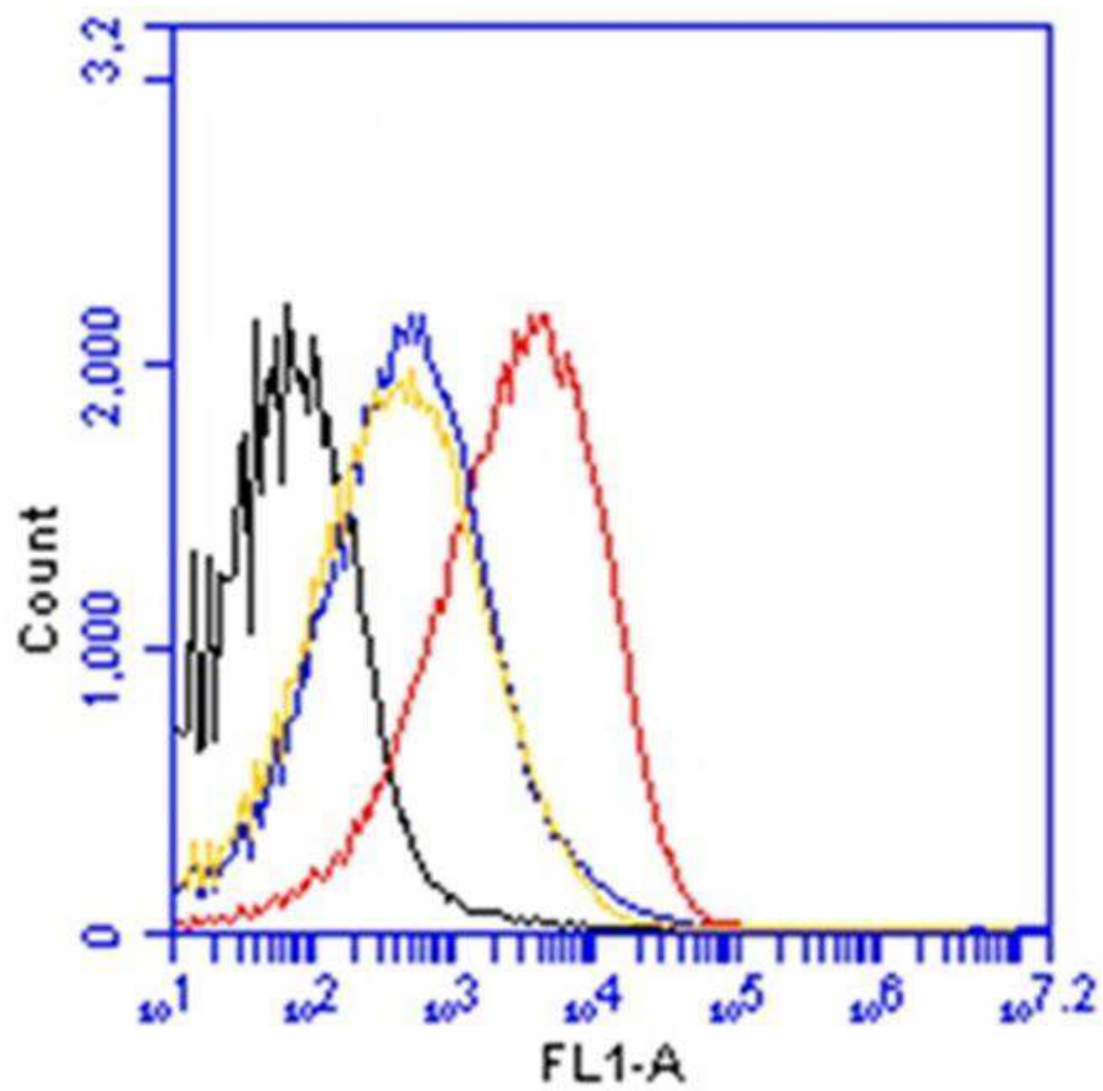
Fig.2 Western blot demonstrating the cytoplasmic and extracellular expression of Lysteriolysin O (LLO) in *Lactococcus lactis* NZ9000. Protein extracts of recombinant or non-recombinant *L. lactis* strain were prepared from cell (left) and

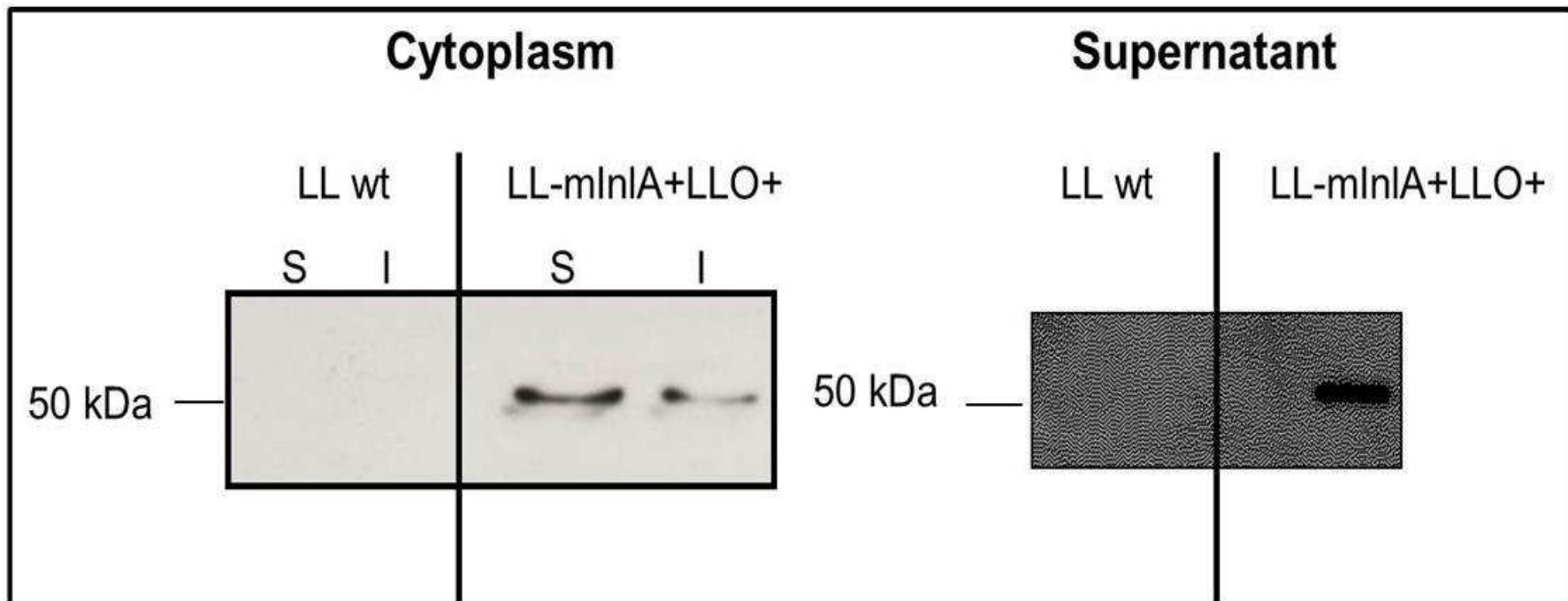
supernatant (right) fractions. LLwt: *L. lactis* NZ9000, LL-mInIA+LLO+: *L. lactis* expressing both *Listeria monocytogenes* mutated InIA and LLO, S: soluble cytoplasmic fraction, I: Insoluble cytoplasmic fraction

