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Lactococcus lactis as a vector for oral vaccine delivery: the case of enterohemorrhagic *Escherichia coli*

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"Success consists of going from failure to failure without loss of enthusiasm"

Winston Churchill

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LIST OF ABBREVIATIONS

A/E	Attaching and effacement
ANOVA	Analysis of variance
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
CCR	Chemokine receptor
CCL	Chemokine ligand
CTB	Cholera toxin subunit B
CFU	Colony forming unit
CTLs	Cytotoxic T lymphocytes
CWA	Cell wall anchor
DCs	Dendritic cells
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EspA	*E. coli* secreted protein A
EspB	*E. coli* secreted protein B
FAE	Follicle-associated epithelium
FDA	Food and drug administration
GALT	Gut-associated lymphoid tissue
Gb_3	globotariosylcermide
GI	Gastrointestinal
GRAS	Generally regarded as safe
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
IG	Intragastric
IgG	Immunoglobulin G
IL	Interleukin
ILFs	Isolated lymphoid follicles
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
L. lactis	*Lactococcus lactis*
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharides
M-cells	Microfold or membranous cells
MLN	Mesenteric lymph nodes
NICE	Nisin-controlled gene expression
OD	Optical density
ON	Overnight
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PP	Peyer's patches
sIgA	Secretory immunoglobulin A
SP	Secretion peptide

Stx	Shiga toxins
T3SS	Type three secretion system
Th	Helper T lymphocytes
Tir	Translocated intimin receptor
TLR	Toll-like recptor
TNF	Tumor necrosis factor
Usp45	Unknown secretory protein

CHAPTER 1

Literature review

1. Oral Vaccines: immunological basis and strategies for vaccine development

1.1. Introduction

Gut-borne illnesses have been responsible for high negative impacts on global public health. The pathogens causing these illnesses include colonizing enteropathogens, whose pathogenicity is confined to the gut mucosa and that can be responsible for life-threatening diarrhea (e.g. *Vibrio cholerae*, pathogenic *Escherichia coli* and rotaviruses), and invasive pathogens, (e.g. polioviruses and *Salmonella Typhi*), which exploit the gut mucosa as a portal of entry to other target organs, resulting in serious systemic diseases (e.g. poliomyelitis and typhoid fever, respectively). The best strategy to protect against these pathogens is the induction of a protective intestinal mucosal immunity. Clinical vaccine development has been largely based on injectable vaccines. However, injectable vaccines typically induce systemic immune responses, and thus can provide protection against invasive pathogens, while being mostly ineffective against colonizing enteric pathogens (Czerkinsky & Holmgren, 2012). Additionally, administration of injectable vaccines results in patient non-compliance and is associated with risks of cross infection by contaminated needle, especially in developing countries. Alternatively, oral vaccines offer important immunologic and practical advantages for protection against enteric pathogens: (1) unlike injectable vaccines, oral vaccines are properly capable of stimulating mucosal immune responses in the gut. These responses can prevent the interactions of pathogens with the gut mucosa and thereby, prevent the associated diseases (Czerkinsky & Holmgren, 2012); (2) comparable to injectable vaccines, oral vaccines are capable of inducing systemic immune responses, and thus provide an extra layer of protection against invasive enteric pathogens; (3) oral vaccines are remarkable for their easy and safe administration, and thus are particularly amenable for application in mass vaccination programs aiming to eradicate certain enteric diseases (e.g. the oral polio vaccine, OVP).

Despite these merits, the development of oral vaccines represents a difficult challenge for immunologists (Neutra & Kozlowski, 2006), for a number of reasons. First, the harsh gastrointestinal conditions, including acidic pH and digestive enzymes, which can cause degradation or denaturation of antigens, in addition to the dilution and dispersion of these antigens within the gastrointestinal contents.

Second, the poor immunogenicity of most orally delivered antigens, which is either due to their poor uptake by mucosal immune cells or to the induction of oral tolerance. The latter refers to the tendency of the gut immune system to maintain a state of hypo- or unresponsiveness to oral antigens, a mechanism by which the undesirable inflammatory responses to dietary antigens and microbiota can be avoided. With the increasing knowledge of the mechanisms underlying the induction of mucosal adaptive immunity and the advances in biotechnology, several vaccine-design strategies have been developed to overcome the described challenges.

In the present chapter we describe the adaptive immune responses induced by oral vaccination. Additionally, we review the strategies employed in oral vaccine development and antigen delivery, in order to effectively elicit mucosal immunity in the gut.

1.2. The gut immune system

The immune system of the gut consists of a number of diffuse and organized lymphoid tissues, which comprise the gut-associated lymphoid tissue (GALT) and the mesenteric lymph nodes (MLNs) (Cesta, 2006) (Figure 1.1). The organized lymphoid tissues of GALT include Peyer's patches (Knappstein *et al.*, 2004a), the appendix and other isolated lymphoid follicles (ILFs). These represent the major inductive sites for mucosal immune responses in the gut. The diffuse lymphoid tissue comprises antigen-presenting cells and lymphocytes, which are scattered throughout the gut mucosal lamina propria to perform both effector and inductive functions (Cesta, 2006).

PPs are large organized lymphoid structures that are situated in the submucosa along the length of the small intestine (Cesta, 2006). They are composed of B cell follicles that contain germinal centers, surrounded by T cell rich interfollicular regions. These follicles are covered with follicle-associated epithelium (FAE), which bears an underlying area of connective tissue known as the subepithelial dome. The FAE comprise specialized antigen-sampling epithelial cells termed, microfold or membranous cells (M-cells), which have a folded luminal surface instead of the microvilli present on conventional enterocytes, and also lack the glycocalyx layer. These adaptations enable M-cells to capture luminal pathogens and particulate

antigens and translocate them to the subepithelial dome. This zone is abundantly rich in dendritic cells (DCs), which immediately take up the translocated antigens.

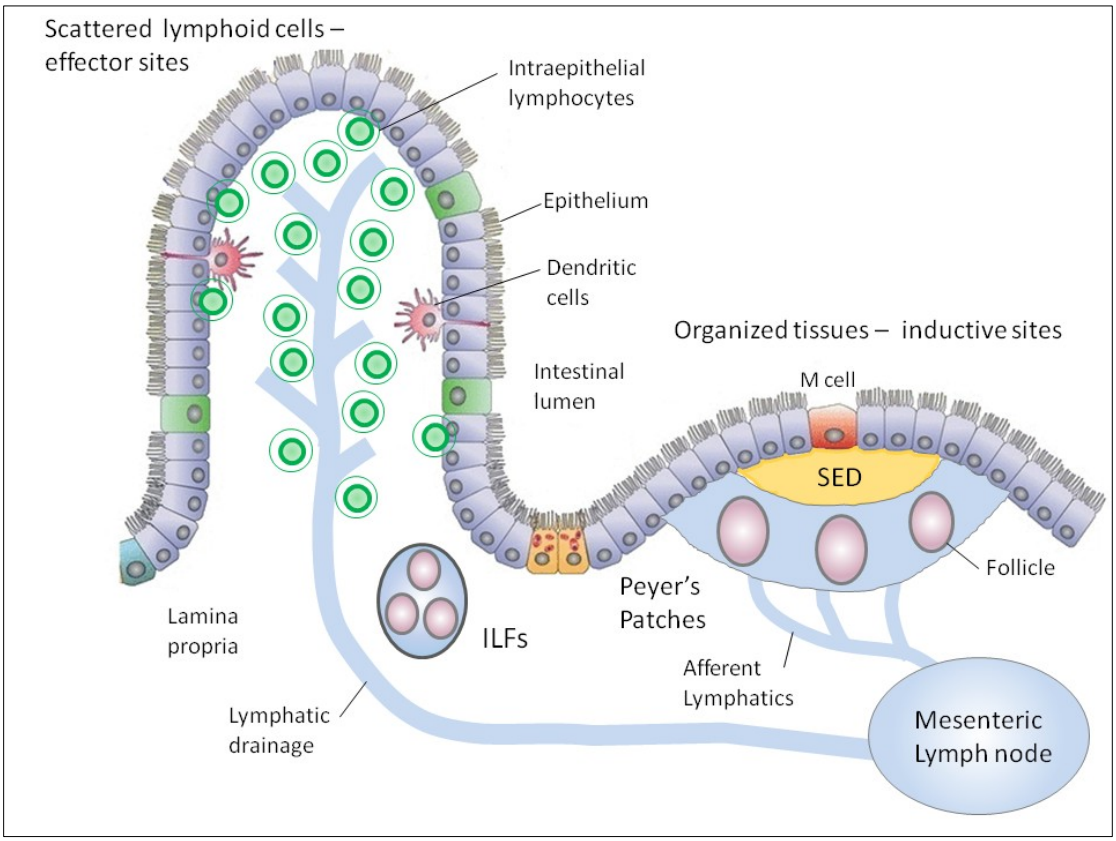

Figure 1.1. Schematic representation of the lymphoid elements of the intestinal immune system. The organized tissues of the Peyer's patches and mesenteric lymph nodes are involved in the induction of immunity. Effector lymphocytes are scattered throughout the lamina propria and epithelium of the mucosa. Both the Peyer's patches and villus lamina propria are drained by afferent lymphatics that go to the MLNs. SED, subepithelial dome. Figure adapted from (Mowat, 2003).

MLNs are also organized lymphoid tissues that constitute a part of the intestinal immune system, but are not considered as a mucosa-associated lymphoid organ, since they do not sample antigen directly from the intestinal lumen. However, they obtain antigens via antigen-loaded DCs that migrate from the PPs or directly from mucosal surfaces (Brandtzaeg & Pabst, 2004). In addition, antigens can drain directly from GALT and mucosa to the MLN via the lymph (Mowat, 2003). In both PPs and MLNs, antigen-loaded DCs migrate to interfollicular regions, where they present

antigenic peptides to $CD4^+$ T cells, which subsequently stimulates the differentiation and maturation of T- and B cells with immune effector functions (Mowat, 2003).

ILFs are small lymphoid aggregates that are scattered throughout the intestinal mucosa (Hamada *et al.*, 2002). They structurally resemble PPs and are similarly covered with FAE, containing M-cells. However, each consists of a single follicle, predominately contains B cells and few DCs and thus, lacks the T cell-rich interfollicular regions (Hamada *et al.*, 2002). This suggests the involvement of a T cell-independent mechanism for the induction of effector immune responses by ILFs (discussed below).

1.3. Induction of mucosal immunity by oral vaccines

Following oral vaccination, a series of cellular events, are initiated in both organized lymphoid tissues and the mucosal lamina propria of the gut, involving DCs, naïve T and B lymphocytes, as to generate antigen-specific antibodies and cytotoxic T lymphocytes (CTLs) for mucosal protection against pathogens. In particular, secretory IgA (sIgA) plays the most important role in protecting the gut mucosal surface against pathogens and/or the toxins they elaborate.

1.3.1. IgA antibody responses

The production of IgA antibodies in the gut is ruled by the coordinating functions of mucosal DCs, T-helper lymphocytes and B lymphocytes (Figure 1.2). Hereto, antigen loaded DCs secrete in PPs and MLNs a subset of cytokines and stimulatory molecules, such as TGFβ, IL-10, IL-6 and retinoic acid (vitamin A derivative), which promote the development of antigen-specific T-helper lymphocytes type 2 (Th2) and $Foxp3^+$T regulatory cells (Treg) (Coombes *et al.*, 2007; Iwasaki & Kelsall, 1999; Sato *et al.*, 2003; Sun *et al.*, 2007). It has been shown that Treg can differentiate into T-follicular helper cells (Tfh) (Tsuji *et al.*, 2009). These T-helper cell subtypes interact with B lymphocytes and induce IgA class switching. The latter is mediated by the signals triggered by the interaction of the B cell receptor, CD40 with the CD40 ligand present on T-helper cells, in addition to the secretion of cytokines such as IL-4, IL-5 and IL-6 produced by the Th2 cells, IL-10 and TGF-β produced by the Treg and Tfh cells, and IL-21 produced by the Tfh cells (Cerutti *et al.*, 1998; Zan *et al.*, 1998; Zhang *et al.*, 1994).

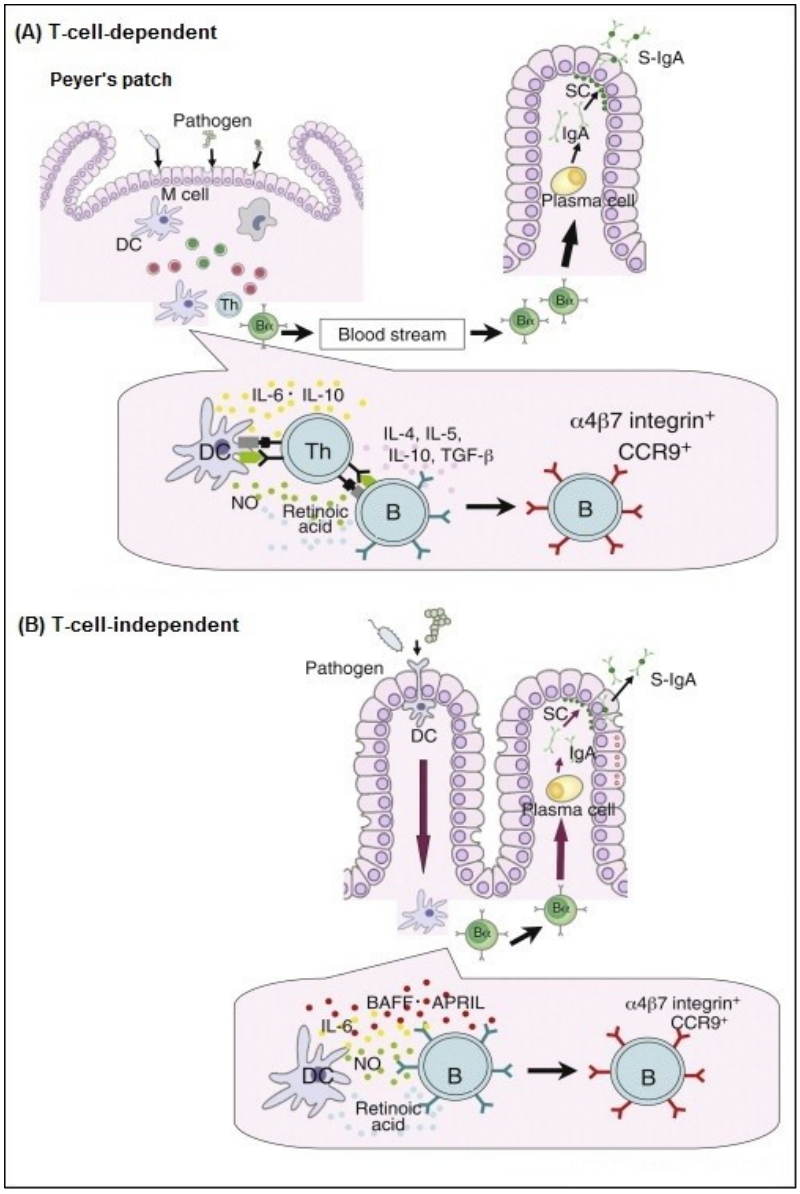

Figure 1.2. Mechanisms of IgA antibody induction in the mouse gut. **(A)** In organized lymphoid follicles such as present in Peyer's patches, a T cell-dependent pathway exists. Orally administered antigens are taken up by M cells in follicle-associated epithelium of PP and then processed and presented by dendritic cells (DCs) and macrophages for the generation of helper T (Th) cells and IgA-committed B cells. IL-4, IL-5, IL-10, and TGF-β produced by Th cells and nitric oxide (NO) and IL-6 produced by PP DCs allow B cells to undergo IgA class switching. Simultaneously, retinoic acid (RA) produced by DCs increases gut homing receptors (α4β7 integrin and CCR9) on antigen-primed Th cells as well as IgA-committed B cells. These antigen-primed Th cells and IgA-committed B cells migrate to effector sites such as intestinal lamina propria for terminal differentiation to IgA producing plasma cells. **(B)** IgA-committed B cells can also be generated via a T cell-independent mechanism. In the latter case, intestinal DCs directly sample antigen from the lumen and present it to the B cells, which under the influence of cytokines such as BAFF, APRIL, and TGF-β1, and NO derived from DCs, start the process of isotype switching and differentiation to IgA producing plasma cells. Figure adapted from (Takahashi *et al.*, 2009).

Besides the role of conventional DCs, PPs possess other specialized DCs types, which enhance the IgA class switching induced by activated T-helper cells. Tumor-necrosis factor (TNF)/ inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) increase the availability of nitric oxide, which in turn increases TGF-β receptor expression on naïve follicular B cells to enhance IgA class switching (Tezuka *et al.*, 2007a). Additionally, Tip-DCs, in addition to other DCs subsets in PPs and MLNs (plasmacytoid and follicular DCs) can induce IgA class switching of B cells in the follicles in a T cell-independent fashion (Suzuki *et al.*, 2010b; Tezuka *et al.*, 2011). This IgA class switching is independent of the B cells CD40/CD40L signaling, but is facilitated by secretion of cytokines such as the B cell activating factor (BAFF) (a member of the TNF-ligand family) and a proliferation-inducing ligand (APRIL) (He *et al.*, 2010; Litinskiy *et al.*, 2002).

Together with PPs and MLNs, ILFs and the lamina propria represent additional important sites for induction of IgA responses. However, IgA class switching in ILFs and the lamina propria only occurs mainly via the T cell-independent pathway (hitherto only described in mice). In ILFs, DCs release active TGF-β1, and in response to microbial toll-like receptor (TLR) signaling can, together with other stromal cells, release BAFF and APRIL (Tsuji *et al.*, 2008a). In the LP, a unique subset of DCs, expressing TLR5 (TLR5$^+$ DCs), secretes retinoic acid and IL-6 in response to bacterial flagellin (TLR5 ligand) (Uematsu *et al.*, 2008; Uematsu & Akira, 2009). Additionally, TLR signaling also induces the secretion of BAFF and APRIL by lamina propria TNF-α$^+$/iNOS$^+$ DCs (Tezuka *et al.*, 2007b). Epithelial cells can also enhance the release of BAFF and APRIL by DCs in the lamina propria through the release of thymic stromal lymphopoietin (Xu *et al.*, 2007).

In addition, epithelial cells can release BAFF and APRIL in response to bacterial TLR signaling (He *et al.*, 2007). All of these cytokines induce and promote T cell-independent IgA class switching (Fagarasan *et al.*, 2010; Suzuki *et al.*, 2010a; Tsuji *et al.*, 2008b). In humans, however, the anatomic location of the T cell-independent immunoglobulin class-switch recombination is still controversial (Berkowska *et al.*, 2011).

Generated IgA-committed B cells, via both T cell-dependent and -independent pathways, finally differentiate into dimeric IgA producing plasma cells in the lamina propria, under the influence of another group of IgA inducing cytokines, the Th2

cytokines IL-5 and IL-6. Dimeric IgA antibodies are transported across epithelial cells into mucosal secretions via polymeric Ig-receptor-mediated transcytosis which results in the release of sIgA in the gut lumen (Brandtzaeg, 2013).

One of the main roles of sIgA is to serve as an immunological barrier, preventing the attachment of pathogens to the mucosal surfaces (Brandtzaeg, 2007). IgA binds surface proteins that mediate the attachment of enteric pathogens to enterocytes and thereby prevents colonization and invasion. The large complexes of the sIgA-bound pathogens are easily entrapped in the mucus and subject to peristaltic or ciliary clearance, a process known as "immune exclusion" (Corthesy, 2013). In addition to pathogens, sIgA can neutralize bacterial enterotoxins. Furthermore, IgA can trap incoming organisms within the epithelial cell vesicular compartments, exporting them back into the lumen (Hutchings *et al.*, 2004). Finally, it can also neutralize viruses intracellularly, and this mechanism of defense is particularly relevant for enteric viruses (Hutchings *et al.*, 2004; Mazanec *et al.*, 1992; Schwartz-Cornil *et al.*, 2002).

1.3.2. IgG and other antibody isotypes

Although IgA is the main isotype in the mucosal secretions, IgG is also present and may contribute to the adaptive immune defenses in the gut. IgG is believed to reach luminal secretions mainly by transudation of serum, although small amounts are also synthesized locally (Brandtzaeg, 2007). However, receptor-mediated IgG transport can occur. Indeed, it has been shown that a neonatal IgG-specific receptor, FcRn is expressed by epithelial cells in the intestine and can mediate IgG transport in both directions across the epithelium (Dickinson *et al.*, 1999). Expression of FcRn is downregulated to nearly undetectable levels after weaning in rodents. However, in human, pigs and cattle, absorptive intestinal epithelial cells continue to express FcRn in adult life (Israel *et al.*, 1997). Interestingly, FcRn can also transport IgG-antigen complexes from lumen across the intestinal barrier into the lamina propria, where it can be delivered to antigen-presenting cells (Yoshida *et al.*, 2004). Mucosal sIgM is less abundant than sIgA but is also present in gut secretions. In addition, IgE can be produced by plasma cells in the lamina propria and is believed to mediate protection against certain intestinal helminthes through the activation of local mast cells (Brandtzaeg, 2007).

1.3.3. Cytotoxic T lymphocytes (CTLs)

Mucosal CTLs constitute a second response that can be induced by oral vaccination (Klavinskis *et al.*, 1996). These cells play an important role against invasive pathogens that exploit the mucosal surface to enter the body, such as viruses and intracellular bacteria. CTLs are generated in the PPs from $CD8^+$ lymphocytes by a subset of $CD8\alpha^+$ DCs, and/or by activated Th1 cells. Of the various $CD8^+$ lymphocyte populations found in mucosal tissues, those expressing the T cell receptors αβ (TCRαβ) and CD8αβ or CD8αβ and CD8αα have mostly effector/cytotoxic activity (Cheroutre & Madakamutil, 2004). For instance, $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ CTLs exhibit potent cytotoxic activity against cells infected with viruses and intracellular protozoan parasites (Cheroutre & Madakamutil, 2004; Muller *et al.*, 2000). Also, IFN-γ and TNF-α secreted by $CD8^+$ T cells may play a key role in enhancing pro-inflammatory responses in the lamina propria, enabling phagocytic cells to scavenger microorganisms that have breached the mucosal barrier.

1.3.4. Gut-homing potential of mucosally primed effector cells

Effector B and T cells primed in the gut mucosal inductive sites are specifically programmed to home to gut mucosal effector sites (Agace, 2010; Mora *et al.*, 2006). After priming, these cells lose their adhesion to the stromal cells, leaving the organized lymphoid tissues. Cells primed in PPs, disseminate via draining lymph to MLNs, where they undergo further differentiation and amplification. Later, mucosally primed cells disseminate via the blood circulation to home back to gut mucosal lamina propria (Mowat, 2003). The gut-homing specificity of effector lymphocytes is determined by complex interactions between lymphocyte homing receptors and their respective ligands expressed on the gut mucosal vascular endothelium. Gut-homing receptors are upregulated on antigen-primed B and T lymphocytes by intestinal DCs in a mechanism involving DCs release of retinoic acid. These receptors include α4β7 integrin, which binds to mucosal addressin cell adhesion molecule-1 (MadCam-1), and the CCR9 and CCR10 chemokine receptors, which bind to the intestinal chemokines CCL25 and CCL28, respectively (Agace, 2010; Mora *et al.*, 2006).

This pattern of adhesion-molecule and chemokine-receptor expression is distinct from that of lymphocytes primed in peripheral lymphoid organs. Those

acquire the α4β1 integrin, L-selectin receptor and the chemokine receptor CCR4 and therefore, cannot migrate to mucosal surfaces (Campbell & Butcher, 2002). This is the molecular explanation as to why oral vaccines, but not injectable counterparts, induce mucosal immunity.

1.4. Systemic antibody responses elicited by oral vaccines

As described above, B and T lymphocytes primed in the gut preferentially undergo IgA class switching and selectively home to gut mucosal effector sites. However, it is well established that oral vaccines are capable of inducing systemic IgG responses. In this regard, mucosal immunization via the intranasal route has been shown to induce activated B cells with a "mixed" gut and systemic lymphoid tissue homing-specificity (El-Kamary *et al.*, 2010). Therefore, a similar outcome presumably occurs after oral vaccination. In such a case, MLNs, which are anatomically recognized as a crossroad between gut mucosal and systemic immune compartments, are likely the site where lymphocytes with mixed homing potential are produced (Mowat, 2003). Presumably, MLNs DCs, probably in cooperation with other local stimuli, can promote the differentiation of B lymphocyte subsets that express homing- and chemokine-receptors specific to both gut mucosal and peripheral lymphoid tissues. These subsets should also show parallel IgA and IgG class switching.

1.5. Strategies for oral vaccine development

As discussed above, the harsh gastrointestinal environment and the tolerogenic arm of the gut immune system are bottlenecks for oral vaccine development. Therefore, several strategies have been explored to design effective oral vaccines against enteric infections.

1.5.1. Live-attenuated vaccines

Live-attenuated vaccines are composed of live bacteria or viruses that are made much less virulent than the pathogenic wild type. This class of vaccines notably mimics the routes of natural infection, provide a high level of antigen exposure, and effectively stimulate innate and adaptive immunity via the pathogen-associated molecular patterns (Manicassamy & Pulendran, 2009). The majority of the currently licensed oral vaccines are based on attenuated pathogens (Holmgren & Svennerholm, 2012). These vaccines were successfully used to prevent various

gut-borne diseases, such as poliomyelitis, typhoid fever and rotavirus-induced diarrhea (Holmgren & Svennerholm, 2012). Nevertheless, the safety and stability of live-attenuated vaccines remains major concern. Attenuated vaccines are developed from wildtype pathogens that were subjected to multiple or prolonged passages in foreign host or tissue (mainly for viruses) or to genetic deletion or modification of major virulence genes. However, reversion to virulence can occur as a result of acquisition of wildtype virulence genes or chromosomal rearrangements that may lead to gene duplication or the activation of dormant genes. Additionally, attenuated vaccines can still be pathogenic to immunocompromised individuals such as the elderly and infants (National Center for Immunization and Respiratory Diseases, 2011). For instance, a rotavirus live-attenuated quadrivalent oral vaccine (RotaShield) based on a Rhesus monkey rotavirus strain equipped with human rotavirus genes (Clements-Mann *et al.*, 2001), was withdrawn from the market after being implicated to cause intussusceptions in vaccinated children (Murphy *et al.*, 2001). Therefore, efforts in vaccine design should focus on developing alternatives to live-attenuated vaccines, with higher safety and comparable efficacy.

1.5.2. Subunit vaccines

To improve the safety of oral vaccines, design strategies have been focusing on developing vaccines based on one or more of pathogen-derived antigens. However, individual (soluble) antigens are mostly vulnerable to the gastrointestinal conditions, poorly taken up at the gut mucosal inductive sites, or have low immunogenicity. Therefore, to further improve the efficacy of subunit-based oral vaccines, a variety of delivery systems and mucosal adjuvants have been explored. However, we still lack licensed vaccines based on these approaches.

1.5.3. Delivery systems

Delivery systems primarily serve to protect oral vaccine antigens against the detrimental gastrointestinal conditions and to enhance the antigen uptake by the cellular components of the intestinal immune system. Nevertheless, some delivery systems also possess adjuvant properties. A variety of the common oral vaccine delivery systems are reviewed in Table 1.1, in terms of their nature/composition, immunological characteristics, advantages and disadvantages,

and status of clinical testing. Live-attenuated pathogens, mainly of bacterial and viral origins, have been commonly used as vectors for oral vaccine delivery (Brave *et al.*, 2007; Carleton, 2010). They can be engineered to express antigens from other pathogens and thus deliver these antigens to the intestinal immune system. Enteropathogen-based vaccine vectors are used for their inherent ability to resist the gastrointestinal conditions and to penetrate mucosal epithelial and antigen-presenting cells. Additionally, the pathogen-associated molecular patterns of these vectors stimulate innate immunity, which enhances the adaptive immune responses to the carried antigens (adjuvanticity). Among bacterial vectors, *Salmonella*, which is an invasive enteropathogen, has been widely investigated for oral vaccine delivery (Galen *et al.*, 2009; Paterson & Maskell, 2010; Wang *et al.*, 2013), building on the extensive knowledge available on its physiology and pathogenesis (Basso *et al.*, 2000; Galen *et al.*, 2009; Sirard *et al.*, 1999). Other intracellular bacteria such as *Mycobacterium* (Kawahara *et al.*, 2006; Kawahara, 2008) and *Listeria* (Bruhn *et al.*, 2007; Saklani-Jusforgues *et al.*, 2003) are also considered suitable candidate vectors for oral vaccine delivery. *Listeria*-based vectors are particularly notable for their ability to invade a variety of host cells, including antigen-presenting cells and $CD4^+$ and $CD8^+$ T lymphocytes (Cossart & Mengaud, 1989; Kaufmann, 1993; Pamer *et al.*, 1997). Similarly, various viral vectors have been successfully tested as oral vaccine vectors, generally resulting in the induction of strong CTL responses. In the veterinary field, there are numerous virus-based oral vaccines that are currently licensed for use in livestock and domesticated animals (Meeusen *et al.*, 2007). Based on their safety records in the veterinary field, many viruses have been studied for potential use in humans as oral vaccine delivery vectors. These include attenuated Poxvirus (Esteban, 2009; Weyer *et al.*, 2009), vaccinia virus and adenovirus (Esteban, 2009; Rimmelzwaan & Sutter, 2009). Adenovirus is to date one of the most widely evaluated viral vectors for oral vaccine delivery, due to ease of production, safety profile, genetic stability, the ease of DNA genome manipulation, and the ability to stimulate both innate and adaptive immune responses (Lasaro & Ertl, 2009; Vemula & Mittal, 2010).

As discussed for live-attenuated vaccines, the use of attenuated pathogens as vaccine delivery vectors is similarly associated with the risk of reverted pathogenicity and the potential for disease development in immunocompromised individuals. Additionally, a major disadvantage of pathogen-based vectors is the

preexisting immunity against them in vaccinated individuals, due to previous exposure. Preexisting vector-specific immunity could potentially lower vaccine efficacy, as has been reported with *Salmonella*- (Attridge *et al.*, 1997; Gahan *et al.*, 2008; Sevil Domenech *et al.*, 2007) and adenovirus-based vectors (Tucker *et al.*, 2008).

Protein or peptide antigens as well as live-vaccines can be partially protected from degradation by oral delivery in enteric-coated gelatin capsules (Levine, 2000) or by inclusion in particulate carrier systems such as co-polymeric microparticles (Chablani *et al.*, 2012; Eldridge *et al.*, 1989; Garg *et al.*, 2010; O'Hagan, 1998) and liposomes (Nordly *et al.*, 2009). The latter provide the important advantages of protecting the antigens from degradation and promote their uptake by PPs M-cells following oral administration. The retention of vaccine antigens on mucosal surfaces by delivery in adherent gel-forming polymers, such as chitosan, has been shown to increase antigen uptake and immune responses (Garg *et al.*, 2010; van der Lubben *et al.*, 2001). Immune-stimulating complexes (ISCOMs) are another type of adhesive delivery systems, which also has adjuvant effects and known to particularly stimulate CTLs responses (Sanders *et al.*, 2005; Sun *et al.*, 2009). Virus-like particles are another delivery system based on recombinant production of non-replicating viral core structures, typically from non-enveloped viruses. They are used as both delivery vectors and adjuvants, inducing IgA and CTLs responses (Schneider-Ohrum & Ross, 2012).