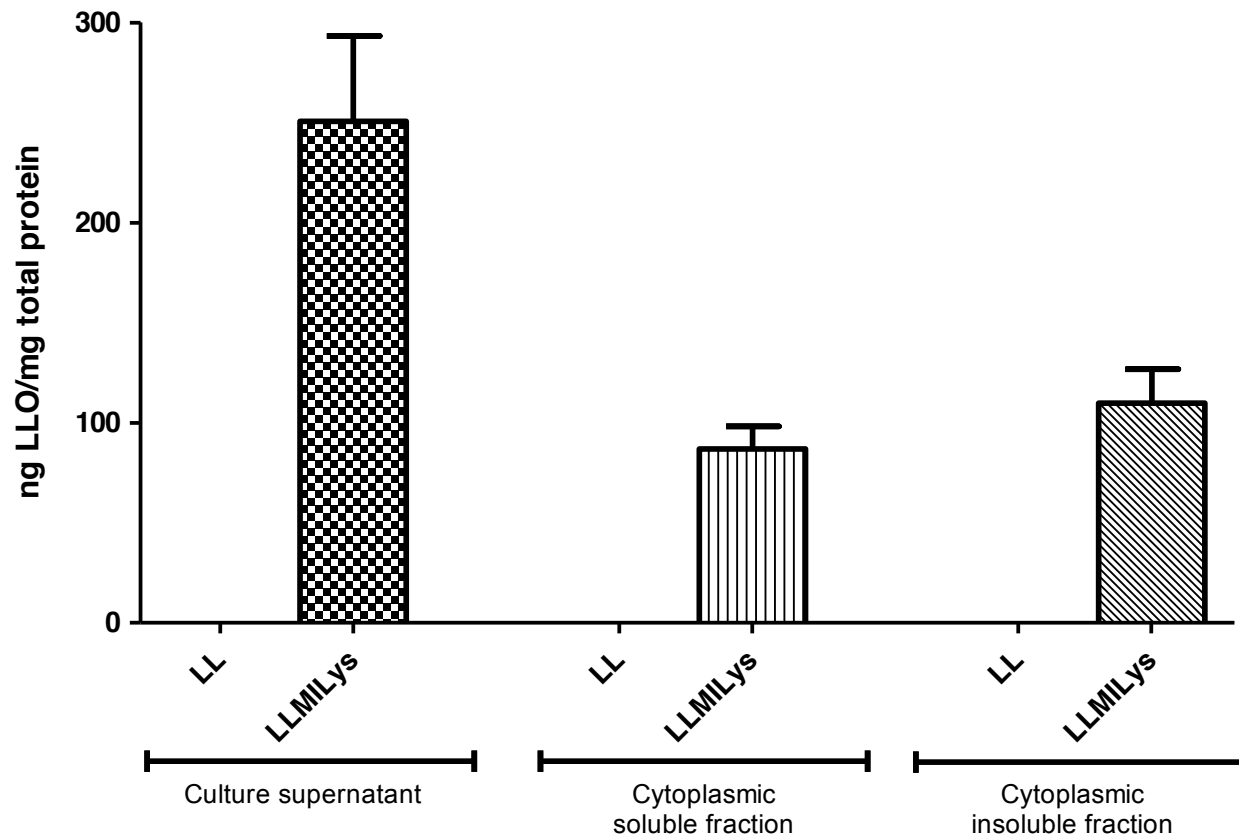
Fig.3 Haemolytic activity of Listeriolysin O (LLO) expressed in *Lactococcus lactis*. Sheep red blood cells were incubated with cytoplasmic extracts and culture supernatants to evaluate LLO pore forming capacity. LLwt: *L. lactis* NZ9000, LLMILys: *L. lactis* expressing both *Listeria monocytogenes* mutated InIA and LLO, S: soluble cytoplasmic fraction, I: Insoluble cytoplasmic fraction

Table 1 Bacterial strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Source/reference
Bacterial strains		
<i>Lactococcus lactis</i> MG1363 (LL wt)	A derivative of <i>L. lactis</i> MG1363 wild type strain generated by the integration of the <i>NisRK</i> genes	26
<i>Lactococcus lactis</i> expressing both mutated internalin A (mInIA) and Lysteriolysin O (LLO) (LL-mInIA+LLO+)	<i>L. lactis</i> NZ9000 strain containing pLL31 plasmid	This work
Plasmids		
pLL31	<i>L. lactis</i> - <i>Escherichia coli</i> shuttle vector carrying the mInIA gene under the control of the constitutive PrfA promoter protein and harboring the native cell wall anchoring signal and hlyA gene (encoding LLO), containing its constitutive promoter and own signal peptide.	MORU VAZE Company







APPENDICE 2-

**Immunotherapy of allergic diseases using probiotics
or recombinant probiotics**

IMMUNOTHERAPY OF ALLERGIC DISEASES USING PROBIOTICS OR RECOMBINANT PROBIOTICS

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Key words: Recombinant probiotics, Lactic Acid Bacteria, allergic diseases, β -lactoglobulin, allergy treatment, Immunotherapy

SUMMARY Allergic diseases affect up to 30% of the western population and their prevalence is increasing. Probiotics are able to modulate the mucosal immune response and clinical trials demonstrated that specific strains, especially Lactic Acid Bacteria (LAB) ones, reduce allergic symptoms. Moreover, the use of recombinant probiotics has been evaluated as possible strategies for the immunotherapy of allergic diseases. The production and delivery of allergens by recombinant LAB in concert with their ability to induce a Th1-type immune response have been shown to be a promising mucosal vaccination strategy in mouse model. Aim of this paper is to review the applications of probiotics and recombinant LAB in allergy immunotherapy with a special focus on beta-lactoglobulin cow's milk allergy model.

IMMUNOLOGICAL BASIS OF ALLERGIC DISEASES

Allergic manifestations are ranked as the fourth most important disease in the world and they are responsible for a substantial health-care burden on society-(Hong et al. 2012). The World Health Organization estimates that over 20% of the world's population suffers from IgE-mediated allergic diseases and that their prevalence over the last 20-30 years is increasing dramatically (WHO/WAO 2002).

Allergies usually occur when the immune system reacts against harmless substances present in the environment. These substances called allergens can enter in contact with the Immune System through various routes such as inhalation, ingestion, skin contact or enter directly into the body through an insect bite. The exposure to environmental allergens can cause a variety of symptoms associated with a range of conditions including allergic asthma, allergic rhinitis, food allergies, and dermatological problems such as allergic eczema and urticarial (Weiner et al. 2011).

The allergic immune response is a complex process beginning with the allergen being presented to allergen-specific naïve CD4+ T lymphocytes by antigen presenting cells (APCs). After antigen

presentation, these cells become activated and can differentiate to T helper type 2 (Th2) cells. At the second exposure to the same allergen, activated Th2 cells secrete interleukins such as IL-4, IL-5 and IL-13. These interleukins are able to stimulate B cell activation leading to the secretion of allergen-specific IgE antibodies which induce cross-linking of the high-affinity IgE receptor (FcεRI) on mast-cells and basophiles. Th2 cell cytokines and IgE activated cells of the innate immune system (eosinophils, mast cells, and basophiles) release histamine and other inflammatory mediators into surrounding tissues. The resulting pathophysiological response includes vasodilation, mucous secretion, nerve stimulation and smooth muscle contraction that manifests clinically as itchiness, rhinorrhea, dyspnea, and anaphylaxis characterizing the type I allergic reaction (Gould and Sutton 2008; Bosnjak et al. 2011). The role of the Th2 cell-mediated immune response against innocuous environmental allergens is well documented. Furthermore, it is important to mention that the expression of the allergic phenotype also depends on the individual predisposition and the gene-environment interactions (Romagnani 2004).

Overall, allergic manifestations are complex diseases that result on the deregulation of the IgE system homeostasis (Gould and Sutton 2008). The development of type I allergy reflects an imbalance in the T-lymphocytes immunity associated with the dominance of Th2 response. Th2 cells secrete IL-4, IL-5 and IL-13 that trigger IgE production by B lymphocytes, as mentioned above. In contrast, Th1 lymphocytes secrete IFN γ which antagonize IL-4 production and consequently, Th2 cells development. IFN γ inhibits IgE switching on B lymphocytes and promotes the differentiation of naïve T cells towards Th1 subsets preventing Th2 cells proliferation (Pène et al. 1988).

In normal circumstances, allergic reactions against commensal micro-organisms or innocuous antigens contained in food are prevented by the induction of immunological tolerance as the peripheral immune system has evolved many strategies to maintain a state of tolerance to

harmless antigens (Akbari et al. 2001). In allergic individuals, the unresponsiveness to innocuous antigens is breakdown. One of the major mechanisms of oral tolerance is the induction of Regulatory T cells (Tregs cells), favoured by Resident Dendritic Cells (DC) of the mucosal tissue, and the production by mucosal epithelial cells of TGF- β and IL-10, that have been implicated in the down-regulation of immune responses and, therefore, are considered to be key cytokines in tolerance induction via the mucosa (Akbari et al. 2001; Weiner et al. 2011). By the production of IL-10 and TGF- β , Tregs can suppress IgE production and Th1/Th2 proliferation (Akabari et al. 2003). In the general population the prevalence of allergies has been estimated to be around 1-3% in adults and 4-6% in children according to the Center for Disease Control (CDC). More than 70 foods have been described to cause food allergies but several studies indicate that among children 75% of allergic reactions are due to only a limited number of foods, namely: egg, peanut, milk, fish and nut. Fruits, vegetables, nuts and peanuts are responsible for most allergic reactions among adults. Moreover, the prevalence of reported food allergy increased 18% among children under age 18 from 1997 to 2007. Asthma is estimated by the World Health Organization to affect about 150 million people worldwide and is a major cause of hospitalization for chronic diseases in children in the western population. In more than 50% of adults and in at least 80% of affected children, asthma can be related to an allergic cause. Policies that promote early identification of the disease, to certify adequate treatment and, particularly, improve air quality, are helping to reduce this burden (WHO 2007).

Currently, the only treatment that has been reported to cure allergic diseases is allergen-specific immunotherapy. It involves several injections at increasing doses of the allergen to achieve immunological tolerance over time (Moote and Kim 2011). Even though being considered a potentially disease-modifying therapy, patients' compliance is a problem, as allergen-specific immunotherapy requires the repeated administration of gradually increasing amounts of an

allergen for nearly three years, and carries inherent risks of allergic reactions during the treatment. More recently, a major breakthrough in allergy molecular medicine has been represented by the development of Omalizumab (Xolair), an anti-human IgE monoclonal antibody capable to block and prevent IgE binding to FcεRI (Chang 2000). Yet, also this therapeutic intervention presents the discomfort of high doses injection, and is not sufficient to fully protect from allergic manifestations. Recently, the U.S. Food and Drug Administration (FDA) is conducting a safety review of Xolair due to a possible increased risk of heart attack, abnormal heart rhythm, heart failure, and stroke in patients treated with Xolair (Nowak-Węgrzyn and Sampson 2011).

PROBIOTICS AND ALLERGY TREATMENT

Epidemiological data have demonstrated differences in the intestinal flora between allergic and non-allergic children and, for this reason, it was postulated a possible association between allergy and altered microbiota. In fact, it was observed that gut microbiota plays an important role in the early development of the mucosal immune system and as an environmental factor in the development of allergic diseases. Lack of early microbial stimulation and perturbation of the gastrointestinal microbiota results in aberrant immune responses to innocuous antigens and exerts a negative effect on the development of oral tolerance (Noverr and Huffnagle 2005). Infants that showed higher levels of potentially pathogenic bacteria in their gastrointestinal tract, such as *Clostridium difficile* and *Staphylococcus aureus*, were associated with increased risk of developing allergy. On the other hand, the intestinal flora of non-allergic children was found to be more colonized by Lactic Acid Bacteria (LAB), for example *Lactobacillus* and *Bifidobacterium*, demonstrating that the enhanced presence of these bacteria in the gastrointestinal tract seems to correlate with protection against allergic diseases (Forno et al. 2008; Ozdemir 2010).