Table 1.1. Oral vaccine delivery systems

Delivery system	Nature/ composition	Immunological characteristics			Advantages	disadvantages	Clinical testing	References
		Target	T lymphocyte response	IgA				
Live-attenuated pathogens	**Bacteria:** e.g. *Salmonella Typhimurium* and *Listeria* **Viruses:** e.g. Adenovirus, Poxvirus and Vacinia virus	Epithelial and antigen-presenting cells	Th1, CTLs, Th2	++	- Ability to survive GI conditions - Inherent adjuvanticity - Interaction with, and penetration of host immune cells	- Pathogenicity reversion - Unsafe for immunocompromised individuals - Side effects due to systemic spread and allergy	yes	National Center for Immunization and Respiratory Diseases, 2011; Esteban, 2009; Gahan *et al.*, 2008; Paterson & Maskell, 2010; Tucker *et al.*, 2008; Vemula & Mittal, 2010; Wang *et al.*, 2013

Chitosan	Natural mucoadhesive polymer	Epithelial tight junctions	Th2	++	- Long-term depot and Pulsatile release of antigens - Can mimic prime-boost effects of conventional vaccines	- Issues on antigen stability in microcapsules - Issues antigen dose optimization	yes	Garg *et al.*, 2010; O'Hagan, 1998
Particles:								
PLG	Poly-lactic coglycolic acid micro and nanoparticles	ND	Th1, CTLs	+	- Controlled, sustainable antigen release - Mimic prime-boost effects of vaccines	Issues on antigen loading capacity and stability	yes	Peek *et al.*, 2008; Sharma *et al.*, 2014
Liposomes	Phospholipids microparticles	ND	Th1, Th2	+	Strong immune stimulants	- Issues on formulation stability - Costly production	yes	Kersten & Crommelin, 2003; Nordly *et al.*, 2009
ISCOMS or ISCO-MATRIX	*Quillaia* saponines: Quil A or QS-21	DCs	Th1, Th2, CTLs	++	- Strong CTLs responses - Adjuvant effects	Toxicity due to saponines- induced hemolysis	yes	Kersten & Crommelin, 2003; Sanders *et al.*, 2005; Sun *et al.*, 2009
Virus-like particles	virus envelopes (capsids)	ND	Th1, CTLs, Th2	+	- Easy production - Safer than live viruses	Non-invasive	yes	Schneider-Ohrum & Ross, 2012

Emulsions:								
Cationic DDA or liposomes	Cationic DDA or liposomes	DCs	Th1, CTLs	+	Slow antigen release	Moderate immunostimulatory effect	yes	Christensen *et al.*, 2009

Th, T helper; CTLs, cytotoxic T lymphocytes; GI, gastrointestinal; DCs, dendritic cells; DDA, dimethyldioctadecylammonium; ND, not determine

1.5.4. Mucosal adjuvants

The most potent and well-studied mucosal adjuvants in experimental systems are cholera toxin and *E. coli* heat-labile enterotoxins (Holmgren *et al.*, 2005; Liang & Hajishengallis, 2010; Sanchez & Holmgren, 2008). Much effort has been made to generate safe derivatives of these toxins with retained adjuvant activity for human use. An example of these derivatives is the nontoxic recombinantly produced cholera toxin B subunit (CTB), which promotes mucosal immunity (mainly sIgA) to oral antigens (Holmgren *et al.*, 2005; Sanchez & Holmgren, 2008). Mutant heat-labile enterotoxin or cholera toxin, devoid of the toxic activity of the A subunit were also developed. Some of these toxin mutants retain adjuvanticity and can be used as mucosal adjuvants (Lu *et al.*, 2002; Lycke, 2005; Pizza *et al.*, 2001; Sanchez *et al.*, 2002). A second category of bacterial toxin-based adjuvants is developed as hybrid molecules, in which the fully active cholera toxin A1 subunit, lacking its binding domain, is linked to an engineered specific APC-binding protein derived from *Staphylococcus aureus* protein (Lycke, 2004; Lycke, 2005). The mucosal adjuvanticity of the latter, however, is only proven in mice.

Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated "CpG motifs" represent another type of mucosal adjuvant, which interact with TLR9, initiating an immunomodulating cascade (Holmgren *et al.*, 2003; McCluskie *et al.*, 2001). Although being mainly considered for systemic use, CpG motifs were also found to markedly enhance both innate and adaptive mucosal immunity in animal models after oral administration (Liu *et al.*, 2010a; McCluskie *et al.*, 2001). Also, bacterial cell-wall components such as monophosphoryl lipid A derived from lipopolysaccharides of Gram-negative bacteria has immunomodulatory properties, mainly by interacting with TLR4 or by inducing proinflammatory cytokines (Moore *et al.*, 1999; Reed *et al.*, 2009; Salkowski *et al.*, 1997).

Cytokines are normally secreted during immune responses and they have been used during antigen administration to potentiate, modify or direct the immune response. Several cytokines have been used in mucosal immunization (Villinger, 2003) including, IL-1 (Staats & Ennis, 1999), IL-12 (Boyaka *et al.*, 1999; Lee *et al.*, 2003; Staats *et al.*, 2001) and type I Interferon (Bracci *et al.*, 2005).

2. *Lactococcus lactis* as a live-vector for oral vaccine delivery

2.1. Introduction

Lactococcus lactis (*L. lactis*) is a Gram-positive lactic acid bacterium that has been widely used in dairy and fermented food industry. However, over the last two decades, and with the recent advances in biotechnology and molecular biology, the application of *L. lactis* has been extended into oral vaccine design and delivery. In principle, *L. lactis* can be genetically engineered for the recombinant expression of the target vaccine antigen. The generated recombinant strain is thereafter administered orally for the delivery of the vaccine antigen to the gut immune system. Currently, a significant number of studies have been published describing induction of mucosal and systemic immune responses to antigens orally delivered using recombinant *L. lactis* strains (reviewed below).

L. lactis offers a number of significant advantages as a vector for oral vaccine delivery: (1) it provides a safe alternative to engineered live-attenuated pathogens, which are commonly used to deliver oral vaccines (e.g. *Salmonella Typhimurium*). These vectors are associated with risks of reverted pathogenicity and can induce diseases in immunocompromised individuals, the elderly and infants (National Center for Immunization and Respiratory Diseases, 2011). On the contrary, *L. lactis* is a non-pathogenic bacterium that is traditionally used in the food industry and that is generally regarded as safe for oral consumption (Casalta & Montel, 2008). Additionally, being a Gram-positive bacterium, *L. lactis* lacks the cell wall expression of endotoxic lipopolysaccarides, which exist in vectors based on Gram-negative pathogens. (2) *L. lactis* exhibits immunoadjuvant properties (Yam *et al.*, 2008), which can enhance the immune responses to expressed antigens, despite the weak responses elicited to the bacterium itself (Robinson *et al.*, 1997). The latter is particularly important because preexisting immunity against vaccine vectors can reduce their activity upon consequent use for the delivery of other antigens (Attridge *et al.*, 1997; Roberts *et al.*, 1999). On the other hand, the non-colonizing nature of *L. lactis* minimizes the potential of developing immunotolerance since the bacterium exhibits a short gastrointestinal transit. (3) The oral delivery of antigens vectored on *L. lactis* might enhance their uptake by M cells in the gut. *L. lactis* is similar in size and dimensions to

microparticles, a delivery system known to be taken up by M cells (Challacombe *et al.*, 1992; Robinson *et al.*, 2004).

2.2. *L. lactis*– origin, classification and food-grade applications

L. lactis are non-sporulating, non-motile, facultative anaerobe cocci of 0.5 - 1.5 μm in size and are typically arranged in pairs or short chains. Milk is the common source for isolation of *L. lactis* and therefore, is being considered as a natural habitat for the bacterium (Sandine, 1977). *L. lactis* was isolated in 1873 by Lister, being the first microorganism ever isolated in a pure culture, and the first recognized cause of lactic acid fermentation of milk. The isolated bacterium was designated *Bacterium lactis*. In 1909 and based on morphology, *L. lactis* was included into the genus *Streptococcus*, and thus renamed *Streptococcus lactis*. This name was used till 1985, when a phylogenetic study reclassified the members of the diverse genus *Streptococcus* into the genera *Lactococcus*, *Enterococcus* and *Streptococcus*, under the family *Streptococcaceae* (Ludwig *et al.*, 1985). Also, according to the same study, *Streptococcus lactis* and *Streptococcus cremoris* were designated *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, respectively.

L. lactis is capable of converting hexose sugars (e.g. lactose and glucose), into lactic acid (lactic acid fermentation) (Vandenbergh, 1993). By fermentation, food or milk is being modified to acquire desired flavor and texture. Additionally, fermentation serves as a method of food preservation (Vandenbergh, 1993). Both *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* have been used in single and mixed cultures for the production of different kind of cheeses, fermented milks, cultured butter, casein, vegetables like sauerkraut and olives, wine, beer and meat, like salami (Coffey *et al.*, 1998).

Due to the long history of safe use in the food industry, and since the bacterium has never been associated with infectious diseases, *L. lactis* was granted a "GRAS" (generally recognized as safe) status by the US Food and Drug Administration (Casalta & Montel, 2008). Similarly, in Europe, the safety of *L. lactis* as a food-grade bacterium is authorized according to the QPS (Qualified Presumption of Safety) assessment. The latter is a system adopted by the EU for pre-market safety assessment of microorganisms proposed for use in food production (www.efsa.europe.eu).

2.3. Recombinant protein expression in *L. lactis*

Most of the industrially important characteristics of *L. lactis* strains (e.g. lactose catabolism) are encoded on plasmids, which might constitute a large and often unstable DNA complement to the cell. The development of the plasmid-free *L. lactis* strain MG1363 (a *L. lactis* ssp. *cremoris* strain) in the early 1980's has opened the doors for heterologous protein expression in *L. lactis* (Gasson, 1983). MG1363 is stable and relatively amenable to genetic modification. Additionally, many genetic systems are currently available for *L. lactis*, allowing expression and targeting of antigens to various cellular locations, including secretion and cell-wall display (Le Loir *et al.*, 2005). These tools were utilized for the generation of numerous recombinant *L. lactis* strains, designated for the oral delivery of various bacterial, viral, protozoan and eukaryotic antigens (reviewed in details in section 2.5).

2.3.1. *L. lactis* Promoters

Several lactococcal promoter sequences have been isolated, characterized and made available to establish *L. lactis* gene expression systems for the production of heterologous proteins. Before the availability of the first *L. lactis* full genomic sequence (was identified for *L. lactis* ssp. *lactis* (Bolotin *et al.*, 2001)), a variety of constitutive promoters were randomly isolated from an *L. lactis* genomic library by fusions to promoterless reporter genes (Vandervossen *et al.*, 1987; Waterfield *et al.*, 1995). Strong (e.g. P1, P21, P23 and P59) and weak (e.g. P32 and P44) promoters were distinguished by measuring the activity levels of the reporter gene products (Waterfield *et al.*, 1995).

Other lactococcal promoters were isolated from specific characterized lactococcal genes. These include the promoter of the *usp45* gene (van Asseldonk *et al.*, 1990), which encodes for the unknown secretory protein of *L. lactis* (Usp45), in addition to the PnisA, promoter which regulates the production of nisin, a lactococcal bacteriocin produced by some *L. lactis* strains. Today, with the availability of a full lactococcal genomic sequence (Wegmann *et al.*, 2007), new promoters can easily be identified by screening genome sequences. Microarray data can also be used for selection of promoter sequences (Kim *et al.*, 2009).

2.3.2. Heterologous gene expression systems in *L. lactis*

The first class of expression vectors used in *L. lactis* was based on the endogenous pWVO1 replication group of plasmids isolated from *L. lactis* subsp. *cremoris* (de Vos *et al.*, 1989; Kok *et al.*, 1984; Leenhouts *et al.*, 1998; Vandervossen *et al.*, 1985). For instance, plasmid pGKV2 that utilizes the SOP2 promoter from *Bacillus subtilis* was one of the first lactococcal expression vectors described (Vandervossen *et al.*, 1985). Although pWVO1 plasmids are characterized by a wide host range, they are inherently less stable in structure, because of their rolling circle type replication (Gruss & Ehrlich, 1989). In order to overcome these limitations, a second class of heterologous plasmids was developed, based on heterologous (non-lactococcal) operons. These vectors already encoded antibiotic resistance markers and were found operational in *L. lactis* with respect to both replication stability and functional expression of the selection marker. An example is the pAMβ1 plasmid, which displays a high copy number and shows high structural and segregational stability in *L. lactis*, even when a large DNA sequence is inserted. These features made it the plasmid of first choice for the development of several lineages of cloning vectors for *L. lactis*. A series of expression vectors which incorporate the replicon of pAMβ1 are designated pTREX (for theta replicating expression vectors) (Brehm *et al.*, 1987). The expression cassette in pTREX incorporates the PlacA promoter and the following *E. coli* bacteriophage T7 expression signals: i) a putative RNA stabilizing sequence and ii) the translation initiation region of gene 10 which has been modified to increase the complementarity of the Shine Dalgarno (SD; also known as the ribosomal binding site) sequence to the ribosomal 16S RNA of *L. lactis* and iii) the T7 RNA polymerase transcription terminator.

2.3.2.1. Constitutive expression systems

Constitutive expression of a gene means that the gene product is constantly expressed without regulation. Several constitutive lactococcal promoters have been cloned into pTREX plasmids to develop lactococcal expression systems. For instance, pTREX1 is an expression system that has been developed by replacing the PlacA promoter of pTREX by the strong constitutive lactococcal promoter P1 (Wells and Schofiled, 1996). Since pTREX1 combines the stability of the theta replicons with a strong promoter, a variety of heterologous proteins have been expressed in *L. lactis* using pTREX1, such as tetanus toxin fragment C (TTFC) from *Colistridium tetani* (Robinson *et al.*, 1997), interleukin 2 (IL-2) (Steidler *et al.*, 1995), IL-6 (Steidler *et al.*,

1998) and IL-10 (Schotte *et al.*, 2000). However, continuous high-level production of protein might lead to intracellular aggregation or degradation of the protein in the cytoplasm. These phenomena can be deleterious to the bacterial cell. These adverse effects were resolved by developing expression systems based on inducible promoters.

2.3.2.2. Inducible expression systems

Inducible promoters are generally known to regulate the expression of genes related to adaptation of the bacteria to environmental stresses. Many *L. lactis* promoters were found to be induced by shifts in temperature and pH or the presence of certain sugars and ions in the medium. Inducible expression systems allow the control of heterologous protein production, which can be initiated by the addition of the specific inducer to the culture medium at a certain phase of the bacterial growth. This feature is particularly useful to reduce the stress exerted on bacterial cells by continuous production of the heterologus protein or in case the target protein is toxic to the lactococcal system.

The NICE system

The most commonly used expression system in *L. lactis* today is the nisin-controlled gene expression (Brigotti *et al.*, 2010) system (de Ruyter *et al.*, 1996b; Kuipers *et al.*, 1995). Nisin synthesis is encoded by a cluster of 11 genes, present in some *L. lactis* strains (Ra *et al.*, 1996; Siegers & Entian, 1995). The first gene in the cluster, nisA, encodes the nisin peptide itself. The *nisR* and *nisK* genes constitute a two-component regulatory system; *nisR* is a sensor histidine kinase and *nisK* is the response regulator. Other genes in the cluster are associated with nisin modification, translocation, processing or with the immunity of the bacterial host cell to the peptide (Mierau & Kleerebezem, 2005b).

The NICE system utilizes the promoter of *nisA* gene and requires the presence of the *nisR* and *nisK* genes, either integrated into the genome or carried on the cloning plasmid (Figure 2.1). The transcriptional fusion of the gene of interest to the *nisA* promoter sequence allows transcription initiation by addition of sub-inhibitory levels of nisin to the culture medium (0.01 – 10 ng/ml).

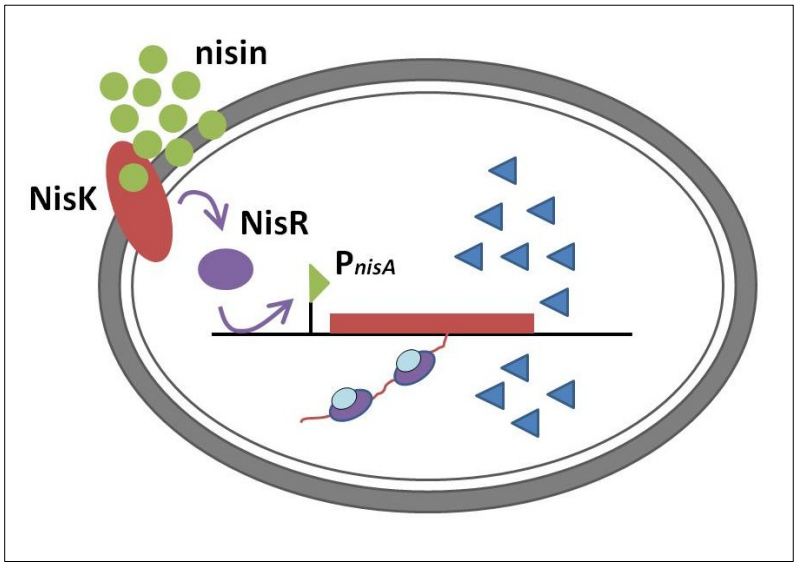

Figure 2.1. Schematic representation of the NICE system. Heterologous gene expression in response to a nisin stimulus involves a membrane-located sensor protein (Rimpilainen *et al.*, 1986) and a cytoplasmic response regulator (NisR) that controls transcriptional activation from the *NisA* gene promoter (PnisA). The auto-induction mechanism of nisin is used as a controlled heterologous gene expression system in *L. lactis*. Figure adapted from (Pontes *et al.*, 2011).

This system for controlled expression has been found to show many desirable characteristics: i) Nisin is a wide spectrum bacteriocin that has been used in food industry and thus, it is an ideal molecule to be used as an inducer (food-grade); ii) protein expression using the NICE system seems to be very tightly controlled, since the protein is undetectable in not induced strains. This even made the expression of lethal proteins in *L. lactis* possible (de Ruyter *et al.*, 1997); iii) very high protein expression levels, since a protein yield of up to 100 mg/L was obtained in the 3000-L scale and the yield was further increased to 300 mg/L in optimized culture conditions (de Ruyter *et al.*, 1996a; Kuipers *et al.*, 1995; Mierau & Kleerebezem, 2005b; Mierau *et al.*, 2005); iv) The availability of various host strains and expression plasmids, including food-grade systems that utilize the lacF marker instead of antibiotics (Eichenbaum *et al.*, 1998; Kuipers *et al.*, 1997). Therefore, the NICE system has been used for the production of variety of proteins of different origins in *L. lactis* and other bacterial hosts (Mierau & Kleerebezem, 2005a; Zhou *et al.*, 2008).

2.3.2.3. Cellular targeting of heterologous proteins

Several systems have been designed to specifically target proteins to the cytoplasm, the cell wall or the extracellular medium in *L. lactis* (Bermudez-Humaran *et al.*, 2003; de Vos, 1999; Dieye *et al.*, 2001). Insertion of the heterologous gene downstream the promoter in absence of secretion or cell-wall targeting motifs designates the antigen expression to the cytoplasm. Cytoplasmic expression of antigens in *L. lactis* has some draw backs for antigen expression yields. *L. lactis* possesses at least two cytoplasmic house-keeping proteases, clpP (Frees & Ingmer, 1999) and FtsH (Nilsson *et al.*, 1994), as well as DnaK, a cytoplasmic chaperon that may promote proteolysis by maintaining the protein in an unfolded state (Koch *et al.*, 1998). The proteolytic activity of these enzymes can negatively influence the cytoplasmic designated expression of heterologus proteins. Additionally, cytoplasmic expression of proteins, which are lethal to the lactococcal cells, might influence cell viability and thus protein expression yield (Le Loir *et al.*, 2005; Miyoshi *et al.*, 2006).

The general secretory pathway in *L. lactis* as well as in other Gram-positive bacteria is the Sec-dependent translocation. Proteins are synthesized in the cytoplasm as precursors containing the mature protein and an N-terminal signal peptide (SP) that is an essential signature for protein secretion (von Heijne, 1990). Absence of the coding sequence of SP upstream the target heterologous gene designates the protein expression to the cytoplasmic compartment. If present, SP is recognized and cleaved by the secretion machinery, allowing the protein to be transferred through the membrane and released into the extracellular medium. For this translocation system a variety of proteins including SecA, SecY and SecE are required. SecY (Koivula *et al.*, 1991) is an essential component of the protein export machinery and interacts with the SP of secretory proteins, but also interacts with the peripheral membrane protein SecA and the integral membrane protein SecE. This, in combination with the action of Sec-specific chaperones retards precursor folding (Pugsley, 1993; Tjalsma *et al.*, 2000). Secretion often allows the heterologous protein to escape the cytoplasmic proteases of *L. lactis* leading to a better expression yield (Le Loir *et al.*, 2005). Additionally, it has been shown that antigen secreting *L. lactis* strains can accumulate the precursor of the antigen to be secreted in their cytoplasm, at levels higher than those of the mature protein expressed by designated cytoplasmic expression. This phenomenon was reported, for example, to the expression of proteins such as the *Staphylococcus* Nuc

and the *Brucella abortus* antigen L7/L12 in *L. lactis* (Bermudez-Humaran *et al.*, 2003; Ribeiro *et al.*, 2002). A possible explanation is that the engagement of the cytoplasmic precursor with the lactococcal secretion machinery in the cytoplasm protects the precursor against proteolysis by cytoplasmic housekeeping enzymes (Le Loir *et al.*, 2005).

To identify SP's for protein secretion in *L. lactis*, several *L. lactis* strains were screened for endogenous secreted proteins in their culture supernatants. One protein, designated as unknown secreted protein of 45 kDa (Usp45), was particularly abundant and its chromosomal located gene *usp45* was isolated from *L. lactis* subsp. *cremoris* MG1363 (van Asseldonk *et al.*, 1990). The secretion efficiency of the Usp45 SP was superior to other new homologous SP's identified in *L. lactis* (Ravn *et al.*, 2003). Only one report showed that a *Lactobacillus brevis* SP drove the secretion of FedF adhesin more efficiently than the Usp45 SP in *L. lactis* (Lindholm *et al.*, 2004).

Even with a homologous SP, secretion may be inefficient, and some heterologous proteins are poorly or not at all secreted (Enouf *et al.*, 2001; Puohiniemi *et al.*, 1992; Ravn *et al.*, 2000). Notably, charges at the N terminus of the mature protein may greatly affect the translocation across the cytoplasmic membrane (Andersson & von Heijne, 1991; Steidler *et al.*, 2003). In this regard, according to several reports, protein conformation rather than protein size seems to influence the efficiency of heterologous protein secretion in *L. lactis* (Enouf *et al.*, 2001; Le Loir *et al.*, 2005; Nouaille *et al.*, 2005).

Heterologous proteins can also be targeted to the cell wall of *L. lactis* by an anchoring signal sequence (cell wall anchor, CWA) containing a conserved LPXTG motif. This anchor is a transmembrane fragment with a charged C terminus which, when fused to the carboxyl terminal of the protein, remains covalently attached to the cell wall by transpeptidation between the LPXTG motif and peptidoglycan. Anchoring of heterologous proteins in *L. lactis* using the CWA of proteins A and M6 of *Staphylococcus aureus* and *Staphylococcus pyogenes* respectively, was demonstrated to be efficient (Dieye *et al.*, 2001; Piard *et al.*, 1997; Ribeiro *et al.*, 2002; Steidler *et al.*, 1998).

2.3.2.4. Codon optimization of heterologous genes

As defined by the degeneracy of the genetic code, the majority of amino acids can be encoded by more than one codon. Different bacterial species vary in their codon usage preference, according to their genomic GC (Guanines/cytosines) content. Bacteria with genomes rich in GC content prefer codons with guanidine and cytosine, whereas bacteria with genomes of lower GC content (including *L. lactis*) prefer codons with adenines and thymines (Fuglsang, 2003). Codon usage preference is an important factor for efficient heterologous protein expression. Heterologous genes with codon sequences rarely used by the host organism will likely result in a poor protein production due to insufficiency of the corresponding tRNA required for proper mRNA translation (Fuglsang, 2003).

The availability of cost-effective tools for codon adaptation and DNA synthesis has facilitated the use of the codon optimization approach in order to enhance heterologous protein expression. Several reports have described the usefulness of this strategy in heterologous protein expression in *L. lactis* (Berlec *et al.*, 2006; Fuglsang, 2003; Steidler *et al.*, 2003; Vandenbroucke *et al.*, 2004). A different approach to overcome codon preference bias of *L. lactis* was used by Hernandez et al., in order to optimize the expression of two chromosomally inserted plant genes related to the production of strawberry flavor in *L. lactis*. A vector plasmid carrying the encoding gene of the rare tRNA AGG was introduced into *L. lactis* to overcome its codon preference bias (Hernandez *et al.*, 2007). Nevertheless, the efficiency of this approach is not superior to codon adaptation (Hernandez *et al.*, 2007).

2.4. Pharmacological and immunological aspects of oral vaccine delivery using *L. lactis*

2.4.1. Modes of antigen delivery by *L. lactis*

Generally live-vaccine delivery vectors can deliver recombinant antigens to the inductive sites of the gut immune system, either via the release of antigens in the intestinal environment, or via the uptake of the vector and the enclosed antigen by gut antigen-presenting cells. Depending on the target cellular location of the antigen, *L. lactis* either actively secretes the antigen in the gut lumen (strains designated for antigen secretion), and/or releases the antigen present in the cytoplasm upon its lysis in the gastrointestinal tract (strains designated for cytoplasmic expression) (Figure

2.2). The uptake of bacteria and other particulate antigens is a characteristic feature of M cells and other antigen-presenting cells in the gut. However, in a study that investigated the interactions of an IL-10-secreting *L. lactis* strain with the intestinal mucosa of mice, the transcellular uptake of the bacterium was described as a rare event (Waeytens *et al.*, 2008). Moreover, although *L. lactis* was found adjacent to M cells, no bacteria were found on the cell membrane or in the cytoplasm. (Waeytens *et al.*, 2008). Thus, an evidence of the cellular uptake of viable *L. lactis* in the gut is currently lacking.

2.4.2. Influence of the antigen cellular location on immunogenicity

The final cellular location of antigens expressed in *L. lactis* (i.e. cytoplasmic, secreted or cell-wall anchored) can influence the immunogenicity of these antigens upon oral delivery. A possible explication is that the cellular location of antigen expression can directly affect the amount of the antigen released and its availability for interaction with the gut immune system. Cytoplasmic expression of antigens often results in weak immune responses (Pei *et al.*, 2005; Pontes *et al.*, 2004; Rodriguez-Diaz *et al.*, 2011). As discussed above, antigens targeted to the cytoplasmic compartment of *L. lactis* can be degraded by the housekeeping proteases, which can result in low antigen-expression levels (Le Loir *et al.*, 2005). Additionally, the release of cytoplasmically expressed antigens in the intestine is subject to lysis of the bacteria in the stomach and duodenum (Figure 2.2).

Secretion can be favored for obtaining higher immune responses to antigens orally delivered using recombinant *L. lactis* (Pei *et al.*, 2005; Perez *et al.*, 2005; Rodriguez-Diaz *et al.*, 2011). Secreted antigens are readily available for interaction with epithelial and antigen-presenting cells in the gut. Additionally, secretion may allow the delivery of higher antigen dose to immune-inductive sites. Following oral administration, viable *L. lactis* cells are metabolically active in the gastrointestinal tract and thus, antigen synthesis and secretion continues in situ (Drouault *et al.*, 1999; Huibregtse *et al.*, 2007; Steidler *et al.*, 2000). Also, antigen secreting strains simultaneously accumulate the antigen in the cytoplasm (the precursor of the secreted protein), usually at higher amounts compared to strains designated to cytoplasmic expression (Bermudez-Humaran *et al.*, 2003; Ribeiro *et al.*, 2002). This enables additional antigen release by lactococcal cells, which will be lysed in the gastrointestinal tract or which are taken up by M cells or sampled by dendritic cells (DCs).

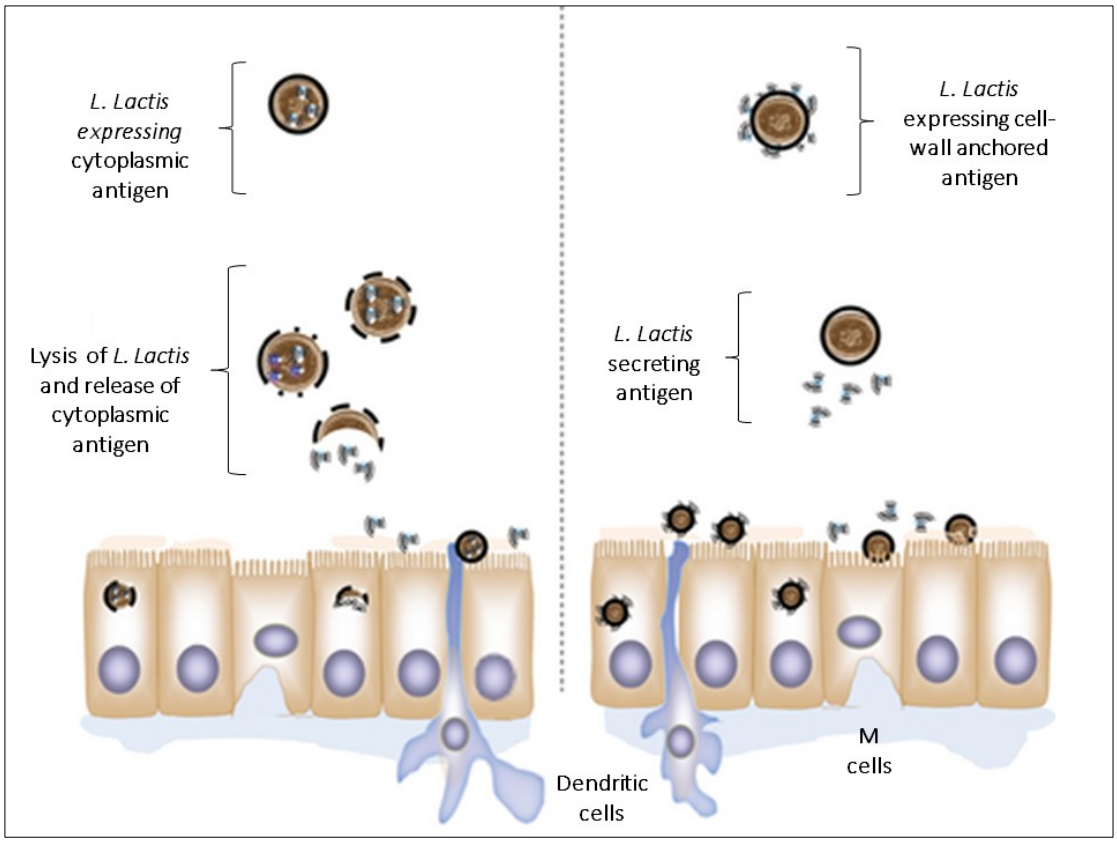

Figure 2.2. Schematic representation of antigen delivery using *L. lactis* vectors in the gut. Recombinant *L. lactis* expressing the antigen reaches the intestinal tract and secretes the antigen or releases it after lysis. The bacteria carrying the antigen and/or the free antigen are taken up by dendritic cells or M cells. Figure adapted from (LeBlanc *et al.*, 2013).

There are only few studies that have compared immune responses to antigens expressed in the different cellular locations of *L. lactis* upon oral immunization (Marelli *et al.*, 2011; Perez *et al.*, 2005). While secretion of Rotavirus VP7 antigen induced higher immune responses than did cytoplasmic expression or cell-wall anchoring (Perez *et al.*, 2005), higher immune responses were obtained by cell-wall anchoring of the Rotavirus VP8 antigen and the staphylococcal Nuc compared to other cell-targeting locations (Dieye *et al.*, 2003; Marelli *et al.*, 2011). Although cell-surface location of antigens is believed to improve the presentation to antigen-presenting cells, in these studies the high immune responses obtained to cell-wall anchored antigens was suggested to be a result of the presence of high amounts of the antigen precursor in the cytoplasm of the recombinant strains (the cytoplasmic precursor of the to-be-anchored antigen).

2.4.3. Adjuvant and immunostimulatory effects of *L. lactis*

Several *in vitro* studies have suggested adjuvant properties of *L. lactis*, which further support the value of the bacterium as a vaccine delivery vector. *L. lactis* ssp. *cremoris* was shown to stimulate the induction of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α upon incubation with a macrophage-like cell line (Suzuki *et al.*, 2008). A second study showed the ability of *L. lactis* MG1363 to induce the secretion of TNF-α, when incubated with murine macrophages (Audouy *et al.*, 2007). Additionally, a recent study performed in a murine air pouch model demonstrated the ability of *L. lactis* MG1363 to recruit leukocytes, which were mainly neutrophils (95%), but also eosinophils, monocytes, macrophages and lymphocytes. These cells were found to contain significant levels of the chemokine legends (McCluskie *et al.*, 2001) CCL1, CCL2, CCL3, CCL4, CXCL2 and CXCL10 mRNA (Yam *et al.*, 2008). The latter are known to mediate the recruitment of the leukocytes (Mantovani *et al.*, 2004). Also, in the same study *L. lactis* was shown to stimulate DCs to upregulate the mRNA expression of cytokines with adjuvant activities such as IL-1β (Yam *et al.*, 2008).

The oral delivery of antigen using recombinant *L. lactis* strains has been shown to induce a mixed Th1/Th2 cell response (Cauchard *et al.*, 2011; Lee *et al.*, 2009; Robinson *et al.*, 2004; Villena *et al.*, 2008; Villena *et al.*, 2010). The type of T-helper cell induced, is largely determined by the function of DCs (Rescigno, 2010). In this context, *in vitro* studies have shown the ability of *L. lactis* ssp. *cremoris* to induce the maturation of DCs (Audouy *et al.*, 2007; Yam *et al.*, 2008), as well as the secretion of cytokines known to direct the differentiation of both Th1 (IFN-γ and IL-12) and Th2 (IL-6 and IL-10) cell types (Latvala *et al.*, 2008; Yam *et al.*, 2008).

2.4.4. Gastrointestinal transit and survival of *L. lactis*.

It should be noted that the two major subspecies of the lactococcus genus (*L. lactis* ssp. *lactis* and ssp. *cremoris*) have different gastrointestinal transit and survival profiles (Kim *et al.*, 1999; Kimoto *et al.*, 2003). Hence, in this section we specifically review the literature on *L. lactis* ssp. *cremoris* to which the vaccine vector strain MG1363 belongs. *L. lactis* is a non-colonizing bacterium and does not integrate with microbiota inhabiting the mammalian intestine. In mice *L. lactis* ssp. *cremoris* has a gastrointestinal transit time of approximately 12 hours (Kimoto *et al.*, 2003). In humans, a trial where *L. lactis* was fed to volunteers twice a day for four days showed that viable bacteria were detected in the feces for three days after the end of the

feeding period, whereas *Bacillus stearothermophilus* (currently known as *Geobacillus stearothermophilus*), which is known for its capacity to survive the gastrointestinal passage, could be detected for up to seven days (Klijn *et al.*, 1995).