In this context, the potential use of LAB to develop effective strategies for the immunotherapy of type I allergies is being explored. LABs are food-grade bacteria with a long record of safe oral consumption and are granted as generally regarded as safe (GRAS) by the FDA. Many LABs are able to colonize the mucosa of the oral, urogenital and gastrointestinal tracts and some species are commensal of mammal microbiota. Specific LAB strains that can provide a health benefit to the host when administered in adequate amount are defined as probiotics (Steidler and Rottiers 2006; Wells and Mercenier 2008). The first study that illustrates the potential of probiotic bacteria to modulate immune response and prevent allergic diseases was conducted by Kalliomäki and colleagues in 2001. The consumption of probiotic *Lactobacillus rhamnosus GG* by either pregnant woman for four weeks prior to labor or newborns, both having high allergy risk factors, decreased significantly the prevalence of atopic eczema. Furthermore, atopic babies that received probiotic strains exhibited reduced symptoms of the disease and higher levels of fecal IgA compared to the control group (Kalliomäki et al. 2001). Although their role in allergy is controversial, IgA are resistant to cleavage by secretory proteases and might block allergens penetration within the mucosal tissue, slowing down the allergic reaction (Novak et al. 2004). A recent work reports that administration of *Bifidobacterium breve* M16V in cow's milk protein intolerance (CMPI) neonates who underwent small intestine surgery could significantly reduce the incidence of CMPI after surgical procedure (Ezaki et al. 2012). Even though *L. rhamnosus GG* is the strain that has been most studied, the potential of several probiotic strains for the prevention or treatment of allergic diseases have been investigated in different clinical trials (*for a review see* Özdemir 2010; Kim and Ji 2012). However, despite these observations, several human probiotic trials against many forms of allergy have yielded inconsistent results; probably reflecting the inherent complexity of the allergic syndrome. Lack of alignment of clinical design e.g. different target populations, countries, intervention schemes and the formulation of probiotic used in the study makes difficult to

compare results obtained with different probiotic strains (Prescott and Bjorksten 2007; Kalliomäki 2010). Moreover, it is important to remark that the variation in the dose, formulation and species of probiotics used in this type of study should be taken into account (Wells 2011a; Kuitunen and Boyle 2012). A recent study investigating the associations of *L. rhamnosus* HN001 and *Bifidobacterium animalis* subsp lactis HN019 with allergic disease and atopy demonstrated that the protective effect was due to the first strain while HN019 did not affect the prevalence of any outcome (Kuitunen and Boyle 2012; Wickens et al. 2012). Indeed, Meijerink et al. showed that the cytokine profile induced by different probiotic strains were highly variable after co-incubation with peripheral blood mononuclear cells (PBMCs). These data demonstrates that probiotics can have distinct immunomodulatory capacities which could affect the type of immune responses elicited in the intestine changing their prophylactic/therapeutic properties (Meijerink et al. 2012).

In attempts to understand the underlying mechanisms of these anti-allergic effects, probiotic bacteria were shown to modulate the balance of the different T-helper cells, skewing the immune response to antigens/allergens from a pro-allergic Th2 toward a Th1 one. High levels of type 1 cytokines IFN γ and IL-12 secretion and up-regulate CD40 and CD86 expression were detected in murine splenocytes co-incubated with *Streptococcus thermophilus* or with *Lactobacillus casei* strains (Ongol et al. 2008) as well as in human myeloid dendritic cells (hmDCs) after co-incubation with *Lactobacillus gasseri*, *Lactobacillus johnsonii* or *Lactobacillus reuteri* strains (Mohamadzadeh et al. 2005). Niers et al. investigated the effect of probiotic-maturated DCs on T cell polarization. DCs which were co-incubated with *Bifidobacteria bifidum* W23 could drive T cells towards a Th1 response leading to a higher secretion of IFN- γ , IL-10 and lower secretion of IL-4, when compared to the control groups. This strain presented to be a good candidate for primary prevention of allergic diseases (Niers et al. 2007). It was also evaluated anti-allergic effects of a Dahi (yogurt) containing probiotic *Lactobacillus acidophilus*, *L. casei* and *Lactococcus lactis* biovar diacetylactis

(named probiotic Dahi) on ovalbumin (OVA) induced allergy in mice. Administration of the probiotic Dahi suppressed the production of total and OVA-specific IgE in mice serum. It was also observed higher amounts of INF- γ and IL-12 and lower amounts of Th2 specific cytokines (IL-4 and IL-6) in the cultured splenocytes from mice fed with probiotic Dahi (Jain et al. 2010). Wang et al. (2012) performed a study using the same OVA-induced allergy mouse model. However, the probiotic chosen for the study was a mixture named GM080 containing *Lactobacillus paracasei*, *L. fermentum* and *L. acidophilus*. It was shown that the OVA treatment increased the signaling proteins of inflammation and apoptosis in cardiomyocytes from mice and administration of GM080 could ameliorate both inflammation and apoptosis in the cells (Wang et al. 2012). By using an allergic poly-sensitization model to birch and grass pollen allergens, Schabussova and collaborators (2011) found that the treatment with *Bifidobacterium longum* NCC 3001 and *L. paracasei* could prevent inflammation in the lungs, a major target organ of allergic disease. Mice administered with the probiotic strains had increased levels of IgA in their Bronchoalveolar lavage (BAL) fluids and both strains induced a general immunosuppression of T cell responses, rather than a shift of the allergen-specific Th2 responses towards the Th1 phenotype. Moreover, IL-10 mRNA expression was elevated in the probiotic treated group and the beneficial effects of *B. longum* NCC 3001 were maintained for a more prolonged period of time (Schabussova et al. 2011). Co-incubation of PBMCs from allergic subjects with a variety of LAB strains inhibited allergen stimulated Th2-cytokine release and increases the Th1-cytokine response (Ghadimi et al. 2008). Recently, it was investigated the prophylactic potential of different probiotic strains using a peanut sensitization model. The prophylactic treatment with both *Lactobacillus salivarius* HMI00 and *L. casei* Shirota (LCS) decreased the production of IL-4 and/or IL-5 by mice splenocytes and mast cell responses leading to a reduction on Th2 immune responses. On the contrary, *L. plantarum* WCFS1 strain appeared to increase the Th2 phenotype (Meijerink et al. 2012). It was also reported that a

mixture of eight live probiotic bacteria (VSL#3) when administered orally in mice pre-sensitized with Shrimp tropomyosin (ST) allergen were capable to suppress established Th2 responses and generate regulatory T cells populations which were able to control allergic inflammation (Schiavi et al. 2010). Thomas et al. showed that pigs pre-sensitized with *Ascaris suum* allergen (ASA) and afterwards feed with *L. rhamnosus* HN001 strain had a decreasing in the severity of allergic skin and lung reactions. This was related to probiotic-induced modulation of Th1 (INF- γ) and regulatory (IL-10) cytokine expression (Thomas et al. 2011).

Many *in vivo* studies using animal models have shown positive effects of probiotics with respect to the prevention of several allergic diseases. In order to transpose this results to humans, further works should be carried out regarding the effective probiotic strains, optimal dose, timing and duration of supplementation as well as the additive/synergistic effects between probiotics and prebiotics. To date, the immunological mechanisms by which a probiotic strain can exert their beneficial effects on humans still remain to be proven (Kim and Ji 2012).

RECOMBINANT PROBIOTICS AND IMMUNOTHERAPY OF TYPE I ALLERGY

It was shown that supplementation trials with probiotics can decrease allergic manifestations in children. Some recent studies are also focusing on the use of selected probiotic strains as mucosal antigen delivery vehicles for recombinant allergens, serving as attractive adjuvant systems for improved allergy treatment (Schabussova and Wiedermann 2008). However, the current work on prevention of allergic sensitization using recombinant LAB have been carried out only using murine models (Wells 2011b).

Charng et al (2006) demonstrated that recombinant LAB is able to inhibit allergen-induced airway allergic inflammation. In this study mice were intraperitoneally sensitized with *Dermatophagoides pteronyssinus* group-5 allergen (Der p 5) and orally treated with recombinant LAB containing a

plasmid-encoded Der p 5 gene. After sensitization and challenge, it was observed a reduction in the synthesis of Der p 5-specific IgE, and hyperreactivity, thus providing a basis for developing a novel therapeutic method for allergic respiratory diseases (Charng et al. 2006). Parental administration of anti-IgE monoclonal antibodies is currently used to treat allergic patients by passive immunization but requires high doses of monoclonal antibodies resulting in high therapy costs. In order to develop vaccine able to induce natural production of IgE auto-antibodies, *L. johnsonii* have been engineered to express either anti-idiotypic scFv or IgE mimotopes fused on its surface. Intranasal administration of the recombinant strains elicits the production of anti-IgE antibodies showing its potential as mucosal vaccine (Scheppeler et al. 2005).

Induction of oral tolerance, which can be defined as the process by which the immune_system does not respond to unwanted and potentially pathogenic innocuous molecules (Maillard and Snapper 2007), is an attractive therapeutic strategy to treat atopic diseases, indeed it represent the natural way for the body to prevent allergy development against food antigens. Therefore, inducing a tolerogenic mechanism is suitable to prevent a hyperactive immune system associated with atopic and autoimmune diseases. Recently, it has been shown that the use of genetically modified *L. lactis* to deliver recombinant autoantigens or allergens can provide a novel therapeutic approach for inducing tolerance (Huibregtse et al. 2007).

The potential of *L. lactis* to induce antigen-specific peripheral tolerance has been evaluated by feeding Ovalbumin-sensitized OVA-T cell receptor transgenic mice with recombinant *L. lactis* expressing OVA. Administration of this strain leads to OVA-specific tolerance by inducing regulatory T cells in a TGF- β dependent manner (Huibregtse et al. 2007). This approach can also be used to develop effective therapeutics for systemic and intestinal immune-mediated inflammatory diseases.

In order to develop mucosal immunotherapy strategies able to modulate the T-cell-mediated response towards a Th1 profile, LAB have been used to produce and deliver allergens at the mucosal surfaces. *Lactobacillus plantarum* has been modified to produce the dust house mite allergen Derp-1. The intranasal administration of the recombinant strain induces Derp-1-T-cell specific proliferative response with low IFN γ production and reduced IL-5 secretion. Although the inhibitory effect on IL-5 production was shown to be specific to the recombinant strain, the effect on IFN γ was due to *L. plantarum* strain. No effect on immunoglobulin production was reported (Kruisselbrink et al. 2001). Unlike, Rigaux et al. showed that prophylactic intranasal pre-treatment of mice with recombinant *L. plantarum* strain producing Derp-1 prevents the development of Th2-biased allergic response by a reduction of specific IgE and the induction of allergen-specific IgG2a antibodies. Moreover, both wild-type and recombinant *L. plantarum* reduce airway eosinophilia following aerosolized allergen exposure and IL-5 secretion upon allergen restimulation (Rigaux et al. 2009).

Recombinant *L. lactis* and *L. plantarum* producing the birch pollen allergen Bet v1 have been also reported to modulate the allergic immune response to Bet v1. In prophylaxis protocols, intranasal administration of mice with recombinant strains expressing Bet v1 induces a non-allergic Th1 immune response specific to Bet v1. After sensitization mice pretreated with the recombinant strains expressing Bet v1 showed a decreased level of specific IgE, of IL-5 detected in bronchoalveolar fluids (BAL) as well as induction of Bet v1 specific IgA and IFN γ . Intranasal delivery was more efficient in modulating the immune response to Betv1, while oral pre-treatment was successful only with recombinant *L. plantarum* strain. The differences in the immune responses induced after oral administration by the two recombinant strains might be explain due to the lower amount of expressed antigen by recombinant *L. lactis*; however differences in immunomodulatory capacities and in gut persistence of the two strains cannot be excluded

(Daniel et al. 2006; Daniel et al. 2007). Schwarzer and co-workers (2011) recently explored the effect of neonatal colonization with a recombinant *L. plantarum* NCIMB8826 strain constitutively producing Bet v 1 in a murine model of type I allergy. It has been shown that mono-colonization with this strain induced a Th1-biased immune response at the cellular level upon stimulation with Bet v 1 (Schwarzer and co-workers 2011), as demonstrated by Daniel and collaborators (Daniel 2006; Daniel 2007). Furthermore, after sensitization with Bet v 1, immunized mice displayed suppressed IL-4 and IL-5 production in spleen and mesenteric lymph node cell cultures as well as decreased allergen-specific antibody responses (IgG1, IgG2a, and IgE) in sera. This suppression was associated with a significant up-regulation of the regulatory marker Foxp3 at the mRNA level in the spleen cells (Schwarzer et al. 2011). The capacity of recombinant *L. plantarum* expressing another allergen has been also investigated. The strain was engineered to express a major Japanese cedar pollen allergen, Cry j 1, and prophylactic effects *in vivo* was recently evaluated. In attempt to facilitate heterologous expression, the codon usage in the Cry j 1 gene was optimized for *L. plantarum* NCL21 strain using the recursive PCR-based exhaustive site-directed mutagenesis. The use of codon-optimized Cry j 1 cDNA and a lactate dehydrogenase gene fusion system led to a successful production of recombinant Cry j 1 in the host strain. Moreover, it was demonstrated that oral administration with *L. plantarum* expressing Cry j 1 suppressed allergen-specific IgE response and nasal symptoms in a murine model of cedar pollinosis (Ohkouchi et al. 2012).