Consistent with its relatively short gastrointestinal transit, *L. lactis* also show a moderate capacity to survive gastrointestinal tract compared to other commensal or probiotic bacteria. In a study performed in pigs, a low survival of *L. lactis* was observed in the stomach and upper jejunum compared to *Bacillus subtilis* spores, which are known for their tolerance to gastrointestinal transit (Drouault *et al.*, 2002). Similarly, data available for humans show a recovery of only 1% of the bacteria from the ileum four hours after ingestion, compared to the probiotic bacterium, *Lactobacillus fermentum* (Vesa *et al.*, 2000). With regard to experimental immunizations in mice, noteworthy that the use of a buffered medium (e.g. BM9) to neutralize gastric acidity and provide nourishment to the bacteria, resulted in the recovery of more than 50% of the bacteria one hour after inoculation (Huibregtse *et al.*, 2007).

2.5. Current status of *L. lactis*-based oral vaccines.

The first attempt to use *L. lactis* as an oral vaccine delivery vector was performed in 1990 by Iwaki et al., (1990). A killed recombinant strain displaying a *Streptococcus mutans* protective antigen (PAc) on the cell-wall was used to immunize mice orally and induced specific serum IgG and intestinal IgA antibodies (Iwaki *et al.*, 1990). These findings provided the first evidence on the feasibility of using *L. lactis* to present antigens to the immune system. Soon after, Robinson et al., reported for the first time the use of live recombinant lactococci for the oral delivery of vaccine antigen to the gut immune system (1997). In their study, a recombinant strain secreting the *Clostridium tetani* TTFC induced specific serum IgG responses and fecal IgA responses, and immunized mice were protected against a lethal challenge with tetanus toxin (Robinson *et al.*, 1997). Currently, a vast collection of published studies are available describing the oral delivery of viral, bacterial, protozoan and eukaryotic antigens using recombinant *L. lactis* as a vector. These studies are reviewed in Table 2.1, with a qualitative overview of the immune responses elicited and the associated pattern of protection, when applicable. Besides the numerous studies describing the efficient oral delivery of antigens by *L. lactis*, few studies have demonstrated the ability of the bacterium to deliver DNA vaccines to the gut immune system (Chatel *et al.*, 2008; Guimaraes *et al.*, 2005; Pontes *et al.*, 2014). The concept of DNA vaccination is based on

the utilization of the host cell translation machinery for the presentation of antigenic epitopes to the immune system. The early study demonstrated the delivery of green fluorescent protein (GFP) encoding DNA (Guimaraes *et al.*, 2005). To allow its penetration into the host cell, the recombinant strain also expressed the *Listeria monocytogenes* internalin on the cell-wall (Guimaraes *et al.*, 2005). However, native non-invasive *L. lactis* were recently shown to delivery DNA encoding bovine β-lactoglobulin, which elicited mucosal and systemic immune responses (Chatel *et al.*, 2008; Pontes *et al.*, 2014).

Despite the extensive preclinical research involving the use of *L. lactis* for the delivery of oral vaccines against infectious agents, to date, there is not a single *L. lactis*-based vaccine under clinical trials. However, at the present time, a patented technology (ActoBiotics™) exists, based on the use of freeze-dried recombinant lactococci for the topical intestinal delivery of biomolecules (TopAct™). These technologies are owned by ActoGeniX (www.ActoGeniX.com), a Belgian biotechnology company that was founded in 2006, as a spin-off of the life science research institute, VIB and the University of Ghent. Interestingly, the safety and tolerability of the ActoBiotics™ was evaluated in a phase I clinical trial, in which patients of moderate colitis were treated orally with a *L. lactis* strain secreting the anti-inflammatory cytokine IL-10 (Braat *et al.*, 2006). The findings of this clinical trial open the doors to the therapeutic application of the technology in humans. Mimopath® is a second technology developed by the Dutch company, Mucosis (www.Mucosis.com), involving the utilization of inactivated *L. lactis* to develop bacterial-like particles for mucosal vaccine delivery. Currently, Mucosis is pursuing the preclinical phase for the development of an oral Mimopath®-based *Shigella* vaccine.

Table 2.1. Summary of oral vaccination studies involving the use of *L. lactis* as a vaccine delivery vector

Infectious agent	Antigen	Bacterial location	Immune responses	Protection pattern	Animal model	References
Bacteria:						
***Clostridium tetani* (Tetanus)**	Tetanus toxin fragment C (TTFC)	CYT	-Serum IgG and fecal IgA -Mixed Th1/Th2 cytokine response in PP and MLN	Survival after a lethal challenge with tetanus toxin	Mouse	Grangette *et al.*, 2002; Grangette *et al.*, 2004; Robinson *et al.*, 1997; Robinson *et al.*, 2004
Brucella abortus	L7/L12	CYT	Fecal IgA	-Partial protection against intraperitoneal challenge with *B. abortus*	Mouse	Pontes *et al.*, 2004
	Cu-Zn superoxide dismutase (SOD)	SEC	-Serum IgM and IgG, fecal IgA -Lymphocyte proliferation -Responses improved by co-administration of *L. lactis* secreting IL-12	-Reduced *B. abortus* counts in spleen after challenge	Mouse	Saez *et al.*, 2012
Helicobacter pylori	Urease B (UreB)	CYT	Serum IgG	-	Mouse	Lee *et al.*, 2001

Helicobacter pylori	Urease B (UreB)	SEC	Serum IgG and fecal IgA	Reduced *H. pylori* gastric counts and urease activity	Mouse	Gu *et al.*, 2009
	Cag12	CYT	Serum IgG	-	Mouse	Kim *et al.*, 2006
	Cholera toxin B subunit plus T and B cell epitopes of UreB	SEC	Serum IgG and fecal IgA	Reduced *H. pylori* gastric counts	Mouse	Li *et al.*, 2014
	UreB and IL-2	CYT	Serum IgG and fecal IgA IFN-γ and IL-4 in serum	Reduced *H. pylori* gastric colonization and urease activity	Mouse	Zhang *et al.*, 2014
Clostridium difficile	nontoxic tetanus toxin fragment C (TETC), and 14 C-terminal repeats (14CDTA) of nontoxic C. difficile toxin A (TcdA)	SEC, CWA	IgG in serum and intestinal fluid	-Increased survival upon lethal challenge with TcdA -Reduced cytotoxicity and enterocyte apoptosis *in vitro* -Reduced expression of mucosal inflammatory cytokines, ICAM-1, MCP-1, IL-6, and Gro-1	Hamster	Yang *et al.*, 2013

Staphylococcus aureus	SEB	CYT, SEC	Serum IgG and fecal IgA	Increased survival after intraperitoneal challenge with SEB-producing *S. aureus* strain	Mouse	Asensi *et al.*, 2013
Enterotoxigenic *Escherichia coli* (ETEC)	F_4 (K88) fimbrial adhesin FaeG	SEC	-Serum IgG and fecal IgA -Antibody secreting cells in spleen, MLN and PP	-	Mouse	Liu *et al.*, 2013
(Neonatal porcine diarrhea)		SEC	Serum IgG	Reduced mortality after challenge	Mouse	Hu *et al.*, 2009
		CYT	-Serum IgG and fecal IgA -Antibody secreting cells in spleen, MLN and PP	-	Mouse	Liu *et al.*, 2010b
Vibrio cholerae	Wzm	SEC	Serum IgG and fecal IgA	Absence of diarrhea after challenge	Rabbit	Zamri *et al.*, 2012
Streptococcus pneumoniae	PppA	CWA	-IgM, IgG and IgA in serum and intestinal and bronchial fluids -Intestinal IgA^+ cells -Mixed Th1/Th2 response (splenocyte cytokines and IgG subtypes analysis)	Reduced pulmonary colonization and bacteremia	Mouse	Villena *et al.*, 2010 Villena *et al.*, 2008

Enterohemorrhagic *Escherichia coli* (EHEC)	EspA	SEC	Serum IgG	Reduced cell cytoskeletal changes *in vitro*	Mouse	Luan *et al.*, 2010
Rhodococcus equi	VapA	SEC	-Serum IgG and fecal IgA -Splenocytes mixed Th1/Th2 cytokine responses	Reduced liver and spleen counts of *R. equi*	Mouse	Cauchard *et al.*, 2011
Virus:						
Human immunodeficiency virus (HIV)	V2-V4 loop of gp120	CWA	-Serum IgG and fecal IgA -IFN-γ producing $CD8^+$ T cells in peritoneal lymph node and spleen	Decreased virus load after a. challenge with HIV Env-expressing vaccinia virus	Mouse	Xin *et al.*, 2003
	Gag	CWA	-IgG and IgA in serum, feces and vaginal fluid - Rectal IL-2, IFN-γ, TNF-α and IL-17 producing $CD4^+$ cells	-	Mouse Rhesus monkey	Chamcha *et al.*, 2012
Rotavirus	VP7	CYT, SEC, CWA	Serum antibodies	Virus neutralization in an *in vitro* assay	Mouse	Perez *et al.*, 2005
	VP8	CYT, SEC, CWA	Serum IgG and fecal IgA	Block infection in an *in vitro* cell line-based assay	Mouse	Marelli *et al.*, 2011
		CYT, SEC	Serum IgG and fecal IgA	-	Mouse	Rodriguez-Diaz *et al.*, 2011

Rotavirus	VP4	CYT	IgA and IgG were found in feces, ophthalmic and vaginal washes and in serum	Virus *in vitro* neutralizing effect	Mouse	Li *et al.*, 2010
Human papilloma virus-16 (HPV-16)	L1 capsid protein	CYT, SEC	Serum IgG and vaginal IgA	-	Mouse	Cho *et al.*, 2007
Sever Acute Respiratory Syndrome (SARS)-coronavirus	Nucleocapsid (N) protein	CYT, SEC	Serum IgG	-	Mouse	Pei *et al.*, 2005
Dengue virus	Envelope protein E domain III (EDIII)	CYT	Serum IgG	Virus neutralization in an *in vitro* assay	Mouse	Sim *et al.*, 2008
Chicken infectious bronchitis virus (IBV)	Multiple epitope antigen EpiC	CWA CYT, SEC	Serum IgG and intestinal IgA	Absence of clinical signs Protection against lethal IBV challenge	Chicken Chicken	Cao *et al.*, 2013 Berlec *et al.*, 2013
Hepatitis A virus	VP1-P2a	CYT, CWA	Serum IgG and fecal IgA	No protection in *in vitro* neutralization assay	Mouse	Berlec *et al.*, 2013
Hepatitis B virus	PreSa	SEC	Serum IgG and fecal IgA	-	Mouse	Zhang *et al.*, 2011a
Avian influenza virus	Hemagglutinin 1 (HA-1)	CYT	-Serum IgG and fecal IgA -Splenocyte secretion of IFN-γ and IL-4	Protection against lethal H5N1 challenge	Mouse	Wang *et al.*, 2012

Avian influenza virus	Hemagglutinin 1 (HA-1)	CWA	Serum IgG and fecal IgA Splenocyte cell proliferation and IFN-γ secreting cells	Protection against lethal H5N1 challenge	Mouse	Lei *et al.*, 2011
Porcine transmissible gastroenteritis virus	SN protein	SEC	Serum IgG and fecal IgA	virus neutralization *in vitro*	Mouse	Tang & Li, 2009
Protozoa:						
***Plasmodium yoelii* (Malaria)**	MSP-1	CYT	-	Reduced parasitemia after challenge with *Plasmodium yoelii*	Mouse	Zhang *et al.*, 2005
	MSA2cP	CYT,CWA	Serum IgG and fecal IgA	-	Mouse	Moorthy *et al.*, 2009
Giardia lamblia	Cyst wall protein-2 (CWP-2)	CWA	Serum IgG and fecal IgA Mixed Th1/Th2 cytokine response in MLN and PP	Reduced cyst fecal output after oral *Giardia lamblia* challenge	Mouse	Lee *et al.*, 2009
Eimeria tenella	3-1E	CYT	-	Reduced fecal oocyst release and cecal lesion score	Chicken	Ma *et al.*, 2013
Leishmania major	LACK plus IL-12	CYT, SEC, CWA	Intestinal IgA Th1 response in spleen and MLN	Reduced food-pad swelling and parasitic burden	Mouse	Hugentobler *et al.*, 2012

- Not performed
CYT, cytoplasmic expression; SEC, secretion; CWA, cell-wall anchoring; MLN, mesenteric lymph nodes; PP, Peyer's Patches

3. Enterohemorrhagic *Escherichia coli*

3.1. Introduction and historical perspective

Enterohemorrhagic *E. coli* (EHEC) are a subset of shiga toxin- (or verocytotoxin-) producing *E. coli* that can cause hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS) (Janot *et al.*, 2009). EHEC are zoonotic enteropathogens transmitted from ruminants to humans through ingestion of contaminated food and water or through direct contact. The disease usually occurs in epidemic outbreaks, which have been frequently reported worldwide. Following infection, patients develop hemorrhagic colitis, which is manifested clinically as bloody diarrhea. In severe cases, HC usually progresses to hemolytic uremic syndrome (Janot *et al.*, 2009), which is a clinical pathological triad consisting of impaired renal functions, microangiopathic hemolytic anaemia and thrombocytopenia (Serna & Boedeker, 2008). HUS causes high fatalities among patients (up to 50% in some outbreaks) (Griffin & Tauxe, 1991a), while a significant percentage of survivors (approximately 30%) suffer a range of permanent disabilities including renal insufficiency, hypertension and neurological deficits (Serna & Boedeker, 2008).

The production of shiga toxins by *E. coli* was first recognized in the late 1970's, when Konowalchuk et al. (Konowalchuk *et al.*, 1977) found that culture filtrates of *E. coli* strains, isolated from infants with diarrhea, exerted irreversible cytopathic effects on Vero cells (Konowalchuk *et al.*, 1977). These effects differed from those caused by the known diarrheagenic *E. coli* heat labile toxin. Soon after, and in 1983, O'Brien and LaVeck could purify the novel toxin from Konowalchuk prototype strain H30, and showed it has a polypeptide nature and that it is similar in structure, antigenicity and biological activity to shiga toxins produced by *Shigella dysenteriae* type 1 (O'Brien & Laveck, 1983). Therefore, they designated the toxin "shiga-like toxin". However, the same group showed that this shiga-like toxin is the same toxin that killed Vero cells and was previously designated "vero cytotoxin" (O'Brien *et al.*, 1983). It was also demonstrated that different types of these toxins existed. Boths terms, Shiga toxin (Stx) and Vero cytotoxin (VT), are interchangeably used.

The clinical association of EHEC with human disease was recognized for the first time in the early 1980's, when reports by Riley et al. (1983) and Karmali et al.

(1983) linked the bacteria to HC and HUS, respectively, diseases of unknown aetiology hitherto. Riley et al. (1983) investigated two outbreaks of HC, which occurred in Oregon and Michigan, USA and found a significant link between the disease, the consumption of undercooked beef from a fast-food chain, and a "rare" *E. coli* serotype, O157:H7. Similarly, Karmali et al. (1983) established a link between various EHEC serotypes, including serotype O157:H7, and sporadic cases of HUS. This link was based on the detection of Stx in stool of all examined HUS patients and the presence of significant antibody responses against Stx in the serum of several of them. These findings were confirmed by Karmali et al. in a later study (Karmali *et al.*, 1985), which has led them to speculate a direct role of Stx in the pathogenesis of HUS.

3.2. Transmission

Various animal species have been reported as reservoirs for EHEC. However, ruminants, particularly cattle, are the main reservoir associated with human infections. Humans typically acquire the infection through the ingestion of meat and dairy products, vegetables and water, which are contaminated with ruminant faeces, in addition to direct contact with animal reservoirs and contaminated objects (Crump *et al.*, 2002; Grant *et al.*, 2008; Swerdlow *et al.*, 1992; Varma *et al.*, 2003). Human fecal contamination of food and water could also play a role in transmission, especially in developing countries. Person-to-person contact seems to play a major role in transmission of EHEC, since up to 20 % of EHEC outbreaks are believed to occur due to secondary transmission rather than to the exposure to the original contaminated source (Snedeker *et al.*, 2009).

3.3. Serotypes and incidence

Over 130 EHEC serotypes have been isolated from humans with diseases, and many of these serotypes have been recovered from animals (Blanco *et al.*, 2003). However, only a limited number of serotypes have been responsible for the majority of human cases. These include the members of the O serogroups 26, 91, 103, 111, 113, 121, 145 and 157 (Karmali *et al.*, 2003). Among these serotypes, *E. coli* O157:H7, the first reported EHEC serotype, has been most commonly associated with severe outbreaks as well as sporadic cases of EHEC infections worldwide (Griffin & Tauxe, 1991b). Recent surveillance data from the US FoodNet (2013) estimate 1.15 infections

per 100, 000 population for the O157 serotype, which is only slightly lower than that of non-O157 serotypes collectively (1.17) (Crim *et al.*, 2014). In Europe, the incidence of EHEC infections has markedly increased in 2011 compared to years from 2008-2010, but this was mainly due to the inclusion of a large outbreak of O104:H4 that occurred in Germany (2.3 per 100, 000 population). However, O104:H4 was an enteroaggregative strain which gained the capacity to produce Stx. The most recent report of the European Food Safety Authority (EFSA) indicates an incidence of 1.15 infections per 100, 000 population, with O157 serotype being the most prevalent (41%). With a particular regard to Belgium, the report estimates incidences of 65 infections per year for the O:157 serotype versus 22 infections with non-O157 serotypes.

3.4. Virulence factors

The pathogenicity induced by EHEC involves two major processes; the intestinal colonization, and the induction of HC and HUS. Therefore, EHEC combines two main types of virulence factors, which are directly responsible for these processes.

3.4.1. Colonization factors

In order to colonize the intestine, EHEC establish an intimate tight adherence to enterocyte, giving rise to a characteristic lesion, known as "attaching and effacing" A/E lesion (Frankel *et al.*, 1998; Kaper *et al.*, 2004; Nataro & Kaper, 1998). This process involves drastic morphological and cytoskeletal changes to enterocytes including the notable loss of enterocyte microvilli (effacement) and the organized accumulation of cytoskeletal actin filaments beneath adherent bacteria, forming what is called the "actin pedestal" (Donnenberg *et al.*, 1997). The virulence factors involved in the formation of A/E lesion are typically categorized on basis of the genetic elements encoding them, and thus can be described as follows:

3.4.2. The locus of enterocyte effacement (LEE)

LEE is a large well-known pathogenicity island (42 kb) that encodes some adhesion factors and a type III secretion system, which are responsible for the formation of the A/E lesion (Frankel *et al.*, 1998). This locus consists of three segments, encoding five operons. The first segment includes the LEE1, LEE2 and LEE3 operons, which contain the genes encoding for the majority of the structural

and chaperon proteins of the T3SS. The second segment contains operon LEE5, which encodes genes of an adherence system consisting of an outer membrane protein, intimin, and its receptor, translocated intimin receptor (Tir). The third segment contains LEE4, which encodes for *E. coli* secreted proteins (Esp) such as EspA, EspB and EspD (Frankel *et al.*, 1998). Together, these secreted proteins forms a complex (EspABD complex), which constitute the translocation apparatus of the T3SS. Nevertheless, some of its members, particularly EspB, are effector proteins themselves, playing a role in mediating enterocyte cytoskeletal changes (discussed below).

3.4.3. Non-LEE pathogenicity islands and plasmid O157

Several EHEC flagellins and fimbrial and outer membrane adhesins encoded on non-LEE pathogenicity islands (mainly the O-islands) have been identified and might significantly contribute to the adherence of the bacteria to enterocytes (Perna *et al.*, 2001). These mainly include the H7, H10 and H21 flagellins (Rogers *et al.*, 2006), the EHEC factor for adherence (Efa1) (Nicholls *et al.*, 2000; Stevens *et al.*, 2002), the outer membrane proteins, Iha (Nicholls *et al.*, 2000) and A (OmpA) (Torres & Kaper, 2003), long polar fimbria variant 1 (Lpf1) (Doughty *et al.*, 2002; Torres *et al.*, 2002; Torres *et al.*, 2004) and the hemorrhagic coli pilus adhesin, HCP (Ledesma *et al.*, 2010). Plasmid O157 is another genetic element that is believed to play a role in EHEC virulence (Levine *et al.*, 1987; Ostroff *et al.*, 1989; Ratnam *et al.*, 1988). The complete pO157 sequence, which has been determined (Burland *et al.*, 1998), has led to the identification of some factors that were suggested to play a role in adherence (e.g. an outer membrane adhesin, ToxB) (Tatarczak *et al.*, 2005; Tatsuno *et al.*, 2001; Toma *et al.*, 2004). However, there have been conflicting *in vitro* and *in vivo* findings with regard to the role of pO157 in adherence to epithelial cells (Fratamico *et al.*, 1993; Karch *et al.*, 1987; Nataro & Kaper, 1998; Tzipori *et al.*, 1987).

3.4.4. Shiga toxins (Stx)

EHEC produce one or more of Stx, whose prototype is produced by *Shigella dysenteriae* type 1 (Strockbine *et al.*, 1986; Takao *et al.*, 1988). Stx1 is almost identical to the latter, differing only in one amino acid, whereas Stx2 shares a 56% of homology with Stx1, and is antigenetically distinct. A number of Stx2 variants have been identified, among which, Stx2c and Stx2d are associated with HC and HUS

(Karch *et al.*, 2006). These variants are encoded as a single transcriptional unit by bacteriophages. Stx molecules have an A1B5 structure, in which one enzymatic active A subunit is linked to a pentamer of receptor binding subunits (B subunits) (Fraser *et al.*, 1994; Stein *et al.*, 1992). The A subunit possesses a RNA N-glycosidase activity that enables the toxin to cleave a specific adenine base (A_{4324}) from the 28 S rRNA of the 60S ribosome (Endo *et al.*, 1988). This process prevents the binding of elongation factor I-dependent aminoacyl-tRNA, inhibits protein synthesis, and induces cell apoptosis as a ribonucleic stress response (Smith *et al.*, 2003) or due to Stx related signaling (Cherla *et al.*, 2003). The B subunits enable the toxin to bind to specific glycolipid receptor, globotariosylcermide (Gb3), which is present on the cell membrane of epithelial and endothelial cells. The toxin binding to Gb3 permits the internalization of STs into target host cell by receptor-mediated endocytosis (Lingwood, 1994; Waddell *et al.*, 1996). Thereafter Stx are transported into a vesicle to the Golgi apparatus and the endoplasmic reticulum by retrograde transport (Sandvig & van Deurs, 2002). In the Golgi apparatus, the A fragment is cleaved by the enzyme furin into two fragments, A1 and A2, which remain covalently linked by a disulphide bond. The latter is thought to be reduced in the endoplasmic reticulum. Then the A1 fragment is transported to the cytoplasm to target the rRNA as described above (O'Loughlin & Robins-Browne, 2001).

Although epithelial cytotoxicity is the main feature of Stx pathogenicity, it is noteworthy to mention the toxin was also found to play a role in adherence of EHEC to enterocytes. Indeed, Stx2 were shown to induce localization of nucleolin in Hep-2 cells (Robinson *et al.*, 2006). Nucleolin is a cell surface molecule which binds to intimin (Sinclair & O'Brien, 2002) before Tir is translocated into the cell membrane.

3.5. Pathogenicity

3.5.1. The A/E lesion

Enterocyte adherence and the formation of the A/E lesion are important characteristics of EHEC pathogenicity. These processes involve three typical stages, which can be described as follows:

3.5.1.1. Initial non-intimate adherence

At first EHEC establish a non-intimate adherence to enterocytes. The mechanisms and the particular virulence factors involved in this process are poorly characterized. However, a role of the H7 flagellin in primary adherence of O157:H7 to epithelial cells have been reported (Mahajan *et al.*, 2009). Other factors such as EfaI, Iha, OmpA and ToxB have been shown to promote adherence of the EHEC to epithelial cell lines and thus, might play a role in early adhesion. Additionally, factors such as Lpf1, have been suggested to be particularly involved in the process of the early adherence (Torres *et al.*, 2002; Torres *et al.*, 2004). However, these speculations require further confirmation.

3.5.1.2. Signal transduction

Following the early adherence, EHEC induces a series of structural and morphological changes in the host cells in order to establish a firm adherence. These changes occur with the aid of the LEE-encoded T3SS, which mediates a contact-dependent translocation of effector proteins into the host cell (Campellone & Leong, 2003; Tobe *et al.*, 2006) (Figure 3.1). The translocated effector proteins induce a cell signalling cascade leading to the formation of the actin pedestal and Tir, which is essential for the establishment of intimate adherence. To translocate effector proteins, EspA forms a needle-like filamentous structure which extends to the host cell membrane (Ebel *et al.*, 1998; Knutton *et al.*, 1998). At the tip of this molecular needle the translocon proteins EspB and EspD, which form a pore in the cell membrane (Ide *et al.*, 2001), through which the translocated proteins are delivered. Tir is one of the first translocated proteins, which is directly injected into the host cell membrane, taking a hairpin loop confirmation that allows its extracellular domain to bind with intimin on the bacterial outer membrane.

EspB is a particular T3SS protein that in addition to its role in translocating other effector proteins plays a major effector role in the formation of the A/E lesion. It binds to host proteins such as α-catenin (Kodama *et al.*, 2002), α1-antitrypsin (Knappstein *et al.*, 2004b) and myosin (Lizumi *et al.*, 2007) in order to regulate the host-cell actin networks, resulting in the formation of the actin pedestal structure and effacement of enterocytes' microvilli (Kodama *et al.*, 2002).

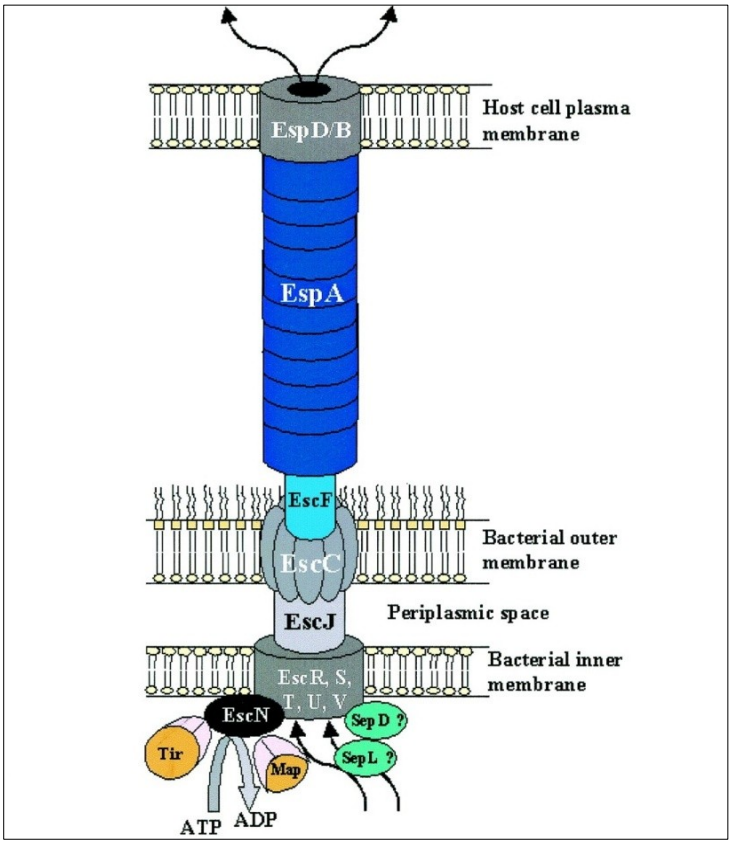

Figure 3.1. Schematic representation of EHEC type 3 secretion apparatus. EspA subunits polymerize to form the needle-like EspA filament. EspB and EspD form the translocation pore in the host cell plasma membrane, connecting the bacteria with enterocyte cell membrane via EspA. Adapted from (Garmendia *et al.*, 2005).

Other translocated effector proteins include EspG, EspF, EspH and MAP (mitochondrial associated protein), which are believed to interfere with host cell signalling (Ritchie & Waldor, 2005). Noteworthy, the T3SS was also found to

translocate other non-LEE encoded effector proteins such as NleH, which alters the transcription process in host cells (Gao *et al.*, 2009b).

3.5.1.3. Intimate adherence

The binding of intimin to its translocated receptor Tir on the cell membrane constitutes the firm attachment of EHEC to enterocytes, which is morphologically characterized by effacement of the microvilli. Additionally, although the role of the cytoskeletal arrangements and actin pedestal formation is hitherto speculative, the establishment of firm anchorage of the bacteria is an obvious possibility.

3.5.2. Inflammation

Inflammation is a prominent feature of the intestinal lesion caused by EHEC. Adhesins such as the HCP and the flagellins (H7, H10, and H21) induce local cytokine production (such as IL8, TNF-alpha, or CCL20) within the intestine (Gobert *et al.*, 2008; Ledesma *et al.*, 2010) or attract subepithelial neutrophils (de Boer *et al.*, 2000) and as a consequence, cause localized inflammation. Additionally, bacteria that produce A/E lesions induce IL22, a cytokine that may increase intestinal damage (Zheng *et al.*, 2008). A possible role of intestinal inflammation in EHEC pathogenicity is to impair the intestinal barrier function, thereby facilitating the passage of Stx to the submucosa. The inflammatory responses extend beyond the intestine to other organs, such as the kidneys. HUS patients have been shown to have high urinary level of IL-8 and monocytes chemoattractant 1 (de Boer *et al.*, 2000). This can be attributed to the role of Stx and EHEC lipopolysaccharides (LPS), which were shown to stimulate microvascular endothelial cells to release these factors (Stahl *et al.*, 2006). Furthermore, binding to and transport of Stx by granulocytes is thought to be responsible for the inflammatory responses and transfer of the toxin to organs such as the kidneys (te Loo *et al.*, 2000).

3.5.3. Vascular injury

After their release, Stx cross the intestinal barrier in order to reach the circulation. The mechanisms of such translocation are different for Stx1 and Stx2, which follow transepithelial and paracellular routes, respectively (Acheson *et al.*, 1996; Hurley *et al.*, 1999; Philpott *et al.*, 1997). In the blood stream, Stx are carried to their Gb3 receptors by polymorphonuclear cells, particularly neutrophils (Brigotti *et*

al., 2010). Gb3 receptors are abundantly expressed by endothelial cells of the kidneys, colon and brain, organs where Stx mainly induce endothelial damage and vascular injury. However, renal glomerular and tubular vessels are considered the main target (Obata *et al.*, 2008; Obrig *et al.*, 1993; Richardson *et al.*, 1992). The vascular injury caused by Stx also involves a pathologic lesion, defined as thrombotic microangiopathy. This lesion is characterized by endothelial cell swelling and detachment from the basement membrane, and thrombosis of the microvasculature of the colon and the kidneys (Richardson *et al.*, 1988). Shiga toxin upregulates the expression of adhesion molecules (e.g. E-selectin) on the endothelial cell surface, and facilitates adherence and subsequent degranulation of neutrophils (Morigi *et al.*, 1995). Degranulated neutrophils release elastase, which can disrupt the extracellular matrix resulting in endothelial cell detachment. Stx also increase cell-surface expression of P-selectin, and both induce the release and prevent the cleavage of the ultra-large von Willebrand factor, and thereby the toxins promote platelet adhesion to the endothelial cell surface, resulting in thrombosis (Morigi *et al.*, 2001; Nolasco *et al.*, 2005). LPS has also been shown to play a role in thrombus formation in HUS patients. They were detectable on platelets surface of preHUS patients before HUS is developed, and additionally, O157 LPS was shown to bind to and activate human platelets. Providing that LPS can also bind to endothelial cells, the former might play a role in thrombus formation by mediating the platelets-endothelial adhesion (Stahl *et al.*, 2006).

3.6. Pathophysiology and clinical presentation of EHEC diseases

The establishment of EHEC disease is thought to require the ingestion of a relatively low dose of the bacteria, ranging from 50 to few hundred organisms (Tilden *et al.*, 1996). However, recent analyses of outbreaks have shown that the dose necessary to cause disease depends on the EHEC strain involved, the matrix in which the bacterium is present when ingested and the host susceptibility (Teunis *et al.*, 2008). Following ingestion, EHEC is well-adapted to endure gastric acidity, which enables the majority of them to reach the intestine. Furthermore, the stimulus resulting from the exposure of EHEC to the acidic pH in the stomach has been shown to induce the upregulation of intimin and T3SS protein expression, which prepares the bacteria for intestinal colonization (House *et al.*, 2009).

As the bacteria reach the intestine, they immediately initiate the colonization process. In most EHEC infections an average incubation period of 3 days, is required before the onset of clinical signs, but the period can vary from 2 to 13 days (Bell *et al.*, 1994). Thereafter, and as a result of enterocyte adherence and the accompanying mucosal inflammation, fluid absorption in the large intestine is disrupted, resulting in non-bloody diarrhea and severe abdominal pain. These symptoms are typically encountered during the first 1 to 3 days of illness and can be accompanied by vomiting (Tarr *et al.*, 2005). Transient fever has also been reported in up to 50% of patients. It usually resolves as the patient is presented to the medical care.

After intestinal colonization, EHEC release Stx, which disseminate through the blood circulation and reach the colon and the kidneys to induce microvascular injuries. The latter is the proposed mechanism leading to bloody diarrhea and the acute renal injury of HUS during EHEC infections. About 10–15% of patients infected with *E. coli* O157, mostly children younger than 5 years old (Garg *et al.*, 2008), develop haemolytic uraemic syndrome 5–13 days after the onset of diarrhea (Scheiring *et al.*, 2008; Tarr *et al.*, 2005). HUS patients are often clinically presented with microangiopathic hemolytic anaemia due to lysis of RBCs (haemoglobin < 10 g/dL) flowing in damaged microvasculature, thrombocytopenia (platelet count < 15×10^9 cells/L) due to excessive platelet activation and precipitation in the damaged vessels and impaired renal function (oliguria or anuria and high concentrations of serum urea and creatinine) due to renal glomerular and tubular vascular damage (Michael *et al.*, 2009). The brain involvement can be a major complication of HUS and may manifest clinically as seizures, coma, and/or dysregulated breathing (Theobald *et al.*, 2001).

3.7. Animal models of EHEC infection

The available knowledge of the pathogenesis of EHEC-mediated diseases in humans is to a great extent limited. Additionally, due to the possible risks that a human volunteer develops HUS, the use of human subjects to study the pathogenicity of EHEC or to evaluate new therapies and vaccines is ethically unacceptable. Therefore, numerous animal models have been developed for these essential purposes. However, studies have evidently shown that not a single animal model is valid to recapitulate the full aspects of EHEC pathogenicity in humans (Mohawk & O'Brien, 2011a). Alternatively, all the available animal models are chosen

based on the desirable study endpoint. Generally, models exist in two main varieties: those reproducing the effects of Stx (in the absence of bacteria) and those that explore the intestinal infection. Models employed to evaluate toxicity rely on injection of Stx, with or without LPS, and often select mortality as a study endpoint (Armstrong *et al.*, 2006; Bentancor *et al.*, 2009; Sauter *et al.*, 2008; Sheoran *et al.*, 2005). However, for the purpose of this thesis, the models reviewed under this section are those essentially mimicking the intestinal colonization and pathology, whether or not showing other aspects of systemic pathogenicity.

3.7.1. Mouse models

As a small animal species, the mouse particularly offers a number of benefits including: relatively low costs of purchase and maintenance, ease of care and handling, availability of numerous immunological reagents, variations in genetic backgrounds among inbred strains, and very importantly, the feasibility of using sufficient numbers of animals in a single study, which allows drawing statistically supported conclusions from the obtained data.

Although in a variety of animal models, EHEC was shown to colonize mice under physiological intestinal conditions (Brando *et al.*, 2008; Conlan & Perry, 1998b; Karpman *et al.*, 1997a; Nagano *et al.*, 2003a), many other researchers adopted various manipulations to enhance EHEC colonization and pathogenicity (Table 3.1). They included strategies that: (1) reduce the facultative commensal flora, to decrease the bacterial competition for the colonizing EHEC (streptomycin treatment prior and during the study) (Lindgren *et al.*, 1993; Wadolkowski *et al.*, 1990b); (2) reduce the bacterial competition and induce phage expression to increase the toxin expression by the bacteria (e.g. by incorporating mitomycin C with a streptomycin treatment) (Fujii *et al.*, 1994; Shimizu *et al.*, 2003; Teel *et al.*, 2002); (3) adopt anexic (germ-free) mice, strictly lacking other competing intestinal bacteria (Aiba *et al.*, 2002; Jeon & Itoh, 2007; Sawamura *et al.*, 1999); (4) induce an underdevelopment of enterocytes to increase predisposition of mice to EHEC colonization (by adoption of low protein-calorie diet) (Kurioka *et al.*, 1998). Whereas high doses of the bacteria were occasionally used to achieve sufficient levels of intestinal colonization and disease in these models, particularly low protein-calorie malnutrition and anexic mice models required lower doses of EHEC to induce the same pathology (Aiba *et al.*, 2002; Jeon & Itoh, 2007; Kurioka *et al.*, 1998; Sawamura *et al.*, 1999).

The influence of mouse strain variation on the capacity of EHEC to colonize the gut has also been studied. Comparing BALB/c (inbred), C57BL/6 (inbred) and CD-1 (or ICR) (outbred) mouse strains for colonization by *E. coli* O157:H, no differences in duration of shedding was observed. Bacterial fecal shedding generally lasted for 1 to 2 weeks (Conlan & Perry, 1998a). However, in another study DC-1 mice remained infected till the end of the study period (28 days), in contrast to BALB/c, C3H/HeN, C3H/HeJ, and A/J mice which stopped shedding one week after the infection (Nagano *et al.*, 2003b). Noteworthy that BALB/c mice showed resistance to reinfection (shorter duration of fecal shedding compared to C57BL/6 (inbred) and CD-1 (outbred) strains) (Conlan & Perry, 1998a). This observation was coupled to the presence of O157-specific IgA (both serum and fecal). This makes BALB/c mice a potential candidate model for EHEC oral vaccination studies (Conlan & Perry, 1998a).

Table 3.1. Examples of commonly used mice models of EHEC intestinal colonization. Adapted from (Mohawk & O'Brien, 2011b).

Model	Mouse strains	Inoculum (CFU)	Inoculation method	Outcomes	Pathology	References
Conventional	C3H/HeN, C3H/HeJ	107, 108	Intragastric	Morbidity, mortality	Kidney, intestine	Karpman *et al.*, 1997
	CD1, BALB/c, C57BL/6	~ 2×10^{10}	Intragastric	Colonization	-	Conlan & Perry, 1998a
	ICR (CD-1)	10^{11}/kg body weight	Intragastric	Colonization	-	Nagano *et al.*, 2003b
	BALB/c	6×10^{9}/kg	Intragastric	Morbidity and mortality	Kidney, intestine	Brando *et al.*, 2008
Streptomycin-treated	CD-1	10^{10}	Feeding	Colonization, morbidity, mortality	Kidney	Wadolkowski *et al.*, 1990a
	CD-1	$\leq 10^{1}$	Feeding	Colonization, morbidity, mortality	Kidney	Lindgren *et al.*, 1993
Mitomycin C and streptomycin	ICR	$> 10^{9}$	Intragastric	Colonization, morbidity, mortality	Kidney, brain	Fujii *et al.*, 1994

Protein-calorie malnutrition	C57BL/6	$2 \times 10^5 - 2 \times 10^7$	Intragastric	Colonization, morbidity, mortality	Kidney, intestine, brain	Kurioka *et al.*, 1998
Germ-free	IQI	5×10^7	Intragastric	Colonization, morbidity, Mortality with hypertoxigenic strain	Kidney, intestine, brain	Taguchi *et al.*, 2002
	Swiss-Webster	10^2–10^6	Intragastric	Colonization, morbidity, mortality	Kidney	Eaton *et al.*, 2008

3.7.2. Pig models

Pigs are a particularly interesting animal species that offers a good model for humans in studies of gastrointestinal physiology, pathology, infection and immunity (Meurens *et al.*, 2012). Indeed, pigs share various aspects of gastrointestinal physiology and mucosal immunology with humans, which distinguish them from other animals as a model for human gastrointestinal infections. Additionally, several enteropathogens can commonly infect pigs and humans. Gnotobiotic piglets can be infected with EHEC strains (Francis *et al.*, 1986; Tzipori *et al.*, 1986; Tzipori *et al.*, 1989; Tzipori *et al.*, 2004), and show a pathogenesis that closely mimics that occurring in humans. In this model, the EHEC infection caused both intestinal lesions and a watery diarrhea. In fact, the A/E lesions evoked by *E. coli* O157:H7 were first described *in vivo* in gnotobiotic piglets (Francis *et al.*, 1986). Renal damage and neurological manifestations are also features of this model, and fatality from systemic complications may occur. The gnotobiotic piglet model is quite well established and has been applied by several groups for the study of *E. coli* O157:H7 pathogenesis as well as in preclinical evaluation of EHEC therapeutics (Tzipori *et al.*, 1988).

3.8. Vaccination against EHEC infection

Unlike the majority of other bacterial diseases, previous clinical studies did not demonstrate beneficial effects of antibiotic therapy on the outcome of EHEC infection (Bell *et al.*, 1997; Ryan *et al.*, 1986). Moreover, several retrospective studies have linked antibiotic therapy to higher rates of HUS development, a prolonged period of symptomatic disease, and a prolonged bacterial shedding (Carter *et al.*, 1987; Pavia *et al.*, 1990; Wong *et al.*, 2000). These outcomes can be attributed to the undesirable release of Stx by destroyed bacterial cells (Wong *et al.*, 2000). Additionally, *in vitro* subinhibitory concentrations of antibiotics may increase production and release of Stx via bacteriophage induction (Zhang *et al.*, 2000). However, animal studies, involving mice (Kurioka *et al.*, 1999) and pigs (Zhang *et al.*, 2009), suggested that the antibiotic fosfomycin could be used to treat human infection. However, only one non-randomized prospective study has been conducted on the use of fosfomycin for prevention of HUS (Ikeda *et al.*, 1999). This study suggested that the use of fosfomycin within the first 3 days and especially within the first two days significantly decreased the risk for HUS. However, the data obtained

have been questioned (Iijima *et al.*, 2008). Recent evidence supported the risk associated with the use of β–lactams and other bactericidal antibiotics in EHEC infections (Smith *et al.*, 2012). Considering these facts, it has been accepted that vaccination could represent the best way to control EHEC infections and prevent the disease outbreaks.

The vast majority of the preclinical vaccination studies performed to develop a human vaccine against EHEC have been performed in mouse models. These studies used several virulence factors as antigens and utilized various vaccination routes. However, there is currently no vaccine available for humans. In this section we will review the studies currently published on EHEC vaccines.

3.8.1. Subunit vaccines

3.8.1.1. Type III secretion system-based vaccines

Humans infected with EHEC show serum antibody responses against T3SS proteins such as Tir, intimin, EspB, EspA and NleA (Asper *et al.*, 2011; Li *et al.*, 2000). Also in animals experimentally infected with EHEC the proteins elicit antibody responses (Asper *et al.*, 2011). This reflects the important role of these antigens in the infection process and their high immunogenicity, making these bacterial surface located and T3SS proteins potential vaccine candidates. The induction of specific immune responses against T3SS proteins is expected to interfere with the intestinal colonization process, and thus prevents the infection.

A mixture of T3SS proteins has been evaluated as an EHEC vaccine in a mouse model using mucosal and systemic vaccination routes. Intranasal (i.n.) immunization using T3SS proteins in combination with an adjuvant resulted in serum and fecal antibody responses that could recognize Tir and EspA and that reduced EHEC fecal shedding (Babiuk *et al.*, 2008). On the other hand, intramuscular (i.m.) immunization of mice eliminated bacterial fecal shedding in the absence of fecal anti-Tir and -EspA antibodies (Babiuk *et al.*, 2008). These promising results have been the basis of a currently available cattle vaccine, commercially known as Econiche® (Potter *et al.*, 2004). The results from the study in mice did not suggest an essential role for fecal secretory IgA in clearance of *E. coli* O157:H7 infection. However, it should be noted that although a mixture of T3SS proteins had been used to immunize mice, the serum and fecal immune responses were tested against only

two antigens. Therefore, the presence of specific fecal IgA responses against other antigens cannot be excluded. In agreement, a recent study in mice using Tir as a vaccine antigen showed that i.n. immunization induced IgG and IgA antibodies in serum and faeces, respectively, reducing shedding and protecting the mice against a lethal dose of *E. coli* O157:H7 (Fan *et al.*, 2012). However, in the same study the subcutaneous (SC) route, which increased serum IgG, but not fecal IgA against Tir, did not induce protection. Similar findings were reported for the KT-12 peptide, an intimin predicted B-cell epitope (Wan *et al.*, 2011).

Intranasal immunization of mice using EspB and the C-terminus of intimin (C-280 γ -intimin), in combination with the mucosal adjuvant MALP-2 induced serum specific IgG and fecal specific IgA responses (Cataldi *et al.*, 2008). These responses were associated with mixed Th1/Th2 cytokine responses as IFN γ -, IL-2- and IL-4-expressing lymphocytes were obtained from EspB- or C280 γ -intimin-vaccinated mice (Cataldi *et al.*, 2008). Oral immunization of mice using intimin-derived vaccine delivered using non-pathogenic carrier strain of *Salmonella enterica* Typhimurium induced specific serum IgG and fecal IgA antibodies and reduced EHEC shedding after challenge (Oliveira *et al.*, 2012).

3.8.1.2. Stx-based vaccines

Since Stx are the main virulence factor of EHEC pathogenicity, vaccination studies aim to induce an immunity which can prevent intoxication. Early work in mice and pigs demonstrated that parenteral vaccination with inactivated Stx effectively induced neutralizing antibodies and in some cases, protection against toxemia (Ishikawa *et al.*, 2003; Marcato *et al.*, 2001; Marcato *et al.*, 2005; Oanh *et al.*, 2012). Also, passive immunization of mice with human or murine antibodies raised against Stx inhibited their activity and resulted in protection (Akiyoshi *et al.*, 2005; Jeong *et al.*, 2010; Mohawk *et al.*, 2010)

Although Stx2 are more associated with human disease than Stx1, the induction of immune responses against both Stx is considered an important criterion of a good vaccine. In this regard, recent studies evaluated hybrid toxins via different immunization routes as vaccine candidates. A hybrid toxoid consisting of an inactivated mutant Stx2A subunit and a native Stx2B subunit induced neutralizing serum antibodies against both Stx in mice that were immunized intraperitoneally

(i.p.) and also protected them from challenge with either toxin types (Smith *et al.*, 2006). Similarly, i.n. immunization of mice with the Stx2B subunit induced systemic IgG and mucosal IgA antibodies, which protected mice against not only Stx2, but also Stx1 (B-subunit cross protection). On the other hand, mice immunized with Stx1B showed protection only against Stx1 (Tsuji *et al.*, 2008c). A fusion protein comprising the B subunits of the both Stx types induced antibodies, which protected mice against a challenge with a lysate of *E. coli* O157:H7 (Gao *et al.*, 2009a). Noteworthy, the antibody responses and the protective effect induced by the fusion construct were higher for Stx2 compared to vaccination with a mixture of non-fused Stx1B and Stx2B. This was thought to be due to an adjuvant effect of the fused Stx1B subunit (Gao *et al.*, 2009a). Furthermore, a derivative of the fusion protein containing an enzymatically inactive Stx2A subunit instead of the Stx2B elicited higher levels of Stx2 neutralizing antibodies and a significantly higher level of protection against a lethal dose of *E. coli* O157:H7 lysate (Cai *et al.*, 2011).

The above studies mainly involved systemic vaccination routes. However, another study has also explored the oral route. Oral immunization of mice with a *Salmonella enterica* strain expressing a mutant Stx2 toxoid induced anti-Stx2B IgG antibodies. Although the anti-toxoid antibodies could neutralize Stx2 *in vitro*, only a limited protection was afforded against an intravenous challenge with Stx2 in mice (Rojas *et al.*, 2010).

3.8.1.3. Subunit fusion vaccines

To improve the efficacy of EHEC vaccines, protein fusion vaccines have been developed combining subunits of T3SS proteins and Stx to achieve multivalent protective effects. A prominent example is the EIS (EspA-Intimin-Stx) fusion, comprising EspA, the C-terminal 300 residues of intimin and the Stx2B subunit. The fusion vaccine was shown to induce specific humoral antibodies against the three antigens and protected mice against EHEC challenge more efficiently than using each of the three antigens individually (Rojas *et al.*, 2010). Further, oral inoculation of mice with a live-attenuated *Salmonella* strain expressing EIS produced specific IgA against EspA, intimin and ST2B (Gu *et al.*, 2011). This immune response was long lasting, since even after 70 days it could be boosted subcutaneously and produced significant protection against a lethal challenge (Gu *et al.*, 2011).

The subcutaneous immunization with a fusion protein consisting of the C-terminal 120 residues of EspA, the C-terminal 282 amino acids of intimin and the Tir site for intimin interaction (EIT fusion), reduced the duration of EHEC shedding. This effect correlated with the generation of specific IgG antibodies, while no specific IgA was detected (Gu *et al.*, 2011). However, specific serum IgG and fecal IgA are induced by a plant-based EIT fusion protein when it is orally delivered. This induction correlated with reduction in EHEC shedding (Amani *et al.*, 2011).

Other fused antigens evaluated in mice include a fusion protein comprising B subunits of the two types of Stx, Tir and the zonula occludens toxin of the *Vibrio cholerae* CTX ϕ bacteriophage, which improves antigen delivery by increasing epithelial permeability (Zhang *et al.*, 2011b). Subcutaneous immunization with the ST2B-Tir-ST1B-Zot fusion protein produced antigen-specific systemic IgG antibodies, while i.n. immunization elicited both serum IgG and IgA. Both immunization routes decreased bacterial shedding after challenge. In another study, an SSI fusion protein, consisting of Stx2B, Stx1B and an intimin fragment, protected mice against large doses of EHEC (Gao *et al.*, 2011). Finally, a fusion of the A1 peptide of Stx2 to EspA was shown to increase mice survival after intravenous challenge with a lethal dose of Stx2 (Cheng *et al.*, 2009).

3.8.2. Bacterial cell-based vaccines

Traditional vaccine approaches using attenuated or killed bacteria are still evaluated for protection against EHEC infection. EHEC strain 86-24 *ler*/*stx* deletion mutant as well as its derivative expressing inactive Stx1 and Stx2 were shown to reduce the bacterial shedding in mice (Liu *et al.*, 2009).

Since attenuated pathogenic microorganisms can be associated with risks of reverted pathogenicity and side effects, a technology based on bacterial cells devoid of their cytoplasmic and nucleic acid contents, has been investigated (Cai *et al.*, 2010; Mayr *et al.*, 2005; Mayr *et al.*, 2012). The bacterial ghosts retains intact cellular morphology and bacterial surface structures and thus, are highly immunogenic, possessing natural adjuvant activity. EHEC N°CIP 105282 bacterial ghosts have been produced. A single oral (Mayr *et al.*, 2005) or rectal (Mayr *et al.*, 2012) dose immunization was able to stimulate humoral and cellular immune responses and protect against a lethal EHEC challenge. Rectal immunization was sufficient to fully

protect against one LD50, whereas the protection obtained by oral immunization was significantly increased after a boost. Similar results were also obtained for oral immunization of mice using EHEC EDL933 bacterial ghosts (Cai *et al.*, 2010), where protection increased after a boost. Bacterial ghosts vaccination induced specific IgG and IgA in serum and colon, accompanied by a mixed Th1/Th2 cytokine response, with Th-2 phenotype dominance (Vilte *et al.*, 2012).

4. References

A

Acheson, D. W., Moore, R., De Breucker, S., Lincicome, L., Jacewicz, M., Skutelsky, E. & Keusch, G. T. (1996). Translocation of Shiga toxin across polarized intestinal cells in tissue culture. *Infection and immunity* **64**, 3294-3300.

Agace, W. (2010). Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunology letters* **128**, 21-23.

Aiba, Y., Ishikawa, H., Shimizu, K., Noda, S., Kitada, Y., Sasaki, M. & Koga, Y. (2002). Role of internalization in the pathogenicity of Shiga toxin-producing *Escherichia coli* infection in a gnotobiotic murine model. *Microbiol Immunol* **46**, 723-731.

Akiyoshi, D. E., Rich, C. M., O'Sullivan-Murphy, S., Richard, L., Dilo, J., Donohue-Rolfe, A., Sheoran, A. S., Chapman-Bonofiglio, S. & Tzipori, S. (2005). Characterization of a human monoclonal antibody against Shiga toxin 2 expressed in Chinese hamster ovary cells. *Infection and immunity* **73**, 4054-4061.

Amani, J., Mousavi, S. L., Rafati, S. & Salmanian, A. H. (2011). Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of *Escherichia coli* O157:H7 in mice. *Plant Sci* **180**, 620-627.

Andersson, H. & von Heijne, G. (1991). A 30-residue-long "export initiation domain" adjacent to the signal sequence is critical for protein translocation across the inner membrane of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 9751-9754.

Armstrong, G. D., Mulvey, G. L., Marcato, P., Griener, T. P., Kahan, M. C., Tennent, G. A., Sabin, C. A., Chart, H. & Pepys, M. B. (2006). Human serum amyloid P component protects against *Escherichia coli* O157 : H7 Shiga toxin 2 *in vivo*: Therapeutic implications for hemolytic-uremic syndrome. *Journal of Infectious Diseases* **193**, 1120-1124.

Asensi, G. F., de Sales, N. F., Dutra, F. F. & other authors (2013). Oral immunization with *Lactococcus lactis* secreting attenuated recombinant staphylococcal enterotoxin B induces a protective immune response in a murine model. *Microb Cell Fact* **12**, 32.

Asper, D. J., Karmali, M. A., Townsend, H., Rogan, D. & Potter, A. A. (2011). Serological Response of Shiga Toxin-Producing *Escherichia coli* Type III Secreted Proteins in Sera from Vaccinated Rabbits, Naturally Infected Cattle, and Humans. *Clinical and Vaccine Immunology* **18**, 1052-1057.

Attridge, S. R., Davies, R. & LaBrooy, J. T. (1997). Oral delivery of foreign antigens by attenuated *Salmonella*: Consequences of prior exposure to the vector strain. *Vaccine* **15**, 155-162.

Audouy, S. A. L., van Selm, S., van Roosmalen, M. L. & other authors (2007). Development of lactococcal GEM-based pneumococcal vaccines. *Vaccine* **25**, 2497-2506.

B

Babiuk, S., Asper, D. J., Rogan, D., Mutwiri, G. K. & Potter, A. A. (2008). Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. *Microbial pathogenesis* **45**, 7-11.

Basso, H., Rohde, M. & Guzman, C. A. (2000). Vectors to achieve selective expression of vaccine antigens within eukaryotic cells using *Salmonella* spp. as carrier strains. *Fems Microbiol Lett* **182**, 219-223.

Bell, B. P., Goldoft, M., Griffin, P. M. & other authors (1994). A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. *JAMA : the journal of the American Medical Association* **272**, 1349-1353.

Bell, B. P., Griffin, P. M., Lozano, P., Christie, D. L., Kobayashi, J. M. & Tarr, P. I. (1997). Predictors of hemolytic uremic syndrome in children during a large outbreak of *Escherichia coli* O157:H7 infections. *Pediatrics* **100**, E12.

Bentancor, L. V., Bilen, M., Brando, R. J. F., Ramos, M. V., Ferreira, L. C. S., Ghiringhelli, P. D. & Palermo, M. S. (2009). A DNA Vaccine Encoding the Enterohemorragic *Escherichia coli* Shiga-Like Toxin 2 A(2) and B Subunits Confers Protective Immunity to Shiga Toxin Challenge in the Murine Model. *Clinical and Vaccine Immunology* **16**, 712-718.

Berkowska, M. A., Driessen, G. J., Bikos, V. & other authors (2011). Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* **118**, 2150-2158.

Berlec, A., Jevnikar, Z., Majhenic, A. C., Rogelj, I. & Strukelj, B. (2006). Expression of the sweet-tasting plant protein brazzein in *Escherichia coli* and *Lactococcus lactis*: a path toward sweet lactic acid bacteria. *Applied microbiology and biotechnology* **73**, 158-165.

Berlec, A., Malovrh, T., Zadravec, P., Steyer, A., Ravnikar, M., Sabotic, J., Poljsak-Prijatelj, M. & Strukelj, B. (2013). Expression of a hepatitis A virus antigen in *Lactococcus lactis* and *Escherichia coli* and evaluation of its immunogenicity. *Applied microbiology and biotechnology* **97**, 4333-4342.

Bermudez-Humaran, L. G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R. & Corthier, G. (2003). Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. *Fems Microbiol Lett* **224**, 307-313.

Blanco, M., Blanco, J. E., Mora, A. & other authors (2003). Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. *Journal of clinical microbiology* **41**, 1351-1356.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D. & Sorokin, A. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* **11**, 731-753.

Boyaka, P. N., Marinaro, M., Jackson, R. J., Menon, S., Kiyono, H., Jirillo, E. & McGhee, J. R. (1999). IL-12 is an effective adjuvant for induction of mucosal immunity. *Journal of immunology* **162**, 122-128.

Braat, H., Rottiers, P., Hommes, D. W. & other authors (2006). A phase I trial with Transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol H* **4**, 754-759.

Bracci, L., Canini, I., Puzelli, S., Sestili, P., Venditti, M., Spada, M., Donatelli, I., Belardelli, F. & Proietti, E. (2005). Type I IFN is a powerful mucosal adjuvant for a selective intranasal vaccination against influenza virus in mice and affects antigen capture at mucosal level. *Vaccine* **23**, 2994-3004.

Brando, R. J., Miliwebsky, E., Bentancor, L. & other authors (2008). Renal damage and death in weaned mice after oral infection with Shiga toxin 2-producing *Escherichia coli* strains. *Clinical and experimental immunology* **153**, 297-306.

Brandtzaeg, P. & Pabst, R. (2004). Let's go mucosal: communication on slippery ground. *Trends Immunol* **25**, 570-577.

Brandtzaeg, P. (2007). Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* **25**, 5467-5484.

Brandtzaeg, P. (2013). Secretory IgA: Designed for Anti-Microbial Defense. *Frontiers in immunology* **4**, 222.

Brave, A., Ljungberg, K., Wahren, B. & Liu, M. A. (2007). Vaccine delivery methods using viral vectors. *Mol Pharmaceut* **4**, 18-32.

Brehm, J., Salmond, G. & Minton, N. (1987). Sequence of the Adenine Methylase Gene of the *Streptococcus Faecalis* Plasmid Pambeta-1. *Nucleic Acids Res* **15**, 3177-3177.

Brigotti, M., Tazzari, P. L., Ravanelli, E. & other authors (2010). Endothelial damage induced by Shiga toxins delivered by neutrophils during transmigration. *Journal of leukocyte biology* **88**, 201-210.

Bruhn, K. W., Craft, N. & Miller, J. F. (2007). *Listeria* as a vaccine vector. *Microbes and Infection* **9**, 1226-1235.

Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J. & Blattner, F. R. (1998). The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157 : H7. *Nucleic Acids Res* **26**, 4196-4204.

C

Cai, K., Gao, X. A., Li, T. & other authors (2010). Intragastric immunization of mice with enterohemorrhagic *Escherichia coli* O157:H7 bacterial ghosts reduces mortality and shedding and induces a Th2-type dominated mixed immune response. *Can J Microbiol* **56**, 389-398.

Cai, K., Gao, X. A., Li, T. & other authors (2011). Enhanced immunogenicity of a novel Stx2Am-Stx1B fusion protein in a mice model of enterohemorrhagic *Escherichia coli* O157:H7 infection. *Vaccine* **29**, 946-952.

Campbell, D. J. & Butcher, E. C. (2002). Rapid acquisition of tissue-specific homing phenotypes by $CD4^+$ T cells activated in cutaneous or mucosal lymphoid tissues. *The Journal of experimental medicine* **195**, 135-141.

Campellone, K. G. & Leong, J. M. (2003). Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157 : H7. *Curr Opin Microbiol* **6**, 82-90.

Cao, H. P., Wang, H. N., Yang, X., Zhang, A. Y., Li, X., Ding, M. D., Liu, S. T., Zhang, Z. K. & Yang, F. (2013). *Lactococcus lactis* anchoring avian infectious bronchitis virus multi-epitope peptide EpiC induced specific immune responses in chickens. *Bioscience, biotechnology, and biochemistry* **77**, 1499-1504.

Carleton, H. A. (2010). Pathogenic bacteria as vaccine vectors: teaching old bugs new tricks. *The Yale journal of biology and medicine* **83**, 217-222.

Carter, A. O., Borczyk, A. A., Carlson, J. A., Harvey, B., Hockin, J. C., Karmali, M. A., Krishnan, C., Korn, D. A. & Lior, H. (1987). A severe outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home. *The New England journal of medicine* **317**, 1496-1500.

Casalta, E. & Montel, M. C. (2008). Safety assessment of dairy microorganisms: The *Lactococcus* genus. *International journal of food microbiology* **126**, 271-273.

Cataldi, A., Yevsa, T., Vilte, D. A., Schulze, K., Castro-Parodi, M., Larzabal, M., Ibarra, C., Mercado, E. C. & Guzman, C. A. (2008). Efficient immune responses against Intimin and EspB of enterohaernorragic *Escherichia coli* after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. *Vaccine* **26**, 5662-5667.

Cauchard, S., Bermudez-Humaran, L. G., Blugeon, S., Laugier, C., Langella, P. & Cauchard, J. (2011). Mucosal co-immunization of mice with recombinant lactococci secreting VapA antigen and leptin elicits a protective immune response against *Rhodococcus equi* infection. *Vaccine* **30**, 95-102.

Cerutti, A., Zan, H., Schaffer, A., Bergsagel, L., Harindranath, N., Max, E. E. & Casali, P. (1998). CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM^+IgD^+ B cell line. *Journal of immunology* **160**, 2145-2157.

Cesta, M. F. (2006). Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicologic pathology* **34**, 599-608.

Chablani, L., Tawde, S. A. & D'Souza, M. J. (2012). Spray-dried microparticles: a potential vehicle for oral delivery of vaccines. *J Microencapsul* **29**, 388-397.

Challacombe, S. J., Rahman, D., Jeffery, H., Davis, S. S. & Ohagan, D. T. (1992). Enhanced Secretory Iga and Systemic Igg Antibody-Responses after Oral Immunization with Biodegradable Microparticles Containing Antigen. *Immunology* **76**, 164-168.

Chamcha, V., Scott, J. R. & Amara, R. (2012). Oral immunization with a recombinant *Lactococcus lactis* expressing HIV-1 Gag on the tip of the pilus induces strong mucosal immune responses. *Retrovirology* **9**.

Chatel, J. M., Pothelune, L., Ah-Leung, S., Corthier, G., Wal, J. M. & Langella, P. (2008). *In vivo* transfer of plasmid from food-grade transiting lactococci to murine epithelial cells. *Gene Ther* **15**, 1184-1190.

Cheng, Y., Feng, Y. J., Luo, P. & other authors (2009). Fusion Expression and Immunogenicity of EHEC EspA-Stx2A1 Protein: Implications for the Vaccine Development. *J Microbiol* **47**, 498-505.

Cherla, R. P., Lee, S. Y. & Tesh, V. L. (2003). Shiga toxins and apoptosis. *Fems Microbiol Lett* **228**, 159-166.

Cheroutre, H. & Madakamutil, L. (2004). Acquired and natural memory T cells join forces at the mucosal front line. *Nature Reviews Immunology* **4**, 290-300.

Cho, H. J., Shin, H. J., Han, I. K., Jung, W. W., Kim, Y. B., Sul, D. & Oh, Y. K. (2007). Induction of mucosal and systemic immune responses following oral immunization of mice with *Lactococcus lactis* expressing human papillomavirus type 16 L1. *Vaccine* **25**, 8049-8057.

Christensen, D., Agger, E. M., Andreasen, L. V., Kirby, D., Andersen, P. & Perrie, Y. (2009). Liposome-based cationic adjuvant formulations (CAF): past, present, and future. *Journal of liposome research* **19**, 2-11.

Clements-Mann, M. L., Dudas, R., Hoshino, Y. & other authors (2001). Safety and immunogenicity of live attenuated quadrivalent human-bovine (UK) reassortant rotavirus vaccine administered with childhood vaccines to infants. *Vaccine* **19**, 4676-4684.

Coffey, A., Ryan, M., Ross, R. P., Hill, C., Arendt, E. & Schwarz, G. (1998). Use of a broad-host-range bacteriocin-producing *Lactococcus lactis* transconjugant as an alternative starter for salami manufacture. *International journal of food microbiology* **43**, 231-235.

Conlan, J. W. & Perry, M. B. (1998a). Susceptibility of three strains of conventional adult mice to intestinal colonization by an isolate of *Escherichia coli* O157:H7. *Can J Microbiol* **44**, 800-805.

Conlan, J. W. & Perry, M. B. (1998b). Susceptibility of three strains of conventional adult mice to intestinal colonization by an isolate of *Escherichia coli* O157 : H7. *Can J Microbiol* **44**, 800-805.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y. & Powrie, F. (2007). A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of experimental medicine* **204**, 1757-1764.

Corthesy, B. (2013). Multi-faceted functions of secretory IgA at mucosal surfaces. *Frontiers in immunology* **4**, 185.

Cossart, P. & Mengaud, J. (1989). *Listeria monocytogenes*. A model system for the molecular study of intracellular parasitism. *Molecular biology & medicine* **6**, 463-474.