Marinho and collaborators also evaluate the immunomodulatory effect of *L. lactis* expressing recombinant IL-10 in a cytoplasmic (LL-CYT) or secreted form (LL-SEC) using a mouse model of ovalbumin (OVA)-induced acute airway inflammation. It was observed that mice immunized with LL-CYT and LL-SEC strains had decreased levels of anti-OVA IgE and IgG1, reduced eosinophils numbers and IL-4 and CCL3 production, when compared to the asthmatic group. Furthermore, LL-CYT strain was more effective to suppress lung inflammation (Marinho et al. 2010). Taken

together, allergen-secreting LAB could be considered as an alternative strategy to prevent food allergy in the future.

PROTEIN DELIVERY BY RECOMBINANT LAB IN COW'S MILK ALLERGY MODEL

Cow's milk protein allergy (CMPA) is a complex disorder that affects 2 to 7.5% of children, with the highest prevalence during the first year of age, and is capable of inducing adverse reactions, which may involve skin, gastrointestinal (GI) tract or respiratory system (Caffarelli et al. 2010; Du Toit 2010). Generally, this allergy is an immunological reaction to four proteins in the casein fraction (α 1-, α 2-, β - and κ -casein) as well as α -lactalbumin and β -lactoglobulin, IgE or non-IgE associated (Wal 2004; Solinas et al. 2010). CMPA develops early in infancy within 12 to 24 month of birth, but 80 to 90% of affected children recover by acquiring tolerance to cow's milk by the age of 5 years (Exl and Fritsché 2001; Crittenden and Bennett 2005) while 51% of adults develop tolerance. Recent studies associated tolerance to cow's milk with a decreased epitope binding to milk peptides by IgE and a simultaneous increase in corresponding epitope binding by IgG4 (Cerecedo et al 2008; Pecora et al. 2009; Savilahti et al. 2010; Wang et al. 2010).

As described above LAB are attractive tools to deliver therapeutic molecules at the mucosal level due to their GRAS status and immunomodulatory capacities (Pontes et al. 2011; Bermudéz-Humaran et al. 2011). Indeed, the model LAB *L. lactis*, was engineered to express the major cow's milk allergen, β -lactoglobulin (BLG). The recombinant allergen was produced predominantly in a soluble, intracellular, and mostly denatured form. Mucosal administration of the recombinant strain induced BLG specific fecal IgA, although allergen-specific IgE, IgA, IgG1 or IgG2a were not detected in mice sera (Chatel et al. 2001). Similar immune response was reported after oral administration of recombinant *L. lactis* secreting a T-cell determinant IgE epitope of BLG (Chatel et al. 2003).

In a food-hypersensitivity mouse model, Adel-Patient and collaborators showed that oral administration of recombinant strains producing different amounts of recombinant BLG partially prevented mice from sensitization. Oral pre-treatment with recombinant strains prevented Th2-type immune response due to a reduction of specific IgE and the induction of allergen-specific IgG2a, immunoglobulins widely recognized as a characteristic of a Th1-type immune response, and fecal IgA antibodies. The intensity of the Th1 immune response induced was correlated with the amount of recombinant BLG produced: the more effective strains were those producing the highest amount of BLG (Adel-Patient et al. 2005).

Intranasal delivery of *L. lactis* recombinant strain did not induce the secretion of BLG specific antibodies but elicited IFN γ production in mice splenocytes after BLG re-stimulation. Intranasal pre-treatment of mice with recombinant *L. lactis* strain producing BLG reduced the airway eosinophilia influx and IL-5 secretion in BAL induced by intranasal allergen challenge. However, unlike after oral administration, no difference in IgE or IgG2a between pre-treated or control mice were detected. In the same study, intranasal co-administration of recombinant *L. lactis* strains producing BLG or IL-12 elicited a protective Th1 immune-response, inhibiting the allergic response in mice without affecting specific BLG IgE secretion (Cortes-Perez et al. 2007). The administration of *L. lactis* expressing cytokines to avoid allergic reactions seems to be a promising approach. After oral challenge with BLG, mice immunized with recombinant *L. lactis* expressing murine IL-10 (LL-mIL10) showed a significant decrease in antigen specific IgE in their gastrointestinal tract preventing anaphylaxis. Moreover, Th2-type response was completely abrogated, suggesting oral tolerance against BLG. Mice that received LL-mIL10 strain had significantly increased levels of IgA antibodies in their feces. Considering that T-cell-dependent secretion of IgA antibodies by B cells is largely induced by IL-10 and TGF- β , it was hypothesized that the active secretion of IL-10 by *L. lactis* up regulated IL-10-secreting cells increasing levels of plasmatic IL-10. This data suggests a

prime role for LL-mIL10 strain in the induction of IL-10 which, consequently, could improve IgA secretion (Frossard et al. 2007). The effects of intranasal administration of recombinant *L. lactis* expressing BLG were also tested in a therapeutic protocol. In oral sensitized mice, intranasal administration of recombinant strain reduced IgG1 production but did not influence specific BLG IgE or IgG2a secretion. After intranasal challenge, mild decreased in IL-4 and IL-5 secreted in BAL was detected (Cortes-Perez et al. 2009).

Hazebrouck et al. (2006) investigated the effect of *L. casei* constitutively producing BLG when established permanently in the gut of gnotiobiotic mice. After oral administration, the immune response against BLG as well as BLG production was monitored for 10 weeks BLG-producing *L. casei* strain was successfully able to colonize the gut of mice and BLG production was detected in the animal feces. Although immunoglobulins were not detected in sera or feces, secretion of IFN- γ and IL-5 was observed in stimulated splenocytes (Hazebrouck et al. 2006).

The influence of the administration route on the immune response elicited by a recombinant BLG *Lb. casei* producing strain was analysed (Hazebrouck et al. 2007; Hazebrouck et al. 2009). Intranasal pre-administration of the BLG-producing *Lb. casei* enhance the BLG-specific IgG2a and IgG1 responses in sensitized mice but did not influence BLG-specific IgE production. Unlikely, oral pre-treatment led to a significant inhibition of BLG-specific IgE production in sensitized mice while IgG1 and IgG2a responses were not elicited. The production of IL-17 cytokine by BLG-reactivated splenocytes was similar after both oral and intranasal administrations. However, BLG-reactivated splenocytes from mice intranasally pretreated showed an enhanced secretion of Th1 cytokines (IFN- γ and IL-12) and Th2 cytokines (IL-4 and IL-5) suggesting a mixed Th1/Th2 cell response, whereas only production of Th1 cytokines, but not Th2 cytokines, was enhanced in BLG-reactivated splenocytes from mice orally pretreated. Those results show that the route of

administration of recombinant LAB may be critical for their immunomodulatory effects (Hazebrouck et al. 2009).