Crim, S. M., Iwamoto, M., Huang, J. Y. & other authors (2014). Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food - Foodborne Diseases Active Surveillance Network, 10 US Sites, 2006-2013. *Mmwr-Morbid Mortal W* **63**, 328-332.

Crump, J. A., Sulka, A. C., Langer, A. J. & other authors (2002). An outbreak of *Escherichia coli* O157 : H7 infections among visitors to a dairy farm. *New Engl J Med* **347**, 555-560.

Czerkinsky, C. & Holmgren, J. (2012). Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. *Current topics in microbiology and immunology* **354**, 1-18.

D

de Boer, W. I., Sont, J. K., van Schadewijk, A., Stolk, J., van Krieken, J. H. & Hiemstra, P. S. (2000). Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *The Journal of pathology* **190**, 619-626.

de Ruyter, P. G., Kuipers, O. P., Beerthuyzen, M. M., van Alen-Boerrigter, I. & de Vos, W. M. (1996a). Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *J Bacteriol* **178**, 3434-3439.

de Ruyter, P. G., Kuipers, O. P. & de Vos, W. M. (1996b). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62**, 3662-3667.

de Ruyter, P. G., Kuipers, O. P., Meijer, W. C. & de Vos, W. M. (1997). Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. *Nature biotechnology* **15**, 976-979.

de Vos, W. M., Vos, P., de Haard, H. & Boerrigter, I. (1989). Cloning and expression of the *Lactococcus lactis* subsp. *cremoris* SK11 gene encoding an extracellular serine proteinase. *Gene* **85**, 169-176.

de Vos, W. M. (1999). Gene expression systems for lactic acid bacteria. *Curr Opin Microbiol* **2**, 289-295.

Dickinson, B. L., Badizadegan, K., Wu, Z., Ahouse, J. C., Zhu, X. P., Simister, N. E., Blumberg, R. S. & Lencer, W. I. (1999). Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell Line. *Journal of Clinical Investigation* **104**, 903-911.

Dieye, Y., Usai, S., Clier, F., Gruss, A. & Piard, J. C. (2001). Design of a protein-targeting system for lactic acid bacteria. *J Bacteriol* **183**, 4157-4166.

Dieye, Y., Hoekman, A. J., Clier, F., Juillard, V., Boot, H. J. & Piard, J. C. (2003). Ability of *Lactococcus lactis* to export viral capsid antigens: a crucial step for development of live vaccines. *Appl Environ Microbiol* **69**, 7281-7288.

Donnenberg, M. S., Kaper, J. B. & Finlay, B. B. (1997). Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol* **5**, 109-114.

Doughty, S., Sloan, J., Bennett-Wood, V., Robertson, M., Robins-Browne, R. M. & Hartland, E. L. (2002). Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli*. *Infection and immunity* **70**, 6761-6769.

Drouault, S., Corthier, G., Ehrlich, S. D. & Renault, P. (1999). Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. *Appl Environ Microb* **65**, 4881-4886.

Drouault, S., Juste, C., Marteau, P., Renault, P. & Corthier, G. (2002). Oral treatment with *Lactococcus lactis* expressing *Staphylococcus hyicus* lipase enhances lipid digestion in pigs with induced pancreatic insufficiency. *Appl Environ Microb* **68**, 3166-3168.

E

Eaton, K. A., Friedman, D. I., Francis, G. J., Tyler, J. S., Young, V. B., Haeger, J., Abu-Ali, G. & Whittam, T. S. (2008). Pathogenesis of renal disease due to enterohemorrhagic *Escherichia coli* in germ-free mice. *Infection and immunity* **76**, 3054-3063.

Ebel, F., Podzadel, T., Rohde, M., Kresse, A. U., Kramer, S., Deibel, C., Guzman, C. A. & Chakraborty, T. (1998). Initial binding of Shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. *Molecular microbiology* **30**, 147-161.

Eichenbaum, Z., Federle, M. J., Marra, D., De Vos, W. M., Kuipers, O. P., Kleerebezem, M. & Scott, J. R. (1998). Use of the lactococcal nisA promoter to regulate gene expression in Gram-positive bacteria: Comparison of induction level and promoter strength. *Appl Environ Microb* **64**, 2763-2769.

El-Kamary, S. S., Pasetti, M. F., Mendelman, P. M. & other authors (2010). Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues. *The Journal of infectious diseases* **202**, 1649-1658.

Eldridge, J. H., Meulbroek, J. A., Staas, J. K., Tice, T. R. & Gilley, R. M. (1989). Vaccine-Containing Biodegradable Microspheres Specifically Enter the Gut-Associated Lymphoid-Tissue Following Oral-Administration and Induce a Disseminated Mucosal Immune-Response. *Immunobiology of Proteins and Peptides V : Vaccines* **251**, 191-202.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. & Igarashi, K. (1988). Site of Action of a Vero Toxin (Vt2) from Escherichia-Coli O157-H7 and of Shiga Toxin on Eukaryotic Ribosomes - Rna N-Glycosidase Activity of the Toxins. *Eur J Biochem* **171**, 45-50.

Enouf, V., Langella, P., Commissaire, J., Cohen, J. & Corthier, G. (2001). Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. *Appl Environ Microbiol* **67**, 1423-1428.

Esteban, M. (2009). Attenuated poxvirus vectors MVA and NYVAC as promising vaccine candidates against HIV/AIDS. *Hum Vaccines* **5**, 867-871.

F

Fagarasan, S., Kawamoto, S., Kanagawa, O. & Suzuki, K. (2010). Adaptive Immune Regulation in the Gut: T Cell-Dependent and T Cell-Independent IgA Synthesis. *Annu Rev Immunol* **28**, 243-273.

Fan, H. Y., Wang, L., Luo, J. & Long, B. G. (2012). Protection against *Escherichia coli* O157:H7 challenge by immunization of mice with purified Tir proteins. *Molecular biology reports* **39**, 989-997.

Francis, D. H., Collins, J. E. & Duimstra, J. R. (1986). Infection of Gnotobiotic Pigs with an *Escherichia. coli* O157-H7 Strain Associated with an Outbreak of Hemorrhagic Colitis. *Infection and immunity* **51**, 953-956.

Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular microbiology* **30**, 911-921.

Fraser, M. E., Chernaia, M. M., Kozlov, Y. V. & James, M. N. (1994). Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 A resolution. *Nature structural biology* **1**, 59-64.

Fratamico, P. M., Bhaduri, S. & Buchanan, R. L. (1993). Studies on *Escherichia coli* Serotype O157/H7 Strains Containing a 60-Mda Plasmid and on 60-Mda Plasmid-Cured Derivatives. *Journal of medical microbiology* **39**, 371-381.

Frees, D. & Ingmer, H. (1999). ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Molecular microbiology* **31**, 79-87.

Fuglsang, A. (2003). Lactic acid bacteria as prime candidates for codon optimization. *Biochemical and biophysical research communications* **312**, 285-291.

Fujii, J., Kita, T., Yoshida, S., Takeda, T., Kobayashi, H., Tanaka, N., Ohsato, K. & Mizuguchi, Y. (1994). Direct evidence of neuron impairment by oral infection with verotoxin-producing *Escherichia coli* O157:H- in mitomycin-treated mice. *Infection and immunity* **62**, 3447-3453.

G

Gahan, M. E., Webster, D. E., Wijburg, O. L., Wesselingh, S. L. & Strugnell, R. A. (2008). Impact of prior immunological exposure on vaccine delivery by *Salmonella enterica serovar Typhimurium*. *Vaccine* **26**, 6212-6220.

Galen, J. E., Pasetti, M. F., Tennant, S., Ruiz-Olvera, P., Sztein, M. B. & Levine, M. M. (2009). *Salmonella enterica serovar Typhi* live vector vaccines finally come of age. *Immunology and cell biology* **87**, 400-412.

Gao, X., Cai, K., Shi, J., Liu, H., Hou, X. J., Tu, W., Xiao, L., Wang, Q. & Wang, H. (2009a). Immunogenicity of a novel Stx2B-Stx1B fusion protein in a mice model of Enterohemorrhagic *Escherichia coli* O157:H7 infection. *Vaccine* **27**, 2070-2076.

Gao, X., Cai, K., Li, T. & other authors (2011). Novel fusion protein protects against adherence and toxicity of enterohemorrhagic *Escherichia coli* O157:H7 in mice. *Vaccine* **29**, 6656-6663.

Gao, X. F., Wan, F. Y., Mateo, K. & other authors (2009b). Bacterial Effector Binding to Ribosomal Protein S3 Subverts NF-kappa B Function. *Plos Pathog* **5**.

Garg, A. X., Salvadori, M., Okell, J. M., Thiessen-Philbrook, H. R., Suri, R. S., Filler, G., Moist, L., Matsell, D. & Clark, W. F. (2008). Albuminuria and estimated GFR 5 years after *Escherichia coli* O157 hemolytic uremic syndrome: an update. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **51**, 435-444.

Garg, N. K., Mangal, S., Khambete, H. & Tyagi, R. K. (2010). Mucosal delivery of vaccines: role of mucoadhesive/biodegradable polymers. *Recent patents on drug delivery & formulation* **4**, 114-128.

Garmendia, J., Frankel, G. & Crepin, V. F. (2005). Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: Translocation, translocation, translocation. *Infection and immunity* **73**, 2573-2585.

Gasson, M. J. (1983). Plasmid complements of Streptococcus *lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**, 1-9.

Gobert, A. P., Coste, A., Guzman, C. A., Vareille, M., Hindre, T., de Sablet, T., Girardeau, J. P. & Martin, C. (2008). Modulation of chemokine gene expression by Shiga-toxin producing *Escherichia coli* belonging to various origins and serotypes. *Microbes and Infection* **10**, 159-165.

Grangette, C., Muller-Alouf, H., Geoffroy, M. C., Goudercourt, D., Turneer, M. & Mercenier, A. (2002). Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. *Vaccine* **20**, 3304-3309.

Grangette, C., Muller-Alouf, H., Hols, P., Goudercourt, D., Delcour, J., Turneer, M. & Mercenier, A. (2004). Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. *Infection and immunity* **72**, 2731-2737.

Grant, J., Wendelboe, A. M., Wendel, A., Jepson, B., Torres, P., Smelser, C. & Rolfs, R. T. (2008). Spinach-associated *Escherichia coli* O157 : H7 outbreak, Utah and New Mexico, 2006. *Emerg Infect Dis* **14**, 1633-1636.

Griffin, P. M. & Tauxe, R. V. (1991a). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic reviews* **13**, 60-98.

Griffin, P. M. & Tauxe, R. V. (1991b). The Epidemiology of Infections Caused by Escherichia-Coli O157-H7, Other Enterohemorrhagic *Escherichia coli*, and the Associated Hemolytic Uremic Syndrome. *Epidemiologic reviews* **13**, 60-98.

Gruss, A. & Ehrlich, S. D. (1989). The Family of Highly Interrelated Single-Stranded Deoxyribonucleic-Acid Plasmids. *Microbiological reviews* **53**, 231-241.

Gu, J., Ning, Y. Y., Wang, H. G. & other authors (2011). Vaccination of attenuated EIS-producing *Salmonella* induces protective immunity against enterohemorrhagic *Escherichia coli* in mice. *Vaccine* **29**, 7395-7403.

Gu, Q., Song, D. F. & Zhu, M. Y. (2009). Oral vaccination of mice against *Helicobacter pylori* with recombinant *Lactococcus lactis* expressing urease subunit B. *Fems Immunol Med Mic* **56**, 197-203.

Guimaraes, V. D., Gabriel, J. E., Lefevre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V. & Langella, P. (2005). Internalin-expres sing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and Infection* **7**, 836-844.

H

Hamada, H., Hiroi, T., Nishiyama, Y. & other authors (2002). Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *Journal of immunology* **168**, 57-64.

He, B., Xu, W., Santini, P. A. & other authors (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* **26**, 812-826.

He, B., Santamaria, R., Xu, W. F. & other authors (2010). The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nature immunology* **11**, 836-U894.

Hernandez, I., Molenaar, D., Beekwilder, J., Bouwmeester, H. & Vlieg, J. E. T. V. (2007). Expression of plant flavor genes in *Lactococcus lactis*. *Appl Environ Microb* **73**, 1544-1552.

Holmgren, J., Czerkinsky, C., Eriksson, K. & Mharandi, A. (2003). Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine* **21**, S89-S95.

Holmgren, J., Adamsson, J., Anjuere, F. & other authors (2005). Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunology letters* **97**, 181-188.

Holmgren, J. & Svennerholm, A. M. (2012). Vaccines against mucosal infections. *Curr Opin Immunol* **24**, 343-353.

House, B., Kus, J. V., Prayitno, N., Mair, R., Que, L., Chingcuanco, F., Gannon, V., Cvitkovitch, D. G. & Barnett Foster, D. (2009). Acid-stress-induced changes in enterohaemorrhagic *Escherichia coli* O157 : H7 virulence. *Microbiology* **155**, 2907-2918.

Hu, C. X., Xu, Z. R., Li, W. F., Niu, D., Lu, P. & Fu, L. L. (2009). Secretory expression of K88 (F4) fimbrial adhesin FaeG by recombinant *Lactococcus lactis* for oral vaccination and its protective immune response in mice. *Biotechnol Lett* **31**, 991-997.

Hugentobler, F., Di Roberto, R. B., Gillard, J. & Cousineau, B. (2012). Oral immunization using live *Lactococcus lactis* co-expressing LACK and IL-12 protects BALB/c mice against Leishmania major infection. *Vaccine* **30**, 5726-5732.

Huibregtse, I. L., Snoeck, V., De Creus, A., Braat, H., De Jong, E. C., Van Deventer, S. J. H. & Rottiers, P. (2007). Induction of ovalbumin-specific tolerance by oral administration of *Lactococcus lactis* secreting ovalbumin. *Gastroenterology* **133**, 517-528.

Hurley, B. P., Jacewicz, M., Thorpe, C. M., Lincicome, L. L., King, A. J., Keusch, G. T. & Acheson, D. W. (1999). Shiga toxins 1 and 2 translocate differently across polarized intestinal epithelial cells. *Infection and immunity* **67**, 6670-6677.

Hutchings, A. B., Helander, A., Silvey, K. J., Chandran, K., Lucas, W. T., Nibert, M. L. & Neutra, M. R. (2004). Secretory immunoglobulin A antibodies against the sigma1 outer capsid protein of reovirus type 1 Lang prevent infection of mouse Peyer's patches. *J Virol* **78**, 947-957.

I

Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner, H. & Schmidt, M. A. (2001). Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cellular microbiology* **3**, 669-679.

Iijima, K., Kamioka, I. & Nozu, K. (2008). Management of diarrhea-associated hemolytic uremic syndrome in children. *Clin Exp Nephrol* **12**, 16-19.

Ikeda, K., Ida, O., Kimoto, K., Takatorige, T., Nakanishi, N. & Tatara, K. (1999). Effect of early fosfomycin treatment on prevention of hemolytic uremic syndrome accompanying *Escherichia coli* O157 : H7 infection. *Clin Nephrol* **52**, 357-362.

Ishikawa, S., Kawahara, K., Kagami, Y., Isshiki, Y., Kaneko, A., Matsui, H., Okada, N. & Danbara, H. (2003). Protection against Shiga toxin 1 challenge by immunization of mice with purified mutant Shiga toxin 1. *Infection and immunity* **71**, 3235-3239.

Israel, E. J., Taylor, S., Wu, Z., Mizoguchi, E., Blumberg, R. S., Bhan, A. & Simister, N. E. (1997). Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells. *Immunology* **92**, 69-74.

Iwaki, M., Okahashi, N., Takahashi, I., Kanamoto, T., Sugitakonishi, Y., Aibara, K. & Koga, T. (1990). Oral Immunization with Recombinant Streptococcus-*Lactis* Carrying the *Streptococcus mutans* Surface Protein Antigen Gene. *Infection and immunity* **58**, 2929-2934.

Iwasaki, A. & Kelsall, B. L. (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *The Journal of experimental medicine* **190**, 229-239.

J-K

Janot, L., Sirard, J. C., Secher, T. & other authors (2009). Radioresistant cells expressing TLR5 control the respiratory epithelium's innate immune responses to flagellin. *Eur J Immunol* **39**, 1587-1596.

Jeon, B. & Itoh, K. (2007). Production of shiga toxin by a luxS mutant of *Escherichia coli* O157:H7 *in vivo* and *in vitro*. *Microbiol Immunol* **51**, 391-396.

Jeong, K. I., Chapman-Bonofiglio, S., Singh, P., Lee, J., Tzipori, S. & Sheoran, A. S. (2010). *In vitro* and *in vivo* protective efficacies of antibodies that neutralize the RNA N-glycosidase activity of Shiga toxin 2. *Bmc Immunol* **11**.

Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123-140.

Karch, H., Heesemann, J., Laufs, R., O'Brien, A. D., Tacket, C. O. & Levine, M. M. (1987). A Plasmid of Enterohemorrhagic *Escherichia coli* O157-H7 is Required for Expression of a New Fimbrial Antigen and for Adhesion to Epithelial-Cells. *Infection and immunity* **55**, 455-461.

Karch, H., Friedrich, A. W., Gerber, A., Zimmerhackl, L. B., Schmidt, M. A. & Bielaszewska, M. (2006). New aspects in the pathogenesis of enteropathic hemolytic uremic syndrome. *Semin Thromb Hemost* **32**, 105-112.

Karmali, M. A., Petric, M., Steele, B. T. & Lim, C. (1983). Sporadic Cases of Hemolytic-Uremic Syndrome Associated with Fecal Cyto-Toxin and Cytotoxin-Producing *Escherichia coli* in Stools. *Lancet* **1**, 619-620.

Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., Arbus, G. S. & Lior, H. (1985). The Association between Idiopathic Hemolytic Uremic Syndrome and Infection by Verotoxin-Producing *Escherichia coli*. *Journal of Infectious Diseases* **151**, 775-782.

Karmali, M. A., Mascarenhas, M., Shen, S. H. & other authors (2003). Association of genomic O-island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *Journal of clinical microbiology* **41**, 4930-4940.

Karpman, D., Connell, H., Svensson, M., Scheutz, F., Alm, P. & Svanborg, C. (1997a). The role of lipopolysaccharide and shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. *Journal of Infectious Diseases* **175**, 611-620.

Karpman, D., Connell, H., Svensson, M., Scheutz, F., Alm, P. & Svanborg, C. (1997b). The role of lipopolysaccharide and Shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. *The Journal of infectious diseases* **175**, 611-620.

Kaufmann, S. H. (1993). Immunity to intracellular bacteria. *Annual review of immunology* **11**, 129-163.

Kawahara, M., Matsuo, K. & Honda, M. (2006). Intradermal and oral immunization with recombinant Mycobacterium bovis BCG expressing the simian immunodeficiency virus Gag protein induces long-lasting, antigen-specific immune responses in guinea pigs. *Clinical immunology* **119**, 67-78.

Kawahara, M. (2008). Recombinant Mycobacterium bovis BCG vector system expressing SIV Gag protein stably and persistently induces antigen-specific humoral immune response concomitant with IFN gamma response, even at three years after immunization. *Clinical immunology* **129**, 492-498.

Kersten, G. F. & Crommelin, D. J. (2003). Liposomes and ISCOMs. *Vaccine* **21**, 915-920.

Kim, E. B., Piao, D. C., Son, J. S. & Choi, Y. J. (2009). Cloning and Characterization of a Novel tuf Promoter from *Lactococcus lactis* subsp *lactis* IL1403. *Current microbiology* **59**, 425-431.

Kim, S. J., Jun, D. Y., Yang, C. H. & Kim, Y. H. (2006). Expression of Helicobacter pylori cag12 gene in *Lactococcus lactis* MG1363 and its oral administration to induce systemic anti-Cag12 immune response in mice. *Applied microbiology and biotechnology* **72**, 462-470.

Kim, W. S., Ren, J. & Dunn, N. W. (1999). Differentiation of *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains by their adaptive response to stresses. *Fems Microbiol Lett* **171**, 57-65.

Kimoto, H., Nomura, M., Kobayashi, M., Mizumachi, K. & Okamoto, T. (2003). Survival of lactococci during passage through mouse digestive tract. *Can J Microbiol* **49**, 707-711.

Klavinskis, L. S., Bergmeier, L. A., Gao, L., Mitchell, E., Ward, R. G., Layton, G., Brookes, R., Meyers, N. J. & Lehner, T. (1996). Mucosal or targeted lymph node immunization of

macaques with a particulate SIVp27 protein elicits virus-specific CTL in the genito-rectal mucosa and draining lymph nodes. *Journal of immunology* **157**, 2521-2527.

Klijn, N., Weerkamp, A. H. & Devos, W. M. (1995). Genetic Marking of *Lactococcus Lactis* Shows Its Survival in the Human Gastrointestinal-Tract. *Appl Environ Microb* **61**, 2771-2774.

Knappstein, S., Ide, T., Schmidt, M. A. & Heusipp, G. (2004a). Alpha 1-antitrypsin binds to and interferes with functionality of EspB from atypical and typical enteropathogenic *Escherichia coli* strains. *Infection and immunity* **72**, 4344-4350.

Knappstein, S., Ide, T., Schmidt, M. A. & Heusipp, G. (2004b). alpha(1)-antitrypsin binds to and interferes with functionality of EspB from atypical and typical enteropathogenic *Escherichia coli* strains. *Infection and immunity* **72**, 4344-4350.

Knutton, S., Rosenshine, I., Pallen, M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G. & Frankel, G. (1998). A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *Embo J* **17**, 2166-2176.

Koch, B., Kilstrup, M., Vogensen, F. K. & Hammer, K. (1998). Induced levels of heat shock proteins in a dnaK mutant of *Lactococcus lactis*. *J Bacteriol* **180**, 3873-3881.

Kodama, T., Akeda, Y., Kono, G., Takahashi, A., Imura, K., Iida, T. & Honda, T. (2002). The EspB protein of enterohaemorrhagic *Escherichia coli* interacts directly with alpha-catenin. *Cellular microbiology* **4**, 213-222.

Koivula, T., Palva, I. & Hemila, H. (1991). Nucleotide sequence of the secY gene from *Lactococcus lactis* and identification of conserved regions by comparison of four SecY proteins. *Febs Lett* **288**, 114-118.

Kok, J., Vandervossen, J. M. B. M. & Venema, G. (1984). Construction of Plasmid Cloning Vectors for Lactic Streptococci Which Also Replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microb* **48**, 726-731.

Konowalchuk, J., Speirs, J. I. & Stavric, S. (1977). Vero Response to a Cytotoxin of *Escherichia coli*. *Infection and immunity* **18**, 775-779.

Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G., Luesink, E. J. & de Vos, W. M. (1995). Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *The Journal of biological chemistry* **270**, 27299-27304.

Kuipers, O. P., de Ruyter, P. G., Kleerebezem, M. & de Vos, W. M. (1997). Controlled overproduction of proteins by lactic acid bacteria. *Trends in biotechnology* **15**, 135-140.

Kurioka, T., Yunou, Y. & Kita, E. (1998). Enhancement of susceptibility to Shiga toxin-producing *Escherichia coli* O157:H7 by protein calorie malnutrition in mice. *Infection and immunity* **66**, 1726-1734.

Kurioka, T., Yunou, Y., Harada, H. & Kita, E. (1999). Efficacy of antibiotic therapy for infection with Shiga-like toxin-producing *Escherichia coli* O157:H7 in mice with protein-calorie malnutrition. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **18**, 561-571.

L

Lasaro, M. O. & Ertl, H. C. J. (2009). New Insights on Adenovirus as Vaccine Vectors. *Mol Ther* **17**, 1333-1339.

Latvala, S., Pietila, T. E., Veckman, V., Kekkonen, R. A., Tynkkynen, S., Korpela, R. & Julkunen, I. (2008). Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World journal of gastroenterology : WJG* **14**, 5570-5583; discussion 5581-5572.

Le Loir, Y., Azevedo, V., Oliveira, S. C. & other authors (2005). Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb Cell Fact* **4**, 2.

LeBlanc, J. G., Aubry, C., Cortes-Perez, N. G. & other authors (2013). Mucosal targeting of therapeutic molecules using genetically modified lactic acid bacteria: an update. *Fems Microbiol Lett* **344**, 1-9.

Ledesma, M. A., Ochoa, S. A., Cruz, A., Rocha-Ramirez, L. M., Mas-Oliva, J., Eslava, C. A., Giron, J. A. & Xicohtencatl-Cortes, J. (2010). The Hemorrhagic Coli Pilus (HCP) of *Escherichia coli* O157:H7 Is an Inducer of Proinflammatory Cytokine Secretion in Intestinal Epithelial Cells. *Plos One* **5**.

Lee, M. H., Roussel, Y., Wilks, M. & Tabaqchali, S. (2001). Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against H-pylori infection in mice. *Vaccine* **19**, 3927-3935.

Lee, P., Abdul-Wahid, A. & Faubert, G. A. (2009). Comparison of the local immune response against *Giardia lamblia* cyst wall protein 2 induced by recombinant *Lactococcus lactis* and Streptococcus gordonii. *Microbes and Infection* **11**, 20-28.

Lee, S., Gierynska, M., Eo, S. K., Kuklin, N. & Rouse, B. T. (2003). Influence of DNA encoding cytokines on systemic and mucosal immunity following genetic vaccination against herpes simplex virus. *Microbes and Infection* **5**, 571-578.

Leenhouts, K., Bolhuis, A., Venema, G. & Kok, J. (1998). Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Applied microbiology and biotechnology* **49**, 417-423.

Lei, H., Sheng, Z. N., Ding, Q., Chen, J., Wei, X. H., Lam, D. M. K. & Xu, Y. H. (2011). Evaluation of Oral Immunization with Recombinant Avian Influenza Virus HA1 Displayed on the *Lactococcus lactis* Surface and Combined with the Mucosal Adjuvant Cholera Toxin Subunit B. *Clinical and Vaccine Immunology* **18**, 1046-1051.

Levine, M. M., Xu, J. G., Kaper, J. B., Lior, H., Prado, V., Tall, B., Nataro, J., Karch, H. & Wachsmuth, K. (1987). A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. *The Journal of infectious diseases* **156**, 175-182.

Levine, M. M. (2000). Immunization against bacterial diseases of the intestine. *J Pediatr Gastr Nutr* **31**, 336-355.

Li, X., Xing, Y., Guo, L., Lv, X., Song, H. & Xi, T. (2014). Oral immunization with recombinant *Lactococcus lactis* delivering a multi-epitope antigen CTB-UE attenuates *Helicobacter pylori* infection in mice. *Pathogens and disease*.

Li, Y. J., Ma, G. P., Li, G. W., Qiao, X. Y., Ge, J. W., Tang, L. J., Liu, M. & Liu, L. W. (2010). Oral Vaccination with the Porcine Rotavirus VP4 Outer Capsid Protein Expressed by *Lactococcus lactis* Induces Specific Antibody Production. *Journal of Biomedicine and Biotechnology*.

Li, Y. L., Frey, E., Mackenzie, A. M. R. & Finlay, B. B. (2000). Human response to *Escherichia coli* O157 : H7 infection: Antibodies to secreted virulence factors. *Infection and immunity* **68**, 5090-5095.

Liang, S. A. & Hajishengallis, G. (2010). Heat-Labile Enterotoxins as Adjuvants or Anti-Inflammatory Agents. *Immunol Invest* **39**, 449-467.

Lindgren, S. W., Melton, A. R. & O'Brien, A. D. (1993). Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model. *Infection and immunity* **61**, 3832-3842.

Lindholm, A., Smeds, A. & Palva, A. (2004). Receptor binding domain of *Escherichia coli* F18 fimbrial adhesin FedF can be both efficiently secreted and surface displayed in a functional form in *Lactococcus lactis*. *Appl Environ Microbiol* **70**, 2061-2071.

Lingwood, C. A. (1994). Verotoxin Recognition of Its Glycolipid Receptor, Globotriaosylceramide - Role in Pathogenesis. *Int Congr Ser* **1072**, 131-137.

Litinskiy, M. B., Nardelli, B., Hilbert, D. M., He, B., Schaffer, A., Casali, P. & Cerutti, A. (2002). DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nature immunology* **3**, 822-829.

Liu, C., Hashizume, T., Kurita-Ochiai, T., Fujihashi, K. & Yamamoto, M. (2010a). Oral immunization with Porphyromonas gingivalis outer membrane protein and CpG oligodeoxynucleotides elicits T helper 1 and 2 cytokines for enhanced protective immunity. *Mol Oral Microbiol* **25**, 178-189.

Liu, J., Sun, Y., Feng, S. Z., Zhu, L. W., Guo, X. J. & Qi, C. (2009). Towards an attenuated enterohemorrhagic *Escherichia coli* O157:H7 vaccine characterized by a deleted ler gene and containing apathogenic Shiga toxins. *Vaccine* **27**, 5929-5935.

Liu, S., Li, Y., Xu, Z. & Wang, Y. (2010b). Immune responses elicited in mice with recombinant *Lactococcus lactis* expressing F4 fimbrial adhesin FaeG by oral immunization. *Veterinary research communications* **34**, 491-502.

Liu, S., Li, Y., Xu, Z. & Wang, Y. (2013). Subcutaneous or oral immunization of mice with *Lactococcus lactis* expressing F4 fimbrial adhesin FaeG. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* **75**, 779-784.

Lizumi, Y., Sagara, H., Kabe, Y. & other authors (2007). The enteropathogenic *E. coli* effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. *Cell host & microbe* **2**, 383-392.

Lu, X. H., Clements, J. D. & Katz, J. M. (2002). Mutant *Escherichia coli* heat-labile enterotoxin [LT(R192G)] enhances protective humoral and cellular immune responses to orally administered inactivated influenza vaccine. *Vaccine* **20**, 1019-1029.

Luan, J. J., Zhuang, Z., Liu, Y., Yun, K., Chen, M. & Wang, P. G. (2010). Expression of EspA in *Lactococcus lactis* NZ9000 and the detection of its immune effect *in vivo* and vitro. *Immunopharm Immunot* **32**, 133-140.

Ludwig, W., Seewaldt, E., Kilpper-Balz, R., Schleifer, K. H., Magrum, L., Woese, C. R., Fox, G. E. & Stackebrandt, E. (1985). The phylogenetic position of Streptococcus and Enterococcus. *Journal of general microbiology* **131**, 543-551.

Lycke, N. (2004). From toxin to adjuvant: the rational design of a vaccine adjuvant vector, CTA1-DD/ISCOM. *Cellular microbiology* **6**, 23-32.

Lycke, N. (2005). Targeted vaccine adjuvants based on modified cholera toxin. *Curr Mol Med* **5**, 591-597.

M

Ma, D., Gao, M., Dalloul, R. A., Ge, J., Ma, C. & Li, J. (2013). Protective effects of oral immunization with live *Lactococcus lactis* expressing Eimeria tenella 3-1E protein. *Parasitology research* **112**, 4161-4167.

Mahajan, A., Currie, C. G., Mackie, S. & other authors (2009). An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157: H7 with bovine intestinal epithelium. *Cellular microbiology* **11**, 121-137.

Manicassamy, S. & Pulendran, B. (2009). Modulation of adaptive immunity with Toll-like receptors. *Seminars in immunology* **21**, 185-193.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25**, 677-686.

Marcato, P., Mulvey, G., Read, R. J., Vander Helm, K., Nation, P. N. & Armstrong, G. D. (2001). Immunoprophylactic potential of cloned shiga toxin 2 B subunit. *Journal of Infectious Diseases* **183**, 435-443.

Marcato, P., Griener, T. P., Mulvey, G. L. & Armstrong, G. D. (2005). Recombinant Shiga toxin B-subunit-keyhole limpet hemocyanin conjugate vaccine protects mice from Shigatoxemia. *Infection and immunity* **73**, 6523-6529.

Marelli, B., Perez, A. R., Banchio, C., de Mendoza, D. & Magni, C. (2011). Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8* subunit induces specific immune response in mice. *J Virol Methods* **175**, 28-37

Mayr, U. B., Haller, C. H., Haidinger, W., Atrasheuskaya, A., Bukin, E., Lubitz, W. & Ignatyev, G. (2005). Bacterial ghosts as an oral vaccine: a single dose of *Escherichia coli* O157 : H7 bacterial ghosts protects mice against lethal challenge. *Infection and immunity* **73**, 4810-4817.

Mayr, U. B., Kudela, P., Atrasheuskaya, A., Bukin, E., Ignatyev, G. & Lubitz, W. (2012). Rectal single dose immunization of mice with *Escherichia coli* O157:H7 bacterial ghosts induces efficient humoral and cellular immune responses and protects against the lethal heterologous challenge. *Microb Biotechnol* **5**, 283-294.

Mazanec, M. B., Kaetzel, C. S., Lamm, M. E., Fletcher, D. & Nedrud, J. G. (1992). Intracellular neutralization of virus by immunoglobulin A antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6901-6905.

McCluskie, M. J., Weeratna, R. D., Payette, P. J. & Davis, H. L. (2001). The potential of CpG oligodeoxynucleotides as mucosal adjuvants. *Crit Rev Immunol* **21**, 103-120.

Meeusen, E. N. T., Walker, J., Peters, A., Pastoret, P. P. & Jungersen, G. (2007). Current status of veterinary vaccines. *Clin Microbiol Rev* **20**, 489-510.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. & Gerdts, V. (2012). The pig: a model for human infectious diseases. *Trends Microbiol* **20**, 50-57.

Michael, M., Elliott, E. J., Ridley, G. F., Hodson, E. M. & Craig, J. C. (2009). Interventions for haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. *The Cochrane database of systematic reviews*, CD003595.

Mierau, I. & Kleerebezem, M. (2005a). 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied microbiology and biotechnology* **68**, 705-717.

Mierau, I. & Kleerebezem, M. (2005b). 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied microbiology and biotechnology* **68**, 705-717.

Mierau, I., Leij, P., van Swam, I., Blommestein, B., Floris, E., Mond, J. & Smid, E. J. (2005). Industrial-scale production and purification of a heterologous protein in *Lactococcus lactis* using the nisin-controlled gene expression system NICE: the case of lysostaphin. *Microb Cell Fact* **4**, 15.

Miyoshi, A., Bermudez-Humaran, L. G., Ribeiro, L. A., Le Loir, Y., Oliveira, S. C., Langella, P. & Azevedo, V. (2006). Heterologous expression of *Brucella abortus* GroEL heat-shock protein in *Lactococcus lactis*. *Microb Cell Fact* **5**, 14.

Mohawk, K. L., Melton-Celsa, A. R., Robinson, C. M. & O'Brien, A. D. (2010). Neutralizing antibodies to Shiga toxin type 2 (Stx2) reduce colonization of mice by Stx2-expressing *Escherichia coli* O157:H7. *Vaccine* **28**, 4777-4785.

Mohawk, K. L. & O'Brien, A. D. (2011a). Mouse Models of *Escherichia coli* O157:H7 Infection and Shiga Toxin Injection. *Journal of Biomedicine and Biotechnology*.

Mohawk, K. L. & O'Brien, A. D. (2011b). Mouse models of *Escherichia coli* O157:H7 infection and shiga toxin injection. *Journal of biomedicine & biotechnology* **2011**, 258185.

Moore, A., McCarthy, L. & Mills, K. H. G. (1999). The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. *Vaccine* **17**, 2517-2527.

Moorthy, S. A. V., Yasawardena, S. G. & Ramasamy, R. (2009). Age-dependent systemic antibody responses and immunisation-associated changes in mice orally and nasally immunised with *Lactococcus lactis* expressing a malaria parasite protein. *Vaccine* **27**, 4947-4952.

Mora, J. R., Iwata, M., Eksteen, B. & other authors (2006). Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* **314**, 1157-1160.

Morigi, M., Micheletti, G., Figliuzzi, M., Imberti, B., Karmali, M. A., Remuzzi, A., Remuzzi, G. & Zoja, C. (1995). Verotoxin-1 promotes leukocyte adhesion to cultured endothelial cells under physiologic flow conditions. *Blood* **86**, 4553-4558.

Morigi, M., Galbusera, M., Binda, E., Imberti, B., Gastoldi, S., Remuzzi, A., Zoja, C. & Remuzzi, G. (2001). Verotoxin-1-induced up-regulation of adhesive molecules renders microvascular endothelial cells thrombogenic at high shear stress. *Blood* **98**, 1828-1835.

Mowat, A. M. (2003). Anatomical basis of tolerance and immunity to intestinal antigens. *Nature reviews Immunology* **3**, 331-341.

Muller, S., Buhler-Jungo, M. & Mueller, C. (2000). Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. *Journal of immunology* **164**, 1986-1994.

Murphy, T. V., Gargiullo, P. M., Massoudi, M. S. & other authors (2001). Intussusception among infants given an oral rotavirus vaccine. *New Engl J Med* **344**, 564-572.

N

National Center for Immunization and Respiratory Diseases (2011). General recommendations on immunization– recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control* **60**, 1-64.

Nagano, K., Taguchi, K., Hara, T., Yokoyama, S., Kawada, K. & Mori, H. (2003a). Adhesion and colonization of enterohemorrhagic *Escherichia coli* O157 : H7 in cecum of mice. *Microbiol Immunol* **47**, 125-132.

Nagano, K., Taguchi, K., Hara, T., Yokoyama, S., Kawada, K. & Mori, H. (2003b). Adhesion and colonization of enterohemorrhagic *Escherichia coli* O157:H7 in cecum of mice. *Microbiol Immunol* **47**, 125-132.

Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142.

Neutra, M. R. & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature reviews Immunology* **6**, 148-158.

Nicholls, L., Grant, T. H. & Robins-Browne, R. M. (2000). Identification of a novel genetic locus that is required for *in vitro* adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. *Molecular microbiology* **35**, 275-288.

Nilsson, D., Lauridsen, A. A., Tomoyasu, T. & Ogura, T. (1994). A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli* ftsH gene product. *Microbiology* **140 (Pt 10)**, 2601-2610.

Nolasco, L. H., Turner, N. A., Bernardo, A., Tao, Z., Cleary, T. G., Dong, J. F. & Moake, J. L. (2005). Hemolytic uremic syndrome-associated Shiga toxins promote endothelial-cell secretion and impair ADAMTS13 cleavage of unusually large von Willebrand factor multimers. *Blood* **106**, 4199-4209.

Nordly, P., Madsen, H. B., Nielsen, H. M. & Foged, C. (2009). Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators. *Expert Opin Drug Del* **6**, 657-672.

Nouaille, S., Bermudez-Humaran, L. G., Adel-Patient, K., Commissaire, J., Gruss, A., Wal, J. M., Azevedo, V., Langella, P. & Chatel, J. M. (2005). Improvement of bovine beta-lactoglobulin production and secretion by *Lactococcus lactis*. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica* **38**, 353-359.

O

O'Brien, A. D., Lively, T. A., Chang, T. W. & Gorbach, S. L. (1983). Purification of *Shigella dysenteriae* 1 (Shiga)-like toxin from *Escherichia coli* O157:H7 strain associated with haemorrhagic colitis. *Lancet* **2**, 573.

O'Hagan, D. T. (1998). Microparticles and polymers for the mucosal delivery of vaccines. *Adv Drug Deliver Rev* **34**, 305-320.

O'Loughlin, E. V. & Robins-Browne, R. M. (2001). Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. *Microbes and Infection* **3**, 493-507.

Oanh, T. K., Nguyen, V. K., De Greve, H. & Goddeeris, B. M. (2012). Protection of piglets against Edema disease by maternal immunization with Stx2e toxoid. *Infection and immunity* **80**, 469-473.

Obata, F., Tohyama, K., Bonev, A. D., Kolling, G. L., Keepers, T. R., Gross, L. K., Nelson, M. T., Sato, S. & Obrig, T. G. (2008). Shiga toxin 2 affects the central nervous system through receptor globotriaosylceramide localized to neurons. *The Journal of infectious diseases* **198**, 1398-1406.

O'B**rien, A. D. & Laveck, G. D. (1983)**. Purification and Characterization of a *Shigella dysenteriae* 1-Like Toxin Produced by *Escherichia coli*. *Infection and immunity* **40**, 675-683.

Obrig, T. G., Louise, C. B., Lingwood, C. A., Boyd, B., Barley-Maloney, L. & Daniel, T. O. (1993). Endothelial heterogeneity in Shiga toxin receptors and responses. *The Journal of biological chemistry* **268**, 15484-15488.

Oliveira, A. F., Cardoso, S. A., Almeida, F. B. D., de Oliveira, L. L., Pitondo-Silva, A., Soares, S. G. & Hanna, E. S. (2012). Oral immunization with attenuated Salmonella vaccine expressing *Escherichia coli* O157:H7 intimin gamma triggers both systemic and mucosal humoral immunity in mice. *Microbiol Immunol* **56**, 513-522.

Ostroff, S. M., Tarr, P. I., Neill, M. A., Lewis, J. H., Hargrett-Bean, N. & Kobayashi, J. M. (1989). Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. *The Journal of infectious diseases* **160**, 994-998.

P

Pamer, E. G., Sijts, A. J., Villanueva, M. S., Busch, D. H. & Vijh, S. (1997). MHC class I antigen processing of *Listeria monocytogenes* proteins: implications for dominant and subdominant CTL responses. *Immunological reviews* **158**, 129-136.

Paterson, G. K. & Maskell, D. J. (2010). Recent advances in the field of *Salmonella Typhi* vaccines. *Hum Vaccin* **6**, 379-384.

Pavia, A. T., Nichols, C. R., Green, D. P. & other authors (1990). Hemolytic-uremic syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations. *The Journal of pediatrics* **116**, 544-551.

Peek, L. J., Middaugh, C. R. & Berkland, C. (2008). Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev* **60**, 915-928.

Pei, H. D., Liu, J. F., Cheng, Y., Sun, C. M., Wang, C., Lu, Y. P., Ding, J., Zhou, J. & Xiang, H. (2005). Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination. *Applied microbiology and biotechnology* **68**, 220-227.

Perez, C. A., Eichwald, C., Burrone, O. & de Mendoza, D. (2005). Rotavirus vp7 antigen produced by *Lactococcus lactis* induces neutralizing antibodies in mice. *J Appl Microbiol* **99**, 1158-1164.

Perna, N. T., Plunkett, G., 3rd, Burland, V. & other authors (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**, 529-533.

Philpott, D. J., Ackerley, C. A., Kiliaan, A. J., Karmali, M. A., Perdue, M. H. & Sherman, P. M. (1997). Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. *Am J Physiol* **273**, G1349-1358.

Piard, J. C., Hautefort, I., Fischetti, V. A., Ehrlich, S. D., Fons, M. & Gruss, A. (1997). Cell wall anchoring of the Streptococcus pyogenes M6 protein in various lactic acid bacteria. *J Bacteriol* **179**, 3068-3072.

Pizza, M., Giuliani, M. M., Fontana, M. R., Monaci, E., Douce, G., Dougan, G., Mills, K. H. G., Rappuoli, R. & Del Giudice, G. (2001). Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* **19**, 2534-2541.

Pontes, D., Azevedo, M., Innocentin, S. & other authors (2014). Immune Response Elicited by DNA Vaccination Using *Lactococcus lactis* Is Modified by the Production of Surface Exposed Pathogenic Protein. *Plos One* **9**.

Pontes, D. S., Dorella, F. A., Ribeiro, L. A., Miyoshi, A., Loir, Y. L., Gruss, A., Oliveira, S. C., Langella, P. & Azevedo, V. (2004). Induction of partial protection in mice after oral administration of *Lactococcus lactis* producing *Brucella abortus* L7/L12 antigen. *J Drug Target* **11**, 489-493.

Pontes, D. S., de Azevedo, M. S., Chatel, J. M., Langella, P., Azevedo, V. & Miyoshi, A. (2011). *Lactococcus lactis* as a live vector: heterologous protein production and DNA delivery systems. *Protein expression and purification* **79**, 165-175.

Potter, A. A., Klashinsky, S., Li, Y. L. & other authors (2004). Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. *Vaccine* **22**, 362-369.

Pugsley, A. P. (1993). The complete general secretory pathway in Gram-negative bacteria. *Microbiological reviews* **57**, 50-108.

Puohiniemi, R., Simonen, M., Muttilainen, S., Himanen, J. P. & Sarvas, M. (1992). Secretion of the *Escherichia coli* outer membrane proteins OmpA and OmpF in Bacillus subtilis is blocked at an early intracellular step. *Molecular microbiology* **6**, 981-990.

R

Ra, S. R., Qiao, M., Immonen, T., Pujana, I. & Saris, E. J. (1996). Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in Lactoccocus *lactis* N8. *Microbiology* **142 (Pt 5)**, 1281-1288.

Ratnam, S., March, S. B., Ahmed, R., Bezanson, G. S. & Kasatiya, S. (1988). Characterization of *Escherichia coli* serotype O157:H7. *Journal of clinical microbiology* **26**, 2006-2012.

Ravn, P., Arnau, J., Madsen, S. M., Vrang, A. & Israelsen, H. (2000). The development of TnNuc and its use for the isolation of novel secretion signals in *Lactococcus lactis*. *Gene* **242**, 347-356.

Ravn, P., Arnau, J., Madsen, S. M., Vrang, A. & Israelsen, H. (2003). Optimization of signal peptide SP310 for heterologous protein production in *Lactococcus lactis*. *Microbiology* **149**, 2193-2201.

Reed, S. G., Bertholet, S., Coler, R. N. & Friede, M. (2009). New horizons in adjuvants for vaccine development. *Trends Immunol* **30**, 23-32.

Rescigno, M. (2010). Intestinal dendritic cells. *Adv Immunol* **107**, 109-138.

Ribeiro, L. A., Azevedo, V., Le Loir, Y., Oliveira, S. C., Dieye, Y., Piard, J. C., Gruss, A. & Langella, P. (2002). Production and targeting of the Brucella abortus antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microb* **68**, 910-916.

Richardson, S. E., Karmali, M. A., Becker, L. E. & Smith, C. R. (1988). The histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. *Human pathology* **19**, 1102-1108.

Richardson, S. E., Rotman, T. A., Jay, V., Smith, C. R., Becker, L. E., Petric, M., Olivieri, N. F. & Karmali, M. A. (1992). Experimental verocytotoxemia in rabbits. *Infection and immunity* **60**, 4154-4167.

Riley, L. W., Remis, R. S., Helgerson, S. D. & other authors (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *The New England journal of medicine* **308**, 681-685.

Rimmelzwaan, G. F. & Sutter, G. (2009). Candidate influenza vaccines based on recombinant modified vaccinia virus Ankara. *Expert Rev Vaccines* **8**, 447-454.

Rimpilainen, M. A., Niskasaari, K., Juutinen, K. M. S., Nurmiaholassila, E. L. & Forsen, R. I. (1986). The Plasma-Membrane of Streptococcus *cremoris*- Isolation and Partial Characterization. *J Appl Bacteriol* **60**, 389-394.

Ritchie, J. M. & Waldor, M. K. (2005). The locus of Enterocyte effacement-encoded effector proteins all promote enterohemorrhagic *Escherichia coli* pathogenicity in infant rabbits. *Infection and immunity* **73**, 1466-1474.

Roberts, M., Bacon, A., Li, J. L. & Chatfield, S. (1999). Prior immunity to homologous and heterologous *Salmonella* serotypes suppresses local and systemic anti-fragment C antibody responses and protection from tetanus toxin in mice immunized with *Salmonella* strains expressing fragment C. *Infection and immunity* **67**, 3810-3815.

Robinson, C. M., Sinclair, J. F., Smith, M. J. & O'Brien, A. D. (2006). Shiga toxin of enterohemorrhagic *Escherichia coli* type O157 : H7 promotes intestinal colonization. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 9667-9672.

Robinson, K., Chamberlain, L. M., Schofield, K. M., Wells, J. M. & LePage, R. W. F. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature biotechnology* **15**, 653-657.

Robinson, K., Chamberlain, L. M., Lopez, M. C., Rush, C. M., Marcotte, H., Le Page, R. W. F. & Wells, J. M. (2004). Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. *Infection and immunity* **72**, 2753-2761.

Rodriguez-Diaz, J., Montava, R., Viana, R., Buesa, J., Perez-Martinez, G. & Monedero, V. (2011). Oral immunization of mice with *Lactococcus lactis* expressing the rotavirus VP8*protein. *Biotechnol Lett* **33**, 1169-1175.

Rogers, T. J., Paton, J. C., Wang, H., Talbot, U. M. & Paton, A. W. (2006). Reduced virulence of an fliC mutant of Shiga-Toxigenic *Escherichia coli* O113 : H21. *Infection and immunity* **74**, 1962-1966.

Rojas, R. L. G., Gomes, P. A. D. P., Bentancor, L. V., Sbrogio-Almeida, M. E., Costa, S. O. P., Massis, L. M., Ferreira, R. C. C., Palermo, M. S. & Ferreira, L. C. S. (2010). *Salmonella enterica Serovar Typhimurium* Vaccine Strains Expressing a Nontoxic Shiga-Like Toxin 2 Derivative Induce Partial Protective Immunity to the Toxin Expressed by Enterohemorrhagic *Escherichia coli*. *Clinical and Vaccine Immunology* **17**, 529-536.

Ryan, C. A., Tauxe, R. V., Hosek, G. W., Wells, J. G., Stoesz, P. A., McFadden, H. W., Jr., Smith, P. W., Wright, G. F. & Blake, P. A. (1986). *Escherichia coli* O157:H7 diarrhea in a nursing home: clinical, epidemiological, and pathological findings. *The Journal of infectious diseases* **154**, 631-638.

S

Saez, D., Fernandez, P., Rivera, A., Andrews, E. & Onate, A. (2012). Oral immunization of mice with recombinant *Lactococcus lactis* expressing Cu,Zn superoxide dismutase of *Brucella abortus* triggers protective immunity. *Vaccine* **30**, 1283-1290.

Saklani-Jusforgues, H., Fontan, E., Soussi, N., Milon, G. & Goossens, P. L. (2003). Enteral immunization with attenuated recombinant *Listeria monocytogenes* as a live vaccine vector: Organ-dependent dynamics of CD4 T lymphocytes reactive to a Leishmania major tracer epitope. *Infection and immunity* **71**, 1083-1090.

Salkowski, C. A., Detore, G. R. & Vogel, S. N. (1997). Lipopolysaccharide and monophosphoryl lipid a differentially regulate interleukin-12, gamma interferon, and interleukin-10 mRNA production in murine macrophages. *Infection and immunity* **65**, 3239-3247.

Sanchez, J., Wallerstrom, G., Fredriksson, M., Angstrom, J. & Holmgren, J. (2002). Detoxification of cholera toxin without removal of its immunoadjuvanticity by the addition of (STa-related) peptides to the catalytic subunit - A potential new strategy to generate immunostimulants for vaccination. *Journal of Biological Chemistry* **277**, 33369-33377.

Sanchez, J. & Holmgren, J. (2008). Cholera toxin structure, gene regulation and pathophysiological and immunological aspects. *Cell Mol Life Sci* **65**, 1347-1360.

Sanders, M. T., Brown, L. E., Deliyannis, G. & Pearse, M. J. (2005). ISCOM (TM)-based vaccines: The second decade. *Immunol Cell Biol* **83**, 119-128.

Sandine, W. E. (1977). New Techniques in Handling Lactic Cultures to Enhance Their Performance. *J Dairy Sci* **60**, 822-828.

Sandvig, K. & van Deurs, B. (2002). Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. *Febs Lett* **529**, 49-53.

Sato, A., Hashiguchi, M., Toda, E., Iwasaki, A., Hachimura, S. & Kaminogawa, S. (2003). $CD11b^{+}$ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. *Journal of immunology* **171**, 3684-3690.

Sauter, K. A. D., Melton-Celsa, A. R., Larkin, K., Troxell, M. L., O'Brien, A. D. & Magun, B. E. (2008). Mouse model of hemolytic-uremic syndrome caused by endotoxin-free Shiga toxin 2 (Stx2) and protection from lethal outcome by anti-Stx2 antibody. *Infection and immunity* **76**, 4469-4478.

Sawamura, S., Tanaka, K. & Koga, Y. (1999). Therapeutic effects of antibiotics against enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (O157) infection: *in vivo* analysis using germfree mice. *Kansenshogaku zasshi The Journal of the Japanese Association for Infectious Diseases* **73**, 1054-1063.

Scheiring, J., Andreoli, S. P. & Zimmerhackl, L. B. (2008). Treatment and outcome of Shiga-toxin-associated hemolytic uremic syndrome (HUS). *Pediatric nephrology* **23**, 1749-1760.

Schneider-Ohrum, K. & Ross, T. M. (2012). Virus-Like Particles for Antigen Delivery at Mucosal Surfaces. *Curr Top Microbiol* **354**, 53-73.

Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme Microb Tech* **27**, 761-765.

Schwartz-Cornil, I., Benureau, Y., Greenberg, H., Hendrickson, B. A. & Cohen, J. (2002). Heterologous protection induced by the inner capsid proteins of rotavirus requires transcytosis of mucosal immunoglobulins. *J Virol* **76**, 8110-8117.

Serna, A. & Boedeker, E. C. (2008). Pathogenesis and treatment of Shiga toxin-producing *Escherichia coli* infections. *Current opinion in gastroenterology* **24**, 38-47.

Sevil Domenech, V. E., Panthel, K., Meinel, K. M., Winter, S. E. & Russmann, H. (2007). Pre-existing anti-*Salmonella* vector immunity prevents the development of protective antigen-specific CD8 T-cell frequencies against murine listeriosis. *Microbes and infection / Institut Pasteur* **9**, 1447-1453.

Sharma, R., Agrawal, U., Mody, N. & Vyas, S. P. (2014). Polymer nanotechnology based approaches in mucosal vaccine delivery: Challenges and opportunities. *Biotechnology advances*.

Sheoran, A. S., Chapman-Bonofiglio, S., Harvey, B. R., Mukherjee, J., Georgiou, G., Donohue-Rolfe, A. & Tzipori, S. (2005). Human antibody against Shiga toxin 2 administered to piglets after the onset of diarrhea due to *Escherichia coli* O157 : H7 prevents fatal systemic complications. *Infection and immunity* **73**, 4607-4613.

Shimizu, K., Asahara, T., Nomoto, K., Tanaka, R., Hamabata, T., Ozawa, A. & Takeda, Y. (2003). Development of a lethal Shiga toxin-producing *Escherichia coli*-infection mouse model using multiple mitomycin C treatment. *Microbial pathogenesis* **35**, 1-9.

Siegers, K. & Entian, K. D. (1995). Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis* 6F3. *Appl Environ Microbiol* **61**, 1082-1089.

Sim, A. C. N., Lin, W., Tan, G. K. X., Sim, M. S. T., Chow, V. T. K. & Alonso, S. (2008). Induction of neutralizing antibodies against dengue virus type 2 upon mucosal administration of a recombinant *Lactococcus lactis* strain expressing envelope domain III antigen. *Vaccine* **26**, 1145-1154.

Sinclair, J. F. & O'Brien, A. D. (2002). Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157 : H7. *Journal of Biological Chemistry* **277**, 2876-2885.

Sirard, J. C., Niedergang, F. & Kraehenbuhl, J. P. (1999). Live attenuated *Salmonella*: a paradigm of mucosal vaccines. *Immunological reviews* **171**, 5-26.

Smith, K. E., Wilker, P. R., Reiter, P. L., Hedican, E. B., Bender, J. B. & Hedberg, C. W. (2012). Antibiotic Treatment of *Escherichia coli* O157 Infection and the Risk of Hemolytic Uremic Syndrome, Minnesota. *Pediatr Infect Dis J* **31**, 37-41.

Smith, M. J., Teel, L. D., Carvalho, H. M., Melton-Celsa, A. R. & O'Brien, A. D. (2006). Development of a hybrid Shiga holotoxoid vaccine to elicit heterologous protection against Shiga toxins types 1 and 2. *Vaccine* **24**, 4122-4129.

Smith, W. E., Kane, A. V., Campbell, S. T., Acheson, D. W. K., Cochran, B. H. & Thorpe, C. M. (2003). Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infection and immunity* **71**, 1497-1504.

Snedeker, K. G., Shaw, D. J., Locking, M. E. & Prescott, R. J. (2009). Primary and secondary cases in *Escherichia coli* O157 outbreaks: a statistical analysis. *Bmc Infect Dis* **9**.

Staats, H. F. & Ennis, F. A. (1999). IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *Journal of immunology* **162**, 6141-6147.

Staats, H. F., Bradney, C. P., Gwinn, W. M., Jackson, S. S., Sempowski, G. D., Liao, H. X., Letvin, N. L. & Haynes, B. F. (2001). Cytokine requirements for induction of systemic and mucosal CTL after nasal immunization. *Journal of immunology* **167**, 5386-5394.

Stahl, A. L., Svensson, M., Morgelin, M., Svanborg, C., Tarr, P. I., Mooney, J. C., Watkins, S. L., Johnson, R. & Karpman, D. (2006). Lipopolysaccharide from enterohemorrhagic *Escherichia coli* binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. *Blood* **108**, 167-176.

Steidler, L., Wells, J. M., Raeymaekers, A., Vandekerckhove, J., Fiers, W. & Remaut, E. (1995). Secretion of Biologically-Active Murine Interleukin-2 by *Lactococcus lactis* Subsp *lactis*. *Appl Environ Microb* **61**, 1627-1629.

Steidler, L., Viaene, J., Fiers, W. & Remaut, E. (1998). Functional display of a heterologous protein on the surface of *Lactococcus lactis* by means of the cell wall anchor of *Staphylococcus aureus* protein A. *Appl Environ Microbiol* **64**, 342-345.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352-1355.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. & Remaut, E. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nature biotechnology* **21**, 785-789.

Stein, P. E., Boodhoo, A., Tyrrell, G. J., Brunton, J. L. & Read, R. J. (1992). Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature* **355**, 748-750.

Stevens, M. P., van Diemen, P. M., Frankel, G., Phillips, A. D. & Wallis, T. S. (2002). Efa1 influences colonization of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infection and immunity* **70**, 5158-5166.

Strockbine, N. A., Marques, L. R., Newland, J. W., Smith, H. W., Holmes, R. K. & O'Brien, A. D. (1986). Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infection and immunity* **53**, 135-140.

Sun, C. M., Hall, J. A., Blank, R. B., Bouladoux, N., Oukka, M., Mora, J. R. & Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *The Journal of experimental medicine* **204**, 1775-1785.

Sun, H. X., Xie, Y. & Ye, Y. P. (2009). ISCOMs and ISCOMATRIX. *Vaccine* **27**, 4388-4401.

Suzuki, C., Kimoto-Nira, H., Kobayashi, M., Nomura, M., Sasaki, K. & Mizurnachi, K. (2008). Immunomodulatory and cytotoxic effects of various *Lactococcus* strains on the murine macrophage cell line J774.1. *International journal of food microbiology* **123**, 159-165.

Suzuki, K., Kawamoto, S., Maruya, M. & Fagarasan, S. (2010a). GALT: Organization and Dynamics Leading to IgA Synthesis. *Adv Immunol* **107**, 153-185.

Suzuki, K., Maruya, M., Kawamoto, S., Sitnik, K., Kitamura, H., Agace, W. W. & Fagarasan, S. (2010b). The Sensing of Environmental Stimuli by Follicular Dendritic Cells Promotes Immunoglobulin A Generation in the Gut. *Immunity* **33**, 71-83.

Swerdlow, D. L., Woodruff, B. A., Brady, R. C. & other authors (1992). A Waterborne Outbreak in Missouri of *Escherichia coli*-O157-H7 Associated with Bloody Diarrhea and Death. *Annals of internal medicine* **117**, 812-819.

T-U

Taguchi, H., Takahashi, M., Yamaguchi, H., Osaki, T., Komatsu, A., Fujioka, Y. & Kamiya, S. (2002). Experimental infection of germ-free mice with hyper-toxigenic enterohaemorrhagic *Escherichia coli* O157:H7, strain 6. *Journal of medical microbiology* **51**, 336-343.

Takahashi, I., Nochi, T., Yuki, Y. & Kiyono, H. (2009). New horizon of mucosal immunity and vaccines. *Curr Opin Immunol* **21**, 352-358.

Takao, T., Tanabe, T., Hong, Y. M., Shimonishi, Y., Kurazono, H., Yutsudo, T., Sasakawa, C., Yoshikawa, M. & Takeda, Y. (1988). Identity of Molecular-Structure of Shiga-Like Toxin-I (Vt1) from *Escherichia coli* O157-H7 with That of Shiga Toxin. *Microbial pathogenesis* **5**, 357-369.

Tang, L. J. & Li, Y. J. (2009). Oral immunization of mice with recombinant *Lactococcus lactis* expressing porcine transmissible gastroenteritis virus spike glycoprotein. *Virus Genes* **39**, 238-245.

Tarr, P. I., Gordon, C. A. & Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **365**, 1073-1086.

Tatarczak, M., Wieczorek, K., Posse, B. & Osek, J. (2005). Identification of putative adhesin genes in shigatoxigenic *Escherichia coli* isolated from different sources. *Vet Microbiol* **110**, 77-85.

Tatsuno, I., Horie, M., Abe, H. & other authors (2001). toxB gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infection and immunity* **69**, 6660-6669.

te Loo, D. M., Monnens, L. A., van Der Velden, T. J., Vermeer, M. A., Preyers, F., Demacker, P. N., van Den Heuvel, L. P. & van Hinsbergh, V. W. (2000). Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood* **95**, 3396-3402.

Teel, L. D., Melton-Celsa, A. R., Schmitt, C. K. & O'Brien, A. D. (2002). One of two copies of the gene for the activatable shiga toxin type 2d in *Escherichia coli* O91:H21 strain B2F1 is associated with an inducible bacteriophage. *Infection and immunity* **70**, 4282-4291.

Teunis, P. F., Ogden, I. D. & Strachan, N. J. (2008). Hierarchical dose response of *E. coli* O157:H7 from human outbreaks incorporating heterogeneity in exposure. *Epidemiology and infection* **136**, 761-770.

Tezuka, H., Abe, Y., Iwata, M., Takeuchi, H., Ishikawa, H., Matsushita, M., Shiohara, T., Akira, S. & Ohteki, T. (2007a). Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* **448**, 929-U927.

Tezuka, H., Abe, Y., Iwata, M., Takeuchi, H., Ishikawa, H., Matsushita, M., Shiohara, T., Akira, S. & Ohteki, T. (2007b). Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* **448**, 929-933.

Tezuka, H., Abe, Y., Asano, J., Sato, T., Liu, J. J., Iwata, M. & Ohteki, T. (2011). Prominent Role for Plasmacytoid Dendritic Cells in Mucosal T Cell-Independent IgA Induction. *Immunity* **34**, 247-257.

Theobald, I., Kuwertz-Broking, E., Schiborr, M. & Heindel, W. (2001). Central nervous system involvement in hemolytic uremic syndrome (HUS)– a retrospective analysis of cerebral CT and MRI studies. *Clin Nephrol* **56**, S3-8.

Tilden, J., Jr., Young, W., McNamara, A. M. & other authors (1996). A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *American journal of public health* **86**, 1142-1145.

Tjalsma, H., Bolhuis, A., Jongbloed, J. D., Bron, S. & van Dijl, J. M. (2000). Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. *Microbiology and molecular biology reviews : MMBR* **64**, 515-547.

Tobe, T., Beatson, S. A., Taniguchi, H. & other authors (2006). An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14941-14946.

Toma, C., Martinez Espinosa, E., Song, T., Miliwebsky, E., Chinen, I., Iyoda, S., Iwanaga, M. & Rivas, M. (2004). Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli*. *Journal of clinical microbiology* **42**, 4937-4946.

Torres, A. G., Giron, J. A., Perna, N. T., Burland, V., Blattner, F. R., Avelino-Flores, F. & Kaper, J. B. (2002). Identification and characterization of lpfABCC ' DE, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157 : H7. *Infection and immunity* **70**, 5416-5427.

Torres, A. G. & Kaper, J. B. (2003). Multiple elements controlling adherence of enterohemorrhagic *Escherichia coli* O157 : H7 to HeLa cells. *Infection and immunity* **71**, 4985-4995.

Torres, A. G., Kanack, K. J., Tutt, C. B., Popov, V. & Kaper, J. B. (2004). Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157 : H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. *Fems Microbiol Lett* **238**, 333-344.

Tsuji, M., Suzuki, K., Kitamura, H., Maruya, M., Kinoshita, K., Ivanov, II, Itoh, K., Littman, D. R. & Fagarasan, S. (2008a). Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity* **29**, 261-271.

Tsuji, M., Suzuki, K., Kitamura, H., Maruya, M., Kinoshita, K., Ivanov, I. I., Itoh, K., Littman, D. R. & Fagarasan, S. (2008b). Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin a generation in the gut. *Immunity* **29**, 261-271.

Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S. & Fagarasan, S. (2009). Preferential generation of follicular B helper T cells from $Foxp3^{+}$ T cells in gut Peyer's patches. *Science* **323**, 1488-1492.

Tsuji, T., Shimizu, T., Sasaki, K. & other authors (2008c). A nasal vaccine comprising B-subunit derivative of Shiga toxin 2 for cross-protection against Shiga toxin types 1 and 2. *Vaccine* **26**, 2092-2099.

Tucker, S. N., Tingley, D. W. & Scallan, C. D. (2008). Oral adenoviral-based vaccines: historical perspective and future opportunity. *Expert Rev Vaccines* **7**, 25-31.

Tzipori, S., Wachsmuth, I. K., Chapman, C., Birner, R., Brittingham, J., Jackson, C. & Hogg, J. (1986). The Pathogenesis of Hemorrhagic Colitis Caused by *Escherichia coli* O157-H7 in Gnotobiotic Piglets. *Journal of Infectious Diseases* **154**, 712-716.

Tzipori, S., Karch, H., Wachsmuth, K. I., Robinsbrowne, R. M., O'Brien, A. D., Lior, H., Cohen, M. L., Smithers, J. & Levine, M. M. (1987). Role of a 60-Megadalton Plasmid and Shiga-Like Toxins in the Pathogenesis of Infection Caused by Enterohemorrhagic *Escherichia coli* O157 - H7 in Gnotobiotic Piglets. *Infection and immunity* **55**, 3117-3125.