DNA DELIVERY BY RECOMBINANT LAB IN COW'S MILK ALLERGY MODEL Recently, the potential of LAB as mucosal DNA delivery vehicles has been investigated. The advantage of DNA vaccine relies in their ability to induce both cellular and humoral Th1 immune responses, resulting in the specific immune activation of the host against the delivered antigen (Tang et al. 1992; Ulmer et al. 1993; Liu 2010). DNA immunization has shown great promise in rodent models of allergic disease and, for this reason, had turned out as a promising novel type of immunotherapy against allergy (Li et al. 2006).

Genetic immunization with a eukaryotic expression cassette encoding the BLG antigen elicited a Th1 immune response in mice. Preventive immunization reduced BLG-specific IgE production and induced IFN γ , IL-10 and BLG-specific IgG2a secretion in sensitized mice (Adel-Patient 2001). Unlike bacterial delivery of recombinant proteins, bacterial mediated DNA delivery leads to the expression by the host of post-translational modified antigens (Fouts 2003). Recombinant BLG is expressed mainly in denaturated form by *E. coli* or *L. lactis*, whereas its production in eukaryotic cells is in the native conformation (Chatel 1999; Chatel 2001). *In vitro* delivery of DNA into mammalian cells by LAB was demonstrated employing *L. lactis* containing a BLG eukaryotic expression plasmid. Production of BLG was detected in Caco-2 cells after co-culture with *L. lactis* harbouring the expression plasmid. Interestingly, no BLG production was detected in cells incubated with the purified plasmid mixed neither with *L. lactis*, suggesting that the expression plasmid should be inside the bacteria to achieve efficient delivery (Guimaraes 2006). The efficiency of DNA delivery to Caco-2 cells was strongly increased by the expression of invasive genes at the surface of *L. lactis*. Recombinant invasive strains expressing either *Listeria monocytogenes*

Internalin A or *Staphylococcus aureus* Fibronectin Binding Protein A genes showed a higher ability to be internalized into mammalian cells compared to the control strain. As a consequence, recombinant invasive strains were more efficient in GFP expression plasmid delivery into Caco-2 cells resulting in higher number of GFP producing cells (Innocentin et al. 2009). *In vivo*, *L. lactis* expressing *L. monocytogenes* InLA was able to invade guinea pig enterocytes after oral administration (Guimaraes et al. 2005).

Chatel et al. demonstrated that *L. lactis* was able to transfer a BLG eukaryotic expression plasmid *in vivo*. After oral administration of BLG delivery strain, BLG cDNA was detected in the epithelial membrane of the small intestine of 40% of mice and BLG protein was produced in 53% of the mice. Although only mild and transitory secretion of BLG specific IgG2a was detected in mice sera after oral administration of *L. lactis* harbouring BLG expression plasmid, a significant decrease in BLG specific IgE in pre-treated sensitized mice sera was reported. Furthermore, BLG reactivated splenocytes of sensitized mice previously orally fed with the *L. lactis* BLG cDNA delivery strain showed an increased IFN γ production and a decreased IL-5 secretion (Chatel et al. 2008).

CONCLUSION

Over the past decades, the use of recombinant LAB to develop possible strategies for mucosal immunotherapy of allergic diseases has strongly increased. Growing amount of data regarding the immuno-modulatory proprieties of specific LAB strains as well as their persistence in the gut tract will provide rationale choice of LAB strain for specific therapeutic application. Furthermore, availability of containment systems for genetically modified LAB (Steidler 2000), might support progress towards human clinical trials of LAB based allergen-specific immunotherapy. Allergen-delivering LAB have been implemented mainly in preventive vaccination protocols, but it remains

to be seen whether a LAB-based vaccine would be successful in a more realistic scenario, closer to human allergic conditions, therefore in atopic individuals.

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AUTHORSHIP

All authors substantial contributed to conception and design of the review; critically revised the manuscript, and approved the final submitted version.

COMPETING INTEREST

Marcela Santiago Pacheco de Azevedo and Silvia Innocentin wrote the review and would like to share the first authorship.

In vitro/Ex-vivo studies with probiotics			
Strain (s)	Experimental model/Disease	Immunological observations	Reference
<i>Lactobacillus gasseri</i> (ATCC no. 19992), <i>Lactobacillus johnsonii</i> (ATCC no. 33200) and <i>Lactobacillus reuteri</i> (ATCC no. 23272)	Human myeloid dendritic cells	Induction of bioactive IL-12, IL-18, IFN γ and T cell proliferation; up-regulation of TOLR-2 in the cells	Mohamadzadeh et al. 2005
<i>Bifidobacteria bifidum</i> W23	Dendritic cells (derived from umbilical cord blood obtained from healthy children); Autologous CD4+ T cells; Chinese hamster ovary (CHO)-cell lines	<i>In vitro</i> -cultured neonatal DC were able to drive Th1 responses; high secretion of IFN- γ , IL-10 and lower secretion of IL-4; TLR activation by probiotic bacteria	Niers et al. 2007
<i>L. rhamnosus</i> GG, <i>Lactobacillus gasseri</i> PA, <i>Bifidobacterium bifidum</i> MF, <i>Bifidobacterium longum</i> SP, and L.gB.bb.I (a mix of <i>L. gasseri</i> , <i>B. bifidum</i> , and <i>B. longum</i>)	PBMCs from allergic or healthy subjects	Modulation of Th1/Th2 response to allergens	Ghadimi et al. 2008
Probiotic effects in animal models of allergic disease			
<i>Streptococcus thermophiles</i> 21072 and <i>Lactobacillus casei</i> subsp. casei 027	C57BL/6 and BALB/c mice	Production of IFN- γ from splenocytes, IL-12p70, IL-10 from peritoneal exudate cells and expression of co-stimulatory molecules in dendritic cells by LAB stimulation;	Ongol et al. 2008
<i>L. acidophilus</i> NCDC14, <i>L. casei</i> NCDC19 and <i>Lactococcus lactis</i> biovar <i>diacetyllactis</i> NCDC-60	Ovalbumin (OVA) induced allergy in mice	OVA-specific IgE in mice serum; higher amounts of INF- γ and IL-12; lower amounts of IL-4 and IL-6 in the cultured splenocytes	Jain et al. 2010
<i>Lactobacillus paracasei</i> , <i>L. fermentum</i> and <i>L. acidophilus</i>	OVA-induced allergy mouse model	Amelioration of both inflammation and apoptosis in cardiomyocytes	Wang et al. 2012
<i>L. paracasei</i> NCC 2461 and <i>B. longum</i> NCC 3001	Mouse model of poly-sensitization	Suppression of Th2 responses, up-regulation of IL-10, TLR2 or TLR4 in the draining lymph nodes	Schabussova et al. 2011