Tzipori, S., Wachsmuth, K. I., Smithers, J. & Jackson, C. (1988). Studies in Gnotobiotic Piglets on Non-O157-H7 *Escherichia coli* Serotypes Isolated from Patients with Hemorrhagic Colitis. *Gastroenterology* **94**, 590-597.

Tzipori, S., Gibson, R. & Montanaro, J. (1989). Nature and Distribution of Mucosal Lesions Associated with Enteropathogenic and Enterohemorrhagic *Escherichia coli* in Piglets and the Role of Plasmid-Mediated Factors. *Infection and immunity* **57**, 1142-1150.

Tzipori, S., Sheoran, A., Akiyoshi, D., Donohue-Rolfe, A. & Trachtman, H. (2004). Antibody therapy in the management of Shiga toxin-induced hemolytic uremic syndrome. *Clin Microbiol Rev* **17**, 926.

Uematsu, S., Fujimoto, K., Jang, M. H. & other authors (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nature immunology* **9**, 769-776.

Uematsu, S. & Akira, S. (2009). Immune responses of $TLR5^{+}$ lamina propria dendritic cells in enterobacterial infection. *Journal of gastroenterology* **44**, 803-811.

V

van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R. J., de Vos, W. M. & Simons, G. (1990). Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* **95**, 155-160.

van der Lubben, I. M., Verhoef, J. C., Borchard, G. & Junginger, H. E. (2001). Chitosan and its derivatives in mucosal drug and vaccine delivery. *Eur J Pharm Sci* **14**, 201-207.

Vandenbergh, P. A. (1993). Lactic-Acid Bacteria, Their Metabolic Products and Interference with Microbial-Growth. *Fems Microbiol Rev* **12**, 221-238.

Vandenbroucke, K., Hans, W., Van Huysse, J., Neirynck, S., Demetter, P., Remaut, E., Rottiers, P. & Steidler, L. (2004). Active delivery of trefoil factors by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice. *Gastroenterology* **127**, 502-513.

Vandervossen, J. M. B. M., Kok, J. & Venema, G. (1985). Construction of Cloning, Promoter-Screening, and Terminator-Screening Shuttle Vectors for *Bacillus subtilis* and *Streptococcus lactis*. *Appl Environ Microb* **50**, 540-542.

Vandervossen, J. M. B. M., Vanderlelie, D. & Venema, G. (1987). Isolation and Characterization of *Streptococcus cremoris* Wg2-Specific Promoters. *Appl Environ Microb* **53**, 2452-2457.

Varma, J. K., Greene, K. D., Reller, M. E. & other authors (2003). An outbreak of *Escherichia coli* O157 infection following exposure to a contaminated building. *Jama-J Am Med Assoc* **290**, 2709-2712.

Vemula, S. V. & Mittal, S. K. (2010). Production of adenovirus vectors and their use as a delivery system for influenza vaccines. *Expert Opin Biol Th* **10**, 1469-1487.

Vesa, T., Pochart, P. & Marteau, P. (2000). Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment Pharm Therap* **14**, 823-828.

Villena, J., Medina, M., Raya, R. & Alvarez, S. (2008). Oral immunization with recombinant *Lactococcus lactis* confers protection against respiratory pneumococcal infection. *Can J Microbiol* **54**, 845-853.

Villena, J., Medina, M., Racedo, S. & Alvarez, S. (2010). Resistance of Young Mice to Pneumococcal Infection can be Improved by Oral Vaccination with Recombinant *Lactococcus lactis*. *J Microbiol Immunol* **43**, 1-10.

Villinger, F. (2003). Cytokines as clinical adjuvants: how far are we? *Expert Rev Vaccines* **2**, 317-326.

Vilte, D. A., Larzabal, M., Mayr, U. B. & other authors (2012). A systemic vaccine based on *Escherichia coli* O157:H7 bacterial ghosts (BGs) reduces the excretion of *E. coli* O157:H7 in calves. *Veterinary immunology and immunopathology* **146**, 169-176.

von Heijne, G. (1990). The signal peptide. *The Journal of membrane biology* **115**, 195-201.

W-X

Waddell, T. E., Lingwood, C. A. & Gyles, C. L. (1996). Interaction of verotoxin 2e with pig intestine. *Infection and immunity* **64**, 1714-1719.

Wadolkowski, E. A., Burris, J. A. & O'Brien, A. D. (1990a). Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. *Infection and immunity* **58**, 2438-2445.

Wadolkowski, E. A., Burris, J. A. & O'Brien, A. D. (1990b). Mouse Model for Colonization and Disease Caused by Enterohemorrhagic *Escherichia coli* O157-H7. *Infection and immunity* **58**, 2438-2445.

Waeytens, A., Ferdinande, L., Neirynck, S., Rottiers, P., De Vos, M., Steidler, L. & Cuvelier, C. A. (2008). Paracellular entry of interleukin-10 producing *Lactococcus lactis* in inflamed intestinal mucosa in mice. *Inflammatory bowel diseases* **14**, 471-479.

Wan, C. S., Zhou, Y., Yu, Y., Peng, L. J., Zhao, W. & Zheng, X. L. (2011). B-cell epitope KT-12 of enterohemorrhagic *Escherichia coli* O157:H7: a novel peptide vaccine candidate. *Microbiol Immunol* **55**, 247-253.

Wang, S. F., Kong, Q. K. & Curtiss, R. (2013). New technologies in developing recombinant attenuated *Salmonella* vaccine vectors. *Microb Pathogenesis* **58**, 17-28.

Wang, Z., Gao, J., Yu, Q. & Yang, Q. (2012). Oral immunization with recombinant *Lactococcus lactis* expressing the hemagglutinin of the avian influenza virus induces mucosal and systemic immune responses. *Future microbiology* **7**, 1003-1010.

Waterfield, N. R., Lepage, R. W. F., Wilson, P. W. & Wells, J. M. (1995). The Isolation of Lactococcal Promoters and Their Use in Investigating Bacterial Luciferase Synthesis in *Lactococcus lactis*. *Gene* **165**, 9-15.

Wegmann, U., O'Connell-Motherwy, M., Zomer, A. & other authors (2007). Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp *cremoris* MG1363. *J Bacteriol* **189**, 3256-3270.

Wells, J.M., and Schofiled, K.M. (1996). Cloning and expression vectors for lactococci. *NATO ASI Series* **H 98**, 37-62.

Weyer, J., Rupprecht, C. E. & Nel, L. H. (2009). Poxvirus-vectored vaccines for rabies-A review. *Vaccine* **27**, 7198-7201.

Wong, C. S., Jelacic, S., Habeeb, R. L., Watkins, S. L. & Tarr, P. I. (2000). The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *The New England journal of medicine* **342**, 1930-1936.

Xin, K. Q., Hoshino, Y., Toda, Y. & other authors (2003). Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. *Blood* **102**, 223-228.

Xu, W. F., He, B., Chiu, A. & other authors (2007). Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nature immunology* **8**, 294-303.

Y-Z

Yam, K. K., Pouliot, P., N'diaye, M. M., Fournier, S., Olivier, M. & Cousineau, B. (2008). Innate inflammatory responses to the Gram-positive bacterium *Lactococcus lactis*. *Vaccine* **26**, 2689-2699.

Yang, X. Q., Zhao, Y. G., Chen, X. Q., Jiang, B. & Sun, D. Y. (2013). The protective effect of recombinant *Lactococcus lactis* oral vaccine on a *Clostridium difficile*-infected animal model. *BMC gastroenterology* **13**, 117.

Yoshida, M., Claypool, S. M., Wagner, J. S., Mizoguchi, E., Mizoguchi, A., Roopenian, P. C., Lencer, W. I. & Blumberg, R. S. (2004). Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Gastroenterology* **126**, A104-A104.

Zamri, H. F., Shamsudin, M. N., Rahim, R. A. & Neela, V. (2012). Oral vaccination with *Lactococcus lactis* expressing the *Vibrio cholerae* Wzm protein to enhance mucosal and systemic immunity. *Vaccine* **30**, 3231-3238.

Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A. & Casali, P. (1998). CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S gamma, S gamma-->S alpha DNA recombination. *Journal of immunology* **161**, 5217-5225.

Zhang, H. X., Qiu, Y. Y., Zhao, Y. H., Liu, X. T., Liu, M. & Yu, A. L. (2014). Immunogenicity of oral vaccination with *Lactococcus lactis* derived vaccine candidate antigen (UreB) of *Helicobacter pylori* fused with the human interleukin 2 as adjuvant. *Molecular and cellular probes* **28**, 25-30.

Zhang, K., Mills, F. C. & Saxon, A. (1994). Switch circles from IL-4-directed epsilon class switching from human B lymphocytes. Evidence for direct, sequential, and multiple step sequential switch from mu to epsilon Ig heavy chain gene. *Journal of immunology* **152**, 3427-3435.

Zhang, Q., Donohue-Rolfe, A., Krautz-Peterson, G., Sevo, M., Parry, N., Abeijon, C. & Tzipori, S. (2009). Gnotobiotic piglet infection model for evaluating the safe use of antibiotics against *Escherichia coli* O157:H7 infection. *The Journal of infectious diseases* **199**, 486-493.

Zhang, Q. X., Zhong, J. & Huan, L. D. (2011a). Expression of hepatitis B virus surface antigen determinants in *Lactococcus lactis* for oral vaccination. *Microbiol Res* **166**, 111-120.

Zhang, X., McDaniel, A. D., Wolf, L. E., Keusch, G. T., Waldor, M. K. & Acheson, D. W. (2000). Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *The Journal of infectious diseases* **181**, 664-670.

Zhang, X. H., He, K. W., Zhang, S. X. & other authors (2011b). Subcutaneous and intranasal immunization with Stx2B-Tir-Stx1B-Zot reduces colonization and shedding of *Escherichia coli* O157:H7 in mice. *Vaccine* **29**, 3923-3929.

Zhang, Z. H., Jiang, P. H., Li, N. J., Shi, M. & Huang, W. D. (2005). Oral vaccination of mice against rodent malaria with recombinant *Lactococcus lactis* expressing MSP-119. *World J Gastroentero* **11**, 6975-6980.

Zheng, Y., Valdez, P. A., Danilenko, D. M. & other authors (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* **14**, 282-289.

Zhou, X. X., Wang, Y. B., Pan, Y. J. & Li, W. F. (2008). Nisin-controlled extracellular production of apidaecin in *Lactococcus lactis*. *Applied microbiology and biotechnology* **78**, 947-953.

SCOPE AND OBJECTIVES

Background:

Lactococcus lactis is a Gram-positive lactic acid bacterium that offers a promising biological tool for the delivery of oral vaccines. The bacterium has been traditionally used in the food industry and is generally regarded as safe for human consumption (FDA "GRAS" status). Additionally, *.L lactis* is amenable to genetic modification and capable of synthesizing heterologous proteins of various origins. Besides its potential for antigen delivery, *.L lactis* possesses adjuvant properties, which are particularly useful to enhance the immune responses to vectored antigens.

In the present thesis, we explored the utilization of *.L lactis* for the development of an oral vaccine against Enterohemorrhagic *Escherichia coli* infection (EHEC) in humans. EHEC infection can result in hemorrhagic colitis and the hemolytic uremic syndrome, potentially life-threatening conditions that represent serious public health concern. Since antibiotic therapy appears to be ineffective in treatment of EHEC disease, preventing infection by vaccination would be the choice for people at higher risk. However, no vaccine is currently available to prevent the infection. Vaccines against enteropathogens should induce immune responses which eliminate or neutralize the microorganism. Toxins or structures involved in colonization are preferred targets. For EHEC, the type 3 secretion system is important for colonization. In this thesis we explored for the first time if intestinal mucosal immunization with EspB, a protein at the tip of the needle formed by the type 3 secretion system, could be sufficient to clear infection. Hereto *L. lactis* strains where constructed that could produce the protein and could be used in oral immunization experiments. For a proof of concept, we evaluated the protective efficacy of our vaccine in a mouse model. However, since pigs offer a more reliable model for EHEC infection as well as for human immunity, we addressed the issue of the gastrointestinal survival of *.L lactis*, one of the most important challenges for the validation of the EHEC *.L lactis*-based vaccine in pig models.

The scope of our research included the following aims:

1. The development of recombinant *L. lactis* strains that properly expresses the vaccine antigen, EspB, for oral vaccination purposes (**Chapters 2 and 3**).

2. Evaluation of the mucosal and systemic antibody and cellular immune responses elicited by the vaccine strains in mice upon oral immunization (**Chapters 2 and 3**).

3. Evaluation of the protective potential of the *L. lactis*-based EspB vaccine against a challenge infection with EHEC in a mouse model (**Chapter 3**).

4. Optimization of the survival of the live vaccine-delivery vector in the gastrointestinal tract of pigs (**Chapter 4**).

CHAPTER 2

Mucosal priming of the murine immune system against enterohemorrhagic *Escherichia coli* O157:H7 using *Lactococcus lactis* expressing the type III secretion system protein EspB

Adapted from

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2.1. Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) have been responsible for multiple outbreaks of hemorrhagic colitis and hemolytic uremic syndrome in humans worldwide. Humans become infected by direct or indirect contact with feces of asymptomatic EHEC shedding ruminants. Currently there is no human vaccine available against EHEC infection. EHEC use a Type III secretion system (T3SS) to colonize the intestine and therefore eliciting mucosal immunity against T3SS proteins could be a potential vaccination strategy. To develop an oral vaccine against EHEC infection, EspB, a significant member of the T3SS, was expressed in the cytoplasm of *Lactococcus lactis*. The latter is a generally regarded as safe (GRAS) bacterium that has been used as a delivery vector for oral vaccines. The constructed recombinant strain (LL-pT:CYT:EspB) was used to immunize BALB/c mice via the oral route. Ten days post-immunization, no specific antibody response was detected in serum or feces of immunized mice. However, significant levels of specific serum Ig and faecal IgA were detected after intraperitoneal boosting of the orally immunized mice with purified EspB ($P < 0.0001$). Our results show that oral administration of LL-pT:CYT:EspB resulted in mucosal priming of mice against the EHEC T3SS protein, EspB. Nevertheless, an optimized EspB expression in *L. lactis* may be required to obtain complete immune response.

2.2. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is one group of colonizing enteropathogens that has been responsible for frequent outbreaks of diarrhea and hemorrhagic colitis (HC) worldwide (Griffin & Tauxe, 1991). HC occasionally progresses to hemolytic-uremic syndrome (Janot *et al.*, 2009), which is the most common cause of acute renal failure in children (Trachtman *et al.*, 2003) and results in fatality rates as high as 50% in the elderly (Carter *et al.*, 1987). HC and HUS are caused by shiga-like toxins (Stxs), which are released by colonizing EHEC into the systemic circulation and induce endothelial damage in intestinal and renal vasculatures (Serna & Boedeker, 2008). Several studies have linked antibiotic therapy to higher rates of HUS development and prolonged duration of the symptomatic disease, probably due to the excessive release of Stxs upon bacterial lysis (Carter *et al.*, 1987; Pavia *et al.*, 1990; Wong *et al.*, 2000; Wong *et al.*, 2012). Therefore, current disease intervention strategies are rather focusing on vaccination. However, no vaccine is currently available for

EHEC infections. Since the Stxs-mediated EHEC diseases develop subsequent to the bacterial intestinal colonization, the latter is considered a key determinant of EHEC pathogenicity and represents a potential target for vaccine design. EHEC colonizes the intestine with the aid of a type III secretion system (T3SS) which enables the bacteria to establish a tight adherence to enterocytes and to modify their cytoskeletal proteins, leading to the characteristic attaching and effacing lesion (Hamada, 2010). EspB is a T3SS protein that plays a central role in mediating the EHEC enterocyte adherence (Frankel *et al.*, 1998; Hamada, 2010; Iizumi *et al.*, 2007; Knappstein *et al.*, 2004; Kodama *et al.*, 2002). Moreover, EspB is highly immunogenic in human patients (Asper *et al.*, 2011; Karpman *et al.*, 2002; Li *et al.*, 2000) and in infected or vaccinated animals (Asper *et al.*, 2011; Cataldi *et al.*, 2008). Therefore, EspB is a potential candidate antigen for vaccination against EHEC. Oral vaccination can be a particularly efficient approach to interfere with EHEC intestinal colonization, as it can effectively induce local immune responses at the intestinal mucosa, and concurrently elicit systemic immune responses (Neutra & Kozlowski, 2006). Nevertheless, oral vaccines are at the risk of being degraded by the harsh gastrointestinal conditions. Thus, vaccine delivery systems represent a useful strategy to ensure the efficient oral delivery of antigens (Lycke, 2012; Pavot *et al.*, 2012). One of the interesting oral antigen delivery systems is based on genetically modified lactic acid bacteria (Yasar *et al.*, 2011) that simultaneously express and deliver antigens to the intestinal mucosa (Berlec *et al.*, 2012; Tarahomjoo, 2012). LAB have been traditionally used in food industry and are generally regarded as safe (GRAS) for human consumption. Therefore, they particularly offer a safe tool for the oral delivery of vaccines. *Lactococcus lactis* is a model LAB that has been extensively studied for oral vaccine delivery. Numerous bacterial, viral and parasitic antigens were expressed in *L. lactis* and the resultant recombinant strains were capable of inducing specific mucosal and systemic immune responses in mice upon oral administration (for a recent review, Wells, 2011). Here, we report the construction of a *L. lactis* strain which constitutively expresses the EHEC antigen, EspB in the cytoplasm (LL-pT:CYT:EspB). Oral immunization of BALB/c mice with LL-EspB has resulted in mucosal priming of the immune system against EspB.

2.3. Material and methods

2.3.1. Bacterial strains and media

L. lactis subspecies *cremoris* MG1363 is a derivative of the dairy starter strain NCDO712, which was cured of all resident plasmids (Gasson, 1983). *L. lactis* was routinely grown overnight as a standing culture at 30°C in liquid M17 medium (Difco, Michigan, USA), supplemented with 0.5% glucose and 5µg/ml erythromycin (GM17E). The bacteria reach a saturation density of 2 x 10^9 cfu/ml within 18 hours of incubation. *E. coli* strains were grown in Luria-Bertani (LB) medium (Difco) at 37°C with vigorous shaking. When required, the medium was supplemented with ampicillin, at a concentration of 100 µg/ml. Stock suspensions of the recombinant strains were made in 50% glycerol and stored at -20°C.

2.3.2. Plasmid construction for the cytoplasmic expression of EspB in *L. lactis*

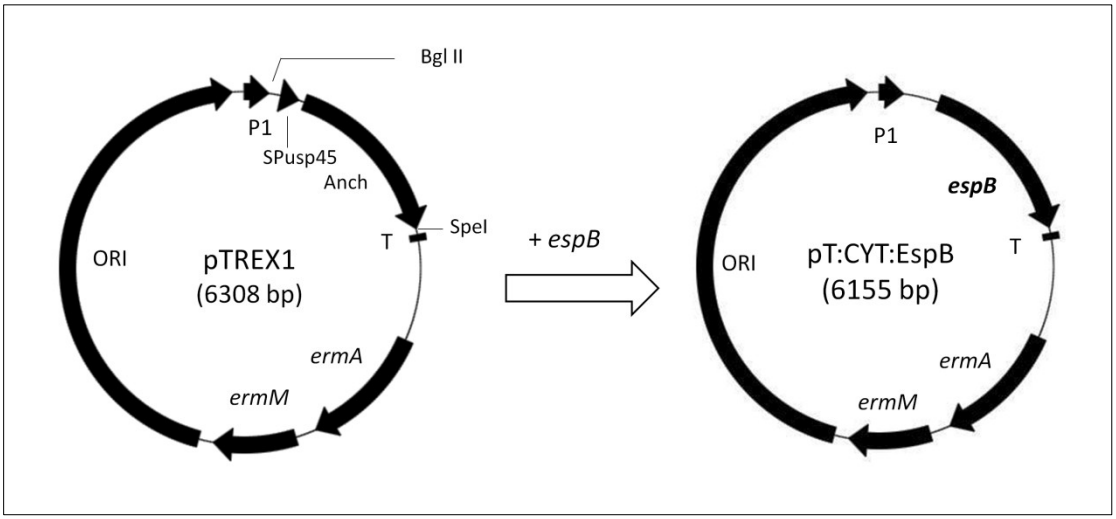

Figure 2.1 Schematic illustration of the lactococcal vector for EspB expression. The EspB gene is inserted into the parent plasmid pTREX1, downstream of the lactococcal promoter P1 between between *Bgl* II and *Spe I* restriction sites.

The DNA sequence encoding the EspB gene of *E. coli* O157:H7 was retrieved from the GenBank (Accession no: NC_002655). This sequence was adapted to the preferential codon use of *L. lactis* and artificially synthesized by Genscript (New York, USA). The EspB coding sequence was then inserted into the lactococcal expression plasmid pTREX1 (Robinson *et al.*, 1997a), downstream of the P1 constitutive promoter,

between *Bgl* II and *Spe I* restriction sites (Figure 2.1). The resulting pT:CYT:EspB plasmid was introduced in *L. lactis* MG1363 by electroporation (Wells *et al.*, 1993). The resulting strain was designated LL-pT:CYT:EspB.

2.3.3. EspB purification

E. coli DH5α harboring the pCVD468 was used for the recombinant EspB expression and purification (Karpman, 2002 #16). Briefly, the transformed bacteria were induced with 1 mM isopropyl-β-d-thiogalactopyranoside and the recombinant his-tagged protein was purified by nickel affinity chromatography.

2.3.4. *L. lactis* protein extraction

A saturated overnight culture of the strain LL-pT:CYT:EspB was grown as described earlier. Bacteria were harvested by centrifugation at 5000 *g* for 2 minutes. The cell wall was disrupted by resuspending the pellet in Tris-EDTA buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA) containing lysozyme (1 mg/ml), and incubating them for 1 hour at 37°C. After incubation, the digested cells were mechanically homogenized using a 24G needle and a clear lysate was obtained by centrifugation of the mixture at 5000 *g* for 2 minutes.

2.3.5. Immunizations

Two groups of 6 week-old BALB/c mice were intragastrically immunized with either LL-pT:CYT:EspB (n=5) or LL-pTREX1 (n=4). The latter is *L. lactis* MG1363 containing the empty vector pTREX1 (sham control). *L. lactis* cultures were grown overnight in GM17E at 30°C. Saturated cultures were harvested by centrifugation and concentrated 10-fold in buffered M9 salts (Schotte *et al.*, 2000). Each group of mice received three cycles of intragastric immunizations with three-week intervals. Each immunization cycle consisted of five inoculations of 2 x 10^9 cfu on five consecutive days. Twenty-one days after the last oral immunization all mice were systemically boosted by the intraperitoneal injection (i.p.) with 50 µg of purified EspB in incomplete Freund's adjuvant (Sigma-Aldrich). Serum and feces were collected 10 days after each oral immunization and after the systemic boost.

2.3.6. Preparation of serum and faecal samples for ELISA

Blood was taken and after 1 hour of incubation at 37°C, serum was collected, inactivated at 56°C for 30 min, and subsequently treated with kaolin (Sigma-Aldrich).

Therefore, 4 volumes of a kaolin suspension (25% [w/v] in PBS) were added to 1 volume of serum and incubated at room temperature for 30 min. The suspension was centrifuged at 5500 g for 10 min, and the supernatant was diluted in ELISA dilution buffer, yielding a final serum dilution of 1/10. Immediately after collection, faecal pellets were suspended at a concentration of 0.1 g/ml in PBS supplemented with 1% (w/v) BSA and complete protease inhibitors (Roche Applied Sciences, Penzberg, Germany). After overnight stirring at 4°C, the suspension was centrifuged at 7200 *g* for 10 minutes and the supernatant was used for faecal specific IgA detection by ELISA.

2.3.7. ELISA for EspB-specific antibodies

A direct ELISA was developed for the detection of EspB-specific antibodies in serum or faecal samples. An ELISA plate (Polysorp, Nunc) was coated with 5 µg/ml of recombinant EspB in PBS and incubated for 2 hours at 37°C. After incubation, the remaining binding sites were blocked by PBS supplemented with 0.2% (v/v) Tween® 80. Two-fold dilution series of serum or faecal suspensions, starting from the dilution 1:10, were prepared in ELISA dilution buffer and were added to the plate, which was incubated for 2 hours at 37°C. Subsequently, the wells were treated for 1 hour with optimal dilutions of rabbit anti-mouse Ig (for serum) or goat anti-mouse IgA (for faeces) labelled with horseradish peroxidase (Dako). The reaction was developed by adding ABTS solution containing H_2O_2 (Roche diagnostics). The optical density was spectrophotometrically measured at 405 nm (OD_{405}). Between each incubation step, plates were washed three times with PBS supplemented with 0.2% Tween® 20. The cut-off values were calculated as the mean OD_{405} –value of all sera or faecal suspensions (dilution 1:10) at day 0, increased with 3 times the standard deviation. The antibody titre is the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

2.3.8. Statistical analysis

Poisson regression, a model for regression analysis, was used to compare serum or faecal titres of individual mice of both groups. Data analysis was performed using STATA software, version 11.

2.4. Results, discussion and conclusion

L. lactis has been used before for delivery of oral vaccines to the intestinal immune system (Kim *et al.*, 2006; Robinson *et al.*, 1997b; Steidler *et al.*, 2003). Here we

investigated the mucosal immune responses in mice after oral immunization with an *L. lactis* strain, genetically modified to express the EHEC antigen EspB (LL-pT:CYT:EspB). EspB expression was quantified in the cytoplasmic contents of LL-pT:CYT:EspB using a specific sandwich ELISA: 113± 42 ng per 2 x 10^9 cfu. This rather low concentration of EspB expression by *L. lactis* could be attributed to the cytoplasmic targeting of the protein expression, since *L. lactis* possesses at least two cytoplasmic house-keeping proteases, clpP (Frees & Ingmer, 1999) and FtsH (Nilsson *et al.*, 1994), as well as DnaK, a cytoplasmic chaperone that may promote proteolysis by maintaining the protein in an unfolded state (Koch *et al.*, 1998).

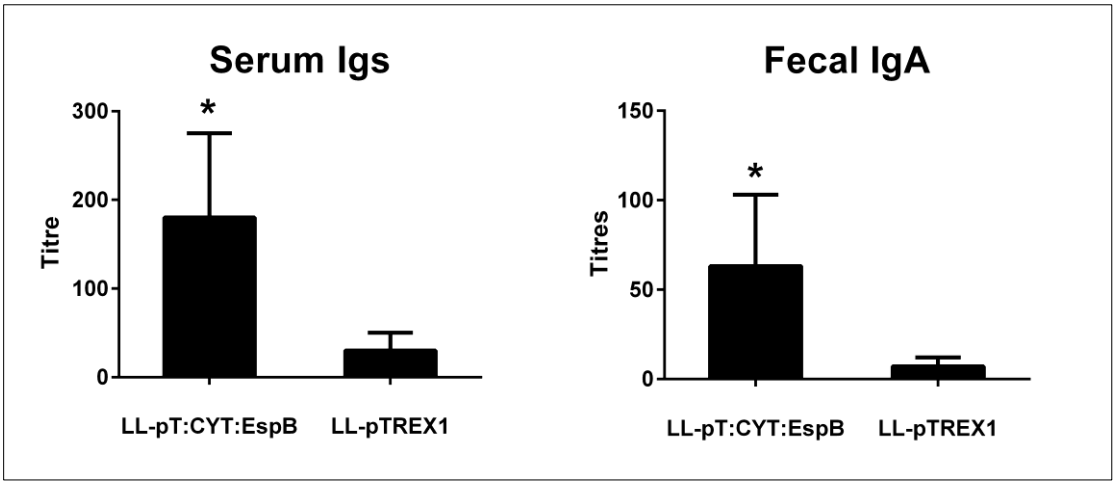

Figure 2.2. EspB-specific antibody titers in serum and feces of the mice immunized orally using either LL-pT:CYT:EspB (n=5) or LL-TREX1 (n=4), 10 days after the intraperitoneal boost with purified EspB. * = P< 0.0001

No specific antibody responses were detected in the serum or the feces following oral immunization with LL-pT:CYT:EspB. Responses remained absent after the third cycle of oral immunization. Hence, we further investigated whether priming or oral tolerance had been induced against EspB upon the oral administration of LL-pT:CYT:EspB. Oral tolerance is a state of local and systemic immune unresponsiveness that is induced by oral uptake of an innocuous antigen such as food proteins (Andre *et al.*, 1975; Iwamoto & Nakao, 1995; Swarbrick *et al.*, 1979). Therefore, the orally preimmunized mice were systemically boosted by an i.p. injection of 50 µg of purified EspB. Ten days after the systemic boost, specific serum total-Igs and faecal IgA (Figure 2.2) were detected in both groups. However, the levels of the specific antibodies were

significantly higher in the group which received LL-pT:CYT:EspB compared to the control group (P< 0.0001). This indicates that oral immunization of BALB/c mice with LL-pT:CYT:EspB resulted in a mucosal priming of the immune system against EspB and not in oral tolerance. A similar finding was reported by Lamont et al. (Lamont *et al.*, 1989), where systemic and intestinal priming of BALB/c was induced by oral administration of small doses (10-50µg) of ovalbumin. In conclusion, our results show the potential of recombinant *L. lactis* expressing EspB to be used in EHEC challenge experiments. Nevertheless, optimization of the antigen expression is required to obtain higher immune responses upon oral immunization.

2.5. Acknowledgment

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2.6. References

A-C

Andre, C., Heremans, J. F., Vaerman, J. P. & Cambiaso, C. L. (1975). A mechanism for the induction of immunological tolerance by antigen feeding: antigen-antibody complexes. *The Journal of experimental medicine* **142**, 1509-1519.

Asper, D. J., Karmali, M. A., Townsend, H., Rogan, D. & Potter, A. A. (2011). Serological response of Shiga toxin-producing *Escherichia coli* type III secreted proteins in sera from vaccinated rabbits, naturally infected cattle, and humans. *Clinical and vaccine immunology : CVI* **18**, 1052-1057.

Berlec, A., Ravnikar, M. & Strukelj, B. (2012). Lactic acid bacteria as oral delivery systems for biomolecules. *Pharmazie* **67**, 891-898.

Carter, A. O., Borczyk, A. A., Carlson, J. A., Harvey, B., Hockin, J. C., Karmali, M. A., Krishnan, C., Korn, D. A. & Lior, H. (1987). A severe outbreak of *Escherichia coli* O157:H7--associated hemorrhagic colitis in a nursing home. *The New England journal of medicine* **317**, 1496-1500.

Cataldi, A., Yevsa, T., Vilte, D. A., Schulze, K., Castro-Parodi, M., Larzabal, M., Ibarra, C., Mercado, E. C. & Guzman, C. A. (2008). Efficient immune responses against Intimin and EspB of enterohaemorragic *Escherichia coli* after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. *Vaccine* **26**, 5662-5667.

F-I

Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular microbiology* **30**, 911-921.

Frees, D. & Ingmer, H. (1999). ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Molecular microbiology* **31**, 79-87.

Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *Journal of bacteriology* **154**, 1-9.

Griffin, P. M. & Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic reviews* **13**, 60-98.

Hamada, D. (2010). Cytoskeleton-modulating effectors of enteropathogenic and enterohemorrhagic *Escherichia coli*. *The FEBS journal* **277**, 2389.

Iizumi, Y., Sagara, H., Kabe, Y. & other authors (2007). The enteropathogenic *E. coli* effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. *Cell host & microbe* **2**, 383-392.

Iwamoto, I. & Nakao, A. (1995). Induction of Th2 cell tolerance to a soluble antigen by blockade of the LFA-1-dependent pathway prevents allergic inflammation. *Immunol Res* **14**, 263-270.

Janot, L., Sirard, J. C., Secher, T. & other authors (2009). Radioresistant cells expressing TLR5 control the respiratory epithelium's innate immune responses to flagellin. *Eur J Immunol* **39**, 1587-1596.

K-N

Karpman, D., Bekassy, Z. D., Sjogren, A. C. & other authors (2002). Antibodies to intimin and *Escherichia coli* secreted proteins A and B in patients with enterohemorrhagic *Escherichia coli* infections. *Pediatric nephrology* **17**, 201-211.

Kim, S. J., Jun, D. Y., Yang, C. H. & Kim, Y. H. (2006). Expression of *Helicobacter pylori* cag12 gene in *Lactococcus lactis* MG1363 and its oral administration to induce systemic anti-Cag12 immune response in mice. *Applied microbiology and biotechnology* **72**, 462-470.

Knappstein, S., Ide, T., Schmidt, M. A. & Heusipp, G. (2004). Alpha 1-antitrypsin binds to and interferes with functionality of EspB from atypical and typical enteropathogenic *Escherichia coli* strains. *Infection and immunity* **72**, 4344-4350.

Koch, B., Kilstrup, M., Vogensen, F. K. & Hammer, K. (1998). Induced levels of heat shock proteins in a dnaK mutant of *Lactococcus lactis*. *J Bacteriol* **180**, 3873-3881.