<i>L. plantarum</i> WCFS1, <i>L. plantarum</i> NCIMB8826, <i>L. salivarius</i> HMI001, <i>L. casei</i> Shirota, 28 different strains comprising 12 species of probiotics	Human PBMC (hPBMC); Mouse peanut (PE) sensitization model	Induction of IL-10, IL-12 and IFN- γ in hPBMC; Modulation of PE-specific antibody responses by treatment with lactobacilli; <i>ex vivo</i> cytokine response (increased amounts of IL-4, IL-5 and IL-10)	Meijerink et al. 2012
Probiotic VSL#3 (<i>Lactobacillus acidophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. casei</i> , <i>L. plantarum</i> , <i>Bifidobacterium longum</i> , <i>B. infantis</i> , <i>B. breve</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>)	Mouse Shrimp tropomyosin (ST) sensitization model	Protection of mice against anaphylactic reactions; suppression of established Th2 responses and generation of regulatory T cells populations	Schiavi et al. 2011
<i>L. rhamnosus</i> HN001	Pig sensitization with <i>Ascaris suum</i> allergen (ASA)	Induction of INF- γ and IL-10 cytokines in PBMCs from the pigs; Diminished allergic skin flare and allergic lung responses	Thomas et al. 2011
Human clinical studies with probiotics			
<i>Lactobacillus ramosus</i> GG	Atopic eczema, allergic rhinitis, or asthma	Reduced symptoms of the disease in patients and higher levels of fecal IgA	Kalliomäki et al. 2001
<i>Bifidobacterium breve</i> M16V	Cow's milk protein intolerance (CMPI)	Reduction of CMPI symptoms in neonates after small intestine surgery	Ezaki et al. 2012
<i>L. rhamnosus</i> HN001	Eczema	Protection against eczema, when given for the first 2 years of life only, extended to at least 4 years of age	Wickens et al. 2012

Table 1: Summary of some research studies concerning the use of different probiotic strains as a potential tool to treat/prevent allergic diseases.

Recombinant probiotic effects in animal models of allergic disease

Strain (s)	Recombinant allergen/ Localization	Experimental model/Disease	Immunological observations	Reference
<i>L. acidophilus</i> ATCC 4356 <i>S. thermophilus</i>	Dermatophagoides pteronyssinus group-5 allergen (Der p 5 /Cytoplasmic expression	BALB/c mice sensitized with Der p 5	Inhibition Der p 5-specific IgE and airway hyperreactivity; eosinophilic and neutrophilic cellular infiltration	Charng et al. 2006
<i>L. johnsonii</i> NCC2754	Anti-idiotypic scFv fragments by anti-human IgE antibodies /anchored to the cell wall	BALB/c mice	Induction of an anti-IgE response after intranasal immunisation	Scheppler et al. 2005
<i>L. lactis</i> MG1363	<i>Gallus gallus</i> ovalbumin (OVA)/secreted to the extracellular medium	OVA-specific T- cell receptor transgenic mice (DO11.10) on a BALB/c background	Suppression of local and systemic OVA-specific T-cell responses	Huibregtse et al. 2007
<i>Lactobacillus</i> <i>plantarum</i> 256	Der p 1 of house dust mites/ Cytoplasmic expression	C57Bl/6 J (H-2b) mice	Inhibition of Th1 components of the response and reduced production of IL-5; Stimulation of immunoregulatory mechan- isms	Kruisselbrink et al. 2001
<i>Lactobacillus</i> <i>plantarum</i> NCIMB8826, <i>L.</i> <i>plantarum</i> EP007	Dust house mite allergen Derp-1/ Cytoplasmic expression	Der p 1- sensitization murine model	Stimulation of dendritic cells (TLR2-, TLR9- and MyD88-dependent mechanism and via MAPK and NF-kB activation)	Rigaux et al. 2009
<i>L. lactis</i> NZ9800 and <i>L. plantarum</i> NCIMB8826	Birch pollen allergen Bet v1/ Cytoplasmic expression	Mouse model of birch pollen allergy	Reduction of allergen-specific IgE; Augmentation of allergen-specific IgA at the mucosae in mice	Daniel et al. 2006
<i>Lactobacillus</i> <i>plantarum</i> NCIMB8826	Birch pollen allergen Bet v1/ Cytoplasmic expression	Murine model of type I allergy	Induction of Th1-biased immune response at the cellular level; Induction of INF- γ by	Schwarzer et al. 2011

			splenocytes and suppression of IL-4 and IL-5 in spleen and mesenteric lymph node	
<i>Lactobacillus plantarum</i> NCL21	Japanese cedar pollen allergen Cry j 1/ Cytoplasmic expression	Murine model of Japanese cedar pollinosis	Suppression of allergen-specific IgE response; Amelioration of the cedar pollinosis like clinical symptoms	Ohkouchi et al. 2012
<i>L. lactis</i> NCDO2118	Full-length <i>Rattus norvegicus</i> IL-10 with the codon usage of <i>L. lactis</i> / Cytoplasmic expression and secreted to the extracellular medium	Mouse model of ovalbumin (OVA)-induced acute airway inflammation	Reduction in eosinophil numbers, EPO, IgE anti-OVA levels, IL-4 and CCL3 levels and pulmonary inflammation and mucus hypersecretion	Marinho et al. 2010
<i>L. lactis</i> NZ9000	Cow's milk allergen, β -lactoglobulin (BLG)/ Cytoplasmic and extracellular locations	BALB/c mice	Induction of BLG specific fecal IgA	Chatel et al. 2001
<i>L. lactis</i> NZ9000	Cow's milk allergen BLG/ cytoplasmic and extracellular expression	BLG sensitization murine model	Induction of specific Th1 response down-regulating Th2 balance; Reduction of specific IgE responses	Adel-Patient et al. 2005
<i>L. lactis</i> NZ9000	IL-12 and Cow's milk allergen BLG fused with the LEISSTCDA Propeptide/secreted to the extracellular medium	BLG sensitization murine model	Induction of a specific Th1 immune response regulating systemic and local Th2 and effectors cells	Cortes-Perez et al. 2007
<i>L. lactis</i> wild type	Murine IL-10/secreted to the extracellular medium	Mouse model of food allergy using BLGn in the presence of cholera toxin	Inhibition of anaphylaxis and antigen-specific serum IgE, IgG1 production; increased production of antigen-specific IgA and IL-10 in the gut	Frossard et al. 2007
<i>L. casei</i> BL23	BLG/secreted to the	Germfree	Influence the maturation of the	Hazebrouck

	extracellular medium	C3H/HeN mice	gut immune system; Increased levels of INF- γ and IL-5	et al. 2006
<i>L. casei</i> BL23	β -lactoglobulin (BLG)	BALB/c mice	Stimulation of systemic IgG1 and IgG2a responses; Suppression of BLG-specific IgE production	Hazebrouck et al. 2009

Table 2: Main research studies about the use of different recombinant probiotic strains to treat/prevent allergic diseases and major effects on the host immune response.