Kodama, T., Akeda, Y., Kono, G., Takahashi, A., Imura, K., Iida, T. & Honda, T. (2002). The EspB protein of enterohaemorrhagic *Escherichia coli* interacts directly with alpha-catenin. *Cellular microbiology* **4**, 213-222.

Lamont, A. G., Mowat, A. M. & Parrott, D. M. (1989). Priming of systemic and local delayed-type hypersensitivity responses by feeding low doses of ovalbumin to mice. *Immunology* **66**, 595-599.

Li, Y., Frey, E., Mackenzie, A. M. & Finlay, B. B. (2000). Human response to *Escherichia coli* O157:H7 infection: antibodies to secreted virulence factors. *Infection and immunity* **68**, 5090-5095.

Lycke, N. (2012). Recent progress in mucosal vaccine development: potential and limitations. *Nature reviews Immunology* **12**, 592-605.

Neutra, M. R. & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature reviews Immunology* **6**, 148-158.

Nilsson, D., Lauridsen, A. A., Tomoyasu, T. & Ogura, T. (1994). A *Lactococcus lactis* Gene Encodes a Membrane-Protein with Putative Atpase Activity That Is Homologous to the Essential Escherichia-Coli Ftsh Gene-Product. *Microbiol-Uk* **140**, 2601-2610.

P-T

Pavia, A. T., Nichols, C. R., Green, D. P. & other authors (1990). Hemolytic-uremic syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations. *The Journal of pediatrics* **116**, 544-551.

Pavot, V., Rochereau, N., Genin, C., Verrier, B. & Paul, S. (2012). New insights in mucosal vaccine development. *Vaccine* **30**, 142-154.

Robinson, K., Chamberlain, L. M., Schofield, K. M., Wells, J. M. & LePage, R. W. F. (1997b). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature biotechnology* **15**, 653-657.

Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme Microb Technol* **27**, 761-765.

Serna, A. t. & Boedeker, E. C. (2008). Pathogenesis and treatment of Shiga toxin-producing *Escherichia coli* infections. *Current opinion in gastroenterology* **24**, 38-47.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. & Remaut, E. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nature biotechnology* **21**, 785-789.

Swarbrick, E. T., Stokes, C. R. & Soothill, J. F. (1979). Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. *Gut* **20**, 121-125.

Tarahomjoo, S. (2012). Development of Vaccine Delivery Vehicles Based on Lactic Acid Bacteria. *Mol Biotechnol* **51**, 183-199.

Trachtman, H., Cnaan, A., Christen, E. & other authors (2003). Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. *JAMA : the journal of the American Medical Association* **290**, 1337-1344.

W-Y

Wells, J. (2011). Mucosal Vaccination and Therapy with Genetically Modified Lactic Acid Bacteria. *Annu Rev Food Sci T* **2**, 423-445.

Wells, J.M., and Schofiled, K.M. (1996). Cloning and expression vectors for lactococci. NATO ASI Series H 98, 37-62.

Wells, J. M., Wilson, P. W. & Le Page, R. W. (1993). Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J Appl Bacteriol* **74**, 629-636.

Wong, C. S., Jelacic, S., Habeeb, R. L., Watkins, S. L. & Tarr, P. I. (2000). The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *The New England journal of medicine* **342**, 1930-1936.

Wong, C. S., Mooney, J. C., Brandt, J. R., Staples, A. O., Jelacic, S., Boster, D. R., Watkins, S. L. & Tarr, P. I. (2012). Risk Factors for the Hemolytic Uremic Syndrome in Children Infected With *Escherichia coli* O157:H7: A Multivariable Analysis. *Clinical Infectious Diseases* **55**, 33-41.

Yasar, E., Vural, D., Safaz, I., Balaban, B., Yilmaz, B., Goktepe, A. S. & Alaca, R. (2011). Which treatment approach is better for hemiplegic shoulder pain in stroke patients: intra-articular steroid or suprascapular nerve block? A randomized controlled trial. *Clin Rehabil* **25**, 60-68.

CHAPTER 3

Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization

Adapted from

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3.1. Abstract

In **Chapter 2**, we described the construction of a recombinant *L. lactis* strain expressing the EHEC antigen, EspB in the cytoplasmic compartment. However, oral immunization of mice with this strain induced weak priming of the immune system. This outcome was attributed to the rather low levels of EspB expressed by this recombinant strain. Therefore, in the present study we optimized the expression of EspB in *L. lactis* by secreting the antigen either under constitutive or nisin-inducible control. Indeed, oral immunization of mice with the EspB-secreting strains successfully induced specific mucosal and systemic antibody responses. These responses were associated with mixed Th1/Th2 cell responses in Peyer's Patches and mesenteric lymph nodes. Moreover, immunized mice exhibited significant protection against *E. coli* O157:H7 colonization, as indicated by the reduced amount and/or duration of the bacterial fecal shedding. Our results demonstrate the protective potential of EspB as an oral vaccine against EHEC infection. Additionally, the study demonstrates the efficient delivery of recombinant EspB by the generally regarded as safe bacterium *L. lactis*. The safety profile of *L. lactis* as a vaccine vehicle can particularly be beneficial to children and elderly as high-risk groups for HUS incidence.

3.2 Introduction

In the previous chapter we described the constitutive cytoplasmic expression of EspB in *L. lactis*. However, oral immunization of mice using this recombinant strain induced weak priming of the immune system. This outcome was attributed to the low levels of EspB expressed by this strain. In the present study, we describe the optimized expression of EspB in *L. lactis*. Additionally, we describe the specific immune responses elicited in mice upon oral immunization with the recombinant strains and the protection conferred against a challenge infection with *E. coli* O157:H7.

3.2. Material and method

3.2.1. Bacteria, plasmids and culture conditions

A summary of the bacterial strains and plasmids used in this study is shown in Table 3.1. *L. lactis* was grown at 30°C in liquid M17 medium (Difco) supplemented with 0.5% glucose. Erythromycin or chloramphenicol was added at concentrations of 5 and

10 μg/ml, respectively. *E. coli* NCTC12900 was grown at 37°C in Luria-Bertani broth (Difco) supplemented with 80, 15 and 100 μg/ml of novobiocin, naladixic acid and streptomycin, respectively.

3.2.2. Constructions of the EspB-expressing *L. lactis* strains

Plasmids pT:SEC-EspB and pNZ:SEC-EspB were constructed for constitutive or nisin-inducible secretion of EspB, respectively (Table 3.1 and Figure 3.1).

Table 3.1. Bacterial strains and plasmid vectors

Plasmid or strain	Description	Reference
***L. lactis* subsp. *cremoris* MG1363**	A derivative of the dairy starter strain NCDO712, which was cured of all resident plasmids	Gasson, 1983
***L. lactis* NZ9000**	*L. lactis* subspecies *cremoris* MG1363 carrying nisR and nisK genes on the chromosome	Kuipers *et al.*, 1998
LL-pTREX	*L. lactis* subspecies *cremoris* MG1363 carrying plasmid pTREX	Chapter 2
LL-pNZ8150	*L. lactis* NZ9000 carrying plasmid pNZ8150	This study
LL-pT:CYT:EspB	*L. lactis* subspecies *cremoris* MG1363 carrying plasmid pT:CYT:EspB	Chapter 2
LL-pT:SEC:EspB	*L. lactis* subspecies *cremoris* MG1363 carrying plasmid pT:SEC:EspB	This study
LL-pNZ:SEC:EspB	*L. lactis* NZ9000 carrying plasmid pNZ:SEC:EspB	This study
***E. coli* NCTC12900**	Shiga toxin-negative mutant of *E. coli* O157:H7, Nov^R, Nal^R and Str^R	Woodward *et al.*, 2003
pTREX1	Lactococcal expression plasmid containing P1 promoter with downstream start codon and	Wells and Schofiled, 1996

	secretion signal (SP_{Usp45}), Em^R	
pNZ8150	Lactococcal expression plasmid containing PnisA promoter with downstream start codon, Cm^R	Mierau & Kleerebezem, 2005
pT:CYT:EspB	Modified pTREX1 containing P_1 promoter with downstream *espB* gene, Em^R	Chapter 2
pT:SEC:EspB	Modified pTREX1 containing P_1 promoter with downstream secretion signal (SP_{Usp45}) and *espB*. Em^R	This study
pNZ:SEC:EspB	Modified pNZ8150 containing P_{nisA} promoter with downstream secretion signal (SP_{Usp45}) and *espB*. Cm^R	This study

Nal^R, Str^R, Cm^R, Em^R is resistance against naladixic acid, streptomycin, chloramphenicol and erythromycin, respectively.

For construction of plasmid pT:SEC-EspB, the *espB* gene was PCR amplified from plasmid pT:CYT-EspB using sequence-specific primer pairs (forward 5′-GGGGGATCCAACACTATCGATAACACTCAAG-3′; reverse: 5′-GGGACTAGTTTAACCAGCAA GACG-3′). pT:CYT-EspB carries a codon optimized *espB* gene of *E. coli* O157:H7 (Genbank Accession no: NC_002655), adapted to the preferential codon use of *L. lactis*. The amplified *espB* gene was inserted downstream of the secretion signal peptide SP_{Usp45} at the BamHI/SpeI ends of pTREX1 (Figure 3.1). For the construction of plasmid pNZ:SEC-EspB, *espB* along with SP_{Usp45} (SP_{Usp45}-*espB*) was PCR amplified from pT:SEC-EspB using sequence-specific primer pairs (forward 5′-ATGAAAAAAAAGATTATCTCAGC-3′; reverse: 5′-GGGACTAGTTTAACCAGCAAGACG-3)′and was inserted at the ScaI/SpeI ends of plasmid pNZ8150 (Figure 3.1). Plasmids pT:SEC-EspB and pNZ:SEC-EspB were used to transform electrocompetent *L. lactis* MG1363 and *L. lactis* NZ9000 (Wells *et al.*, 1993) to obtain the recombinant strains LL- pT:SEC-EspB and LL- pNZ:SEC-EspB, respectively (Table 3.1).

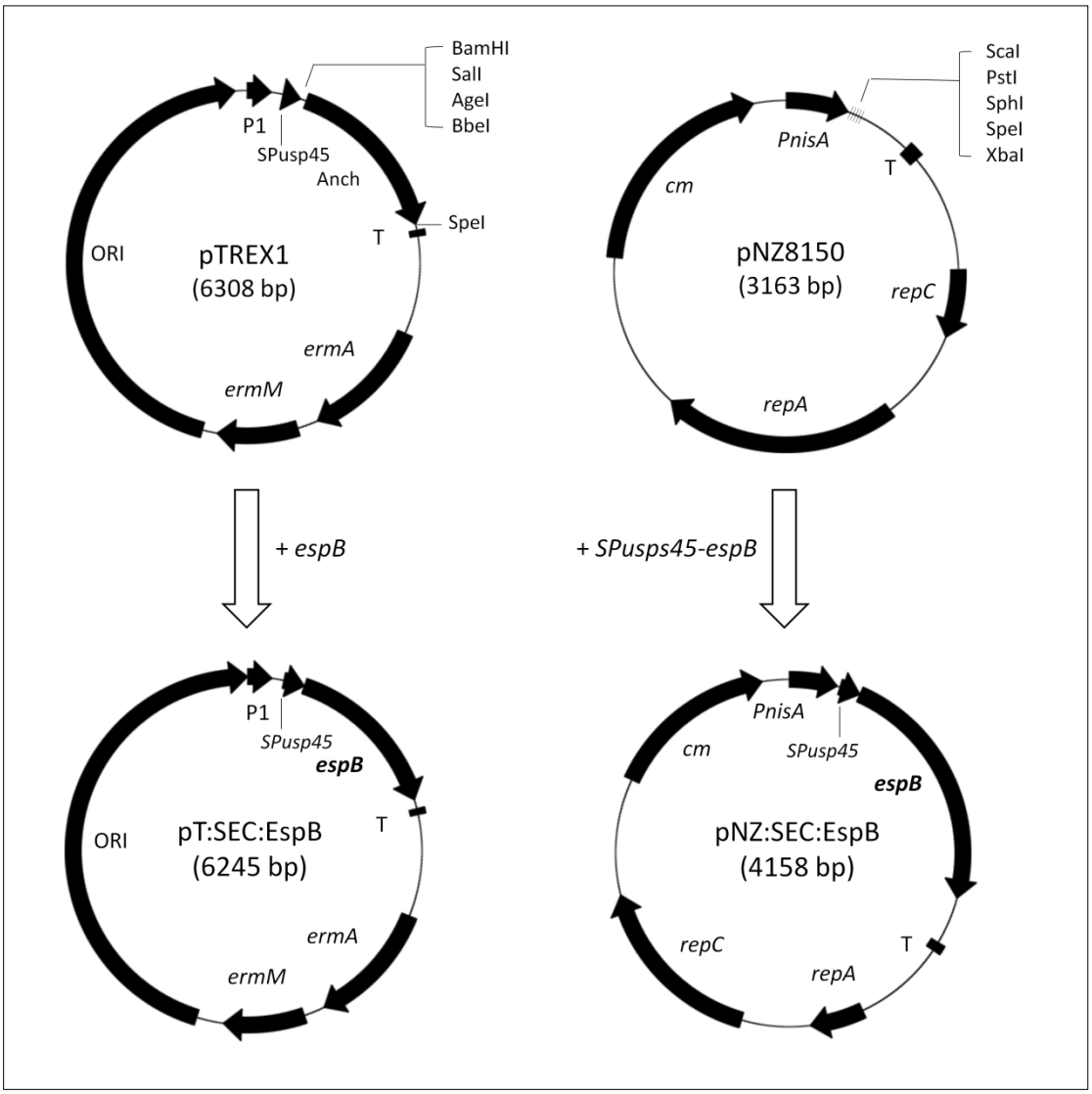

Figure 3.1. *espB* gene cloning for secretion of EspB in *L. lactis*. (A) The expression plasmid pTREX1 containing the constitutive promoter P1 and the secretion peptide signal *SP-Usps45* was used for the insertion of the *espB* coding sequence between BamHI and SpeI restriction sites to form the plasmid pT:SEC:EspB. (B) The expression plasmid pNZ8150 containing the nisin-inducible promoter PnisA was modified by inserting a fragment combining the *espB* gene to an upstream secretion signal (*SPUsp45-espB*) between ScaI and SpeI restriction sites to form the plasmid pNZ:SEC:EspB.

3.2.3. Induction and quantification of EspB expression in *L. lactis*

Overnight cultures of the inducible LL-pNZ:SEC:EspB strain or the constitutive, LL-pT:CYT:EspB and LL-pT:SEC:EspB strains were inoculated in GM17 broth at dilutions of 1:25 and 1:100, respectively. All strains were grown to an optical density at 600 nm of

0.4, at which, nisin (10 ng/ml) was added to the culture medium of LL-pNZ:SEC:EspB. LL-pT:CYT:EspB and LL-pT:SEC:EspB cells were harvested by centrifugation and resuspended in buffered-GM9 (BGM9) medium (Schotte *et al.*, 2000). After a further 4h incubation, cells or supernatant of equal culture volumes were collected for each strain. *L. lactis* cell lysates were enzymatically obtained as described in Chapter 2. EspB was detected and quantified in cell lysates and supernatants of the *L. lactis* cultures using a specific sandwich ELISA, as described in Chapter 2.

3.2.4. Oral immunization of mice with the EspB-expressing *L. lactis* strains

Six-week-old female BALB/c mice were used in the animal experiments. All experimental and animal management procedures were approved by the animal care and ethics committee of Ghent University, Belgium. The lactococcal strains were grown and induced as described above. Cells of the inducible strain were washed twice with sterile PBS to remove the traces of nisin. Groups of nine mice were immunized orally with LL-pT:SEC:EspB, LL-pNZ:SEC:EspB, purified EspB, LL-pTREX1, LL-pNZ8150 or the inoculation medium BGM9 (Schotte *et al.*, 2000). Mice received a primary immunization and a single booster immunization, with three weeks interval. For each of the immunizations, an oral inoculum of 100 µl BGM9 containing 2×10^9 CFU of the proper lactococcal strain or 5 µg of purified EspB was given to each mouse daily for three consecutive days.

3.2.5. EspB-specific antibodies

Total serum EspB-specific immunoglobulins (total-Ig) and faecal IgA were measured 10 days after the booster immunization. Preparation of serum and fecal extracts as well as the ELISA procedures were performed as described in Chapter 2. The cut-off values were calculated as the mean OD_{405} –value of all sera or faecal suspensions (dilutions 1:10 and 1:5, respectively) at day 0, increased with 3 times the standard deviation. The antibody titre is the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

3.2.6. Cytokine ELISA's

Peyer's patches (PP) and mesenteric lymph node (MLN) lymphocytes were isolated from immunized mice (n=3), 10 days after the booster immunization. Cells were resuspended at a concentration of 1.5×10^6 cells/ml in complete RPMI-1640 medium

(Sebastiani *et al.*, 2001) and cultured in 96-well plates. Cells were restimulated with EspB (final concentration: 10 µg/ml) for 48h. Thereafter, supernatants were collected and assayed for murine INF-γ, IL-4 and IL-10 by quantitative ELISA using the mouse Th1/Th2 ELISA Ready-SET-Go® kit (eBioscience), according to the manufacturer's instructions.

3.2.7. **Challenge with *E. coli* O157:H7**

Groups of seven mice were immunized as described above. Ten days after the booster immunization, mice of each group were randomly divided into two subgroups, of which, one subgroup (n=4) received streptomycin (5 g/L) in drinking water 24 hours before infection and till the end of the experiment, while the other subgroup (n=3) was not treated with streptomycin. These subgroups represent two models of varying colonization intensities, since streptomycin treatment enhances EHEC colonization in mice by clearing the competing intestinal flora (Wadolkowski *et al.*, 1990). Mice were challenged by intragastric inoculation of 10^{10} CFU of *E. coli* NCTC12900 in 100 µl of PBS containing 20% sucrose. Faecal excretion of *E. coli* NCTC12900 was monitored at 2-3 days interval, by both conventional bacterial plating and immuno-magnetic separation, as previously described (Walle *et al.*, 2011).

3.2.8. **Statistical analyses**

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc.). One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to analyze the differences in EspB expression levels among the strains as well as the differences in antibody titers, cytokine ratios and duration of fecal shedding among the groups. Pair-wise comparison of individual cytokine levels among immunized and control groups were analyzed using Student's *t*-test. Two-way ANOVA with Bonferroni post-hoc test was used to analyze the differences in fecal loads of *E. coli* NCTC12900 over time among the groups. Differences with a $P < 0.05$ were considered statistically significant.

3.3. Results

3.3.1. ELISA quantification of EspB expression by the recombinant *L. lactis* strains

EspB was detected only in the cell lysate of LL-pT:CYT:EspB, while it was detected in both cell lysates and supernatants of LL-pNZ:SEC:EspB and LL-pT:SEC:EspB (Table 3.2). The detection of EspB in cell lysates of the EspB-secreting strains corresponds to the presence of the cytoplasmic precursor of the mature (secreted) protein. The total amount of EspB expressed by the secreting strains LL-pNZ:SEC:EspB and LL-pT:SEC:EspB (both cytoplasmic and secreted) was respectively 56- and 25-fold higher than that expressed by the cytoplasmic expression strain LL-pT:CYT:EspB ($P< 0.05$). The nisin-inducible LL-pNZ:SEC:EspB strain showed a 2.2-fold higher total EspB expression than the constitutive LL-pT:SEC:EspB strain ($P< 0.05$) (Table 3.2). Both LL-pNZ:SEC:EspB and LL-pT:SEC:EspB were used for the immunization experiments.

Table 3.2. ELISA quantification of EspB production by the recombinant *L. lactis* strains

Strain	Expression features	EspB expression in fractions of cultures (µg/ml)[†]		
		Cell lysate	Supernatant	Total
LL-pT:CYT:EspB	Constitutive cytoplasmic	0.082± 0.02	ND[*]	0.082± 0.02[a]
LL-pNZ:SEC:EspB	Nisin-inducible secretion	0.83± 0.19	3.8± 1.1	4.63± 1.29[a]
LL-pT:SEC:EspB	Constitutive secretion	0.15± 0.05	1.9± 0.4	2.05± 0.45[a]

[†] Culture density approximately equal to 8×10^8 cfu/ml. Data represent means ± standard deviation of three independent measurements.
[a] Significant differences among the strains using one way-ANOVA with Bonferroni post-hoc test ($P < 0.05$).
[*] ND; non detectable

3.3.2. EspB-secreting *L. lactis* strains induce specific antibody responses after oral administration to mice

Mice orally immunized with either LL-pNZ:SEC:EspB or LL-pT:SEC:EspB showed significantly higher levels of EspB-specific serum total-Igs ($P< 0.0001$) and faecal IgA ($P< 0.0001$) compared to those immunized with the corresponding control strains, LL-pNZ8150 and LL-pTREX1, respectively (Figure 3.2). The highest antibody responses were elicited by the nisin-inducible strain LL-pNZ:SEC:EspB compared to the constitutive strain LL-pT:SEC:EspB ($P< 0.0001$). Mice immunized with purified EspB showed no significant difference in total serum total-Ig and faecal IgA compared to those receiving the BGM9 medium.

3.3.3. Oral immunization with *L. lactis*-secreting EspB induces mixed Th1/Th2 immune response

To characterize the cellular immune responses induced by the oral immunization with the EspB-secreting *L. lactis* strains, the production of INF-γ (Th1 cytokine), IL-4 (Th2 cytokine) (Neurath *et al.*, 2002)] and IL-10 (a broadly expressed anti-inflammatory cytokine) (Neurath *et al.*, 2002; Saraiva & O'Garra, 2010) was measured in PP (Figure 3.3A) and MLN (Figure 3.3C) lymphocytes after *in vitro* restimulation with EspB. Lymphocytes of both LL-pT:SEC:EspB and LL-pNZ:SEC:EspB immunized mice showed a significant higher production of all cytokines compared to those immunized with LL-pTREX1 and LL-pNZ8150, respectively ($P< 0.0001$). This finding indicates a mixed Th1/Th2 cell response. Mice immunized with purified EspB showed no significant increase in cytokine production compared to those received the BGM9 medium.

To determine the Th1/Th2 cell-type dominance, the IL-4/INF-γ ratio was further analyzed for PP (Figure 3.3B) and MLN (Figure 3.3D) lymphocytes. The ratio in both LL-pNZ:SEC:EspB and LL-pT:SEC:EspB immunized mice was greater than one and was significantly higher than the baseline ratios shown by control mice immunized with the respective empty vector strain or with the BGM9 medium. This finding indicates a dominance of the Th2 response.

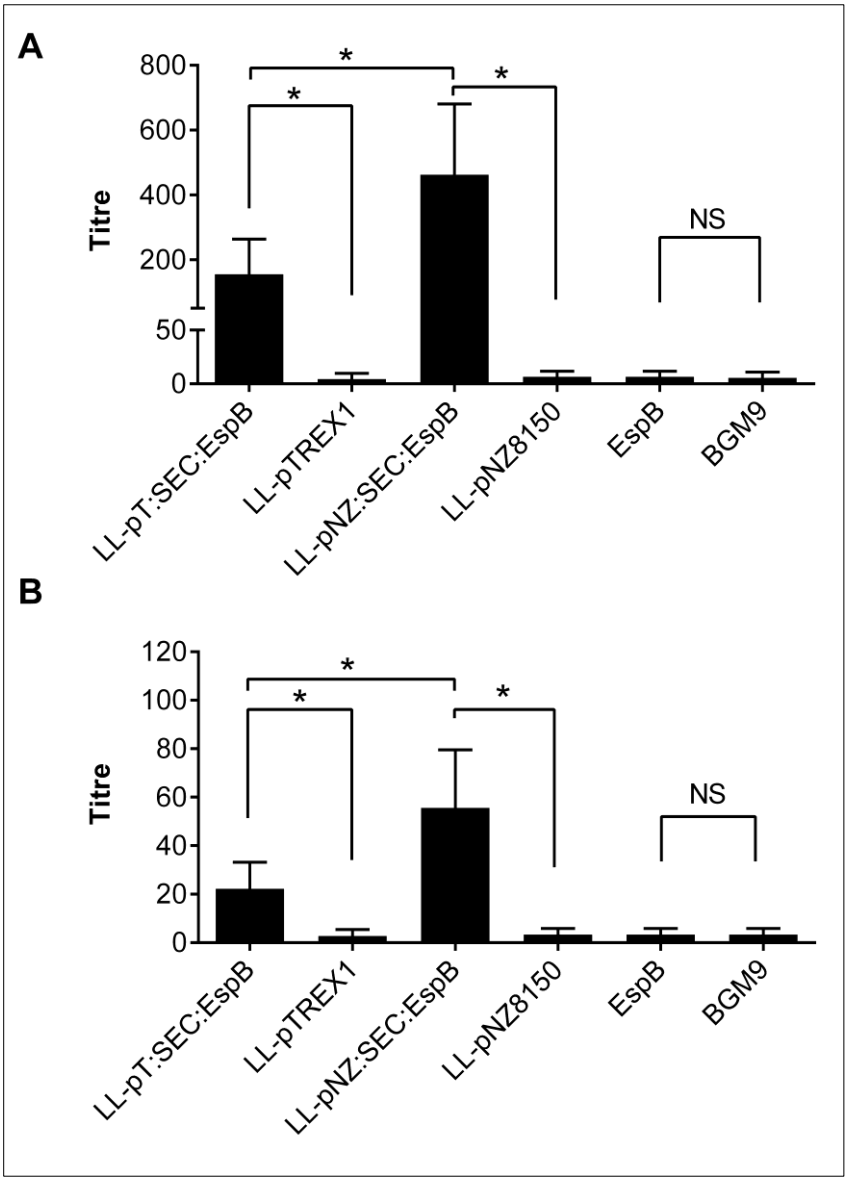

Figure 3.2. Antibody responses in mice immunized with EspB-secreting lactococci. Groups of mice (n=9) were immunized using the constitutive strain LL-pT:SEC:EspB, the inducible strain LL-pNZ:SEC:EspB or purified EspB. Corresponding control groups were immunized using LL-pTREX1, LL-pNZ8150 or the oral inoculation medium, BGM9. Ten days after the booster immunization serum and fecal samples were assayed by ELISA for the presence of EspB-specific total-Ig (A) or IgA (B) respectively. Data present the mean ± SD. Statistical differences were determined using one-way ANOVA with Bonferroni post-hoc test (* $p < 0.0001$; NS= non significant difference).

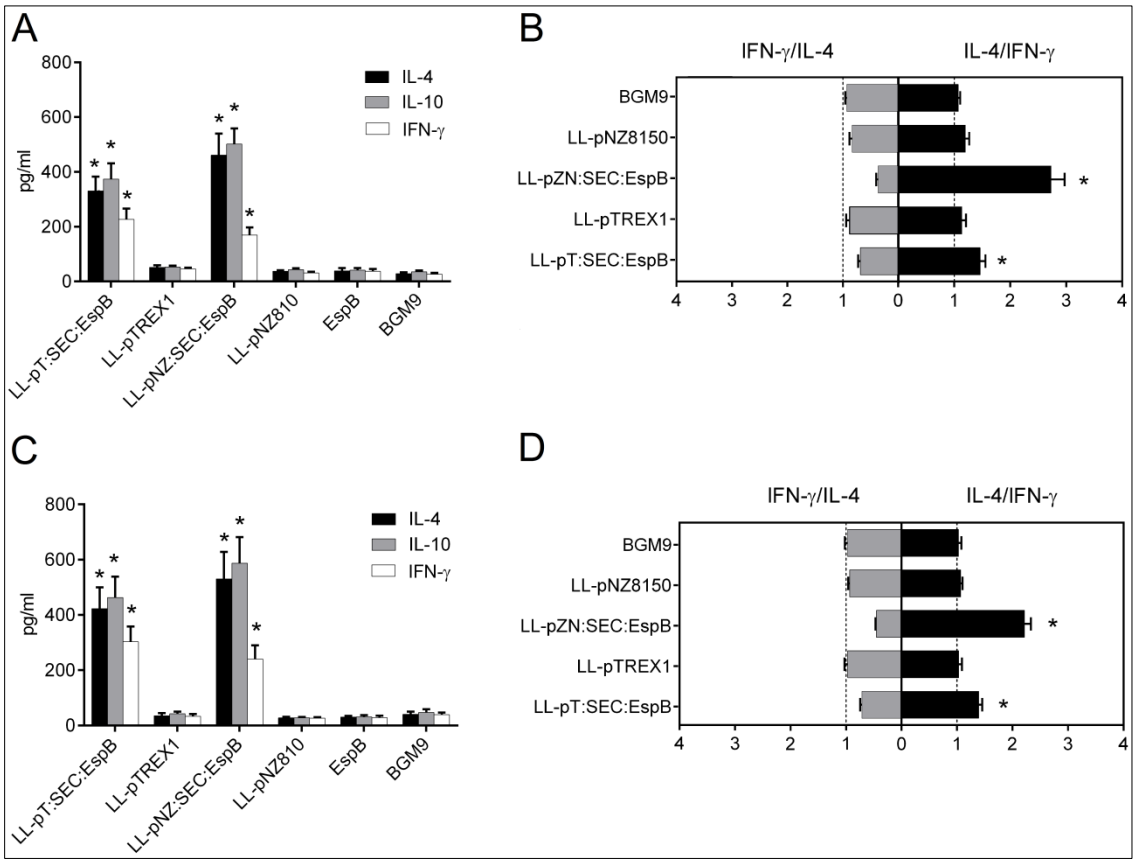

Figure 3.3. Cytokine responses of Peyer's Patches (PP) (A and B) and mesenteric lymph nodes (MLN) (C and D) lymphocytes from mice orally immunized with EspB-secreting *L. lactis*. Ten days after the booster immunization, lymphocytes were isolated from PP and MLN of mice (n=3) of immunized mice; restimulated with EspB, and assayed for production of IFN-γ, IL-4 and IL-10 cytokines. Data present the mean ± SD. Asterisks indicate significant difference between the immunization groups LL-pT:SEC:EspB, LL-pNZ:SEC:EspB and EspB compared to the control groups LL-pTREX1, LL-pNZ8150 and BGM9, respectively (P< 0.05). Statistical differences were determined using Student's *t*-test (P< 0.05). IL-4/INF-γ ratios were calculated to determine the dominant T-helper response in PP (B) and MLN lymphocytes (D). Data present the mean ± SD. Asterisks indicate significant difference between the immunization groups LL-pT:SEC:EspB and LL-pNZ:SEC:EspB compared to the respective control groups LL-pTREX1 and LL-pNZ8150, or to the BMG9 control group (P< 0.05). Statistical differences were determined using one-way ANOVA with Bonferroni post-hoc test (P< 0.05).

3.3.4. Oral immunization with *L. lactis*-secreting EspB protects mice against *E. coli* O157:H7 colonization

Comparisons of *E. coli O157:H7* fecal counts among the immunization groups within either the streptomycin-treated (Figure 3.4A) or the non-streptomycin-treated (Figure 3.4B) challenge groups show significantly reduced counts in LL-pNZ:SEC:EspB and LL-pT:SEC:EspB groups in comparison to LL-pNZ8150 and LL-pTREX1 groups, respectively, or to the BGM9 group, at most of the time points ($P< 0.05$). This reduction was significantly higher in the LL-pNZ:SEC:EspB immunization group compared to the LL-pT:SEC:EspB group ($P< 0.05$) (Figures 3.4 A and B).

Additionally, comparisons of the durations of *E. coli O157:H7* fecal shedding among the streptomycin-treated immunization groups show a significantly shorter duration in the LL-pNZ:SEC:EspB group (11.52 ± 2.87 days) compared to LL-pNZ8150 and BGM9 groups (30.5 ± 1.73 and 29.75 ± 1.5 days, respectively) ($P< 0.05$). However, the duration of fecal shedding shown by the LL-pT:SEC:EspB group was not significantly shorter than that of the LL-pTREX1 or the BGM9 group (26.75 ± 1.5 days versus 29.75 ± 1.5 days). Within the non-streptomycin-treated immunization groups, the duration of fecal shedding shown by the LL-pNZ:SEC:EspB group (11.52 ± 2.87 days) and the LL-pT:SEC:EspB group (16 ± 1.73 days) was significantly shorter than that of the LL-pNZ8150 group (19.3 ± 2.31 days) and the LL-pTREX1 group (22 days), respectively or than that of the BGM9 group (22 days) ($P< 0.05$). The shorter duration of fecal shedding shown by the LL-pNZ:SEC:EspB group in comparison to the LL-pT:SEC:EspB group was significant ($P< 0.05$).

3.4. Discussion and conclusion

We have previously described the constitutive cytoplasmic expression of EspB in *L. lactis* (LL-pT:CYT:EspB) (Chapter 2). However, oral vaccination of BALB/c mice with this strain induced a weak priming the immune system. This outcome was attributed to the low amount of EspB expressed by LL-pT:CYT:EspB, presumably due to the antigen degradation by the lactococcal cytoplasmic proteases (Frees & Ingmer, 1999; Koch *et al.*, 1998; Nilsson *et al.*, 1994). Therefore, in the present study we hypothesized that secretion could allow EspB to escape cytoplasmic proteolysis and thus would result in a higher expression yield.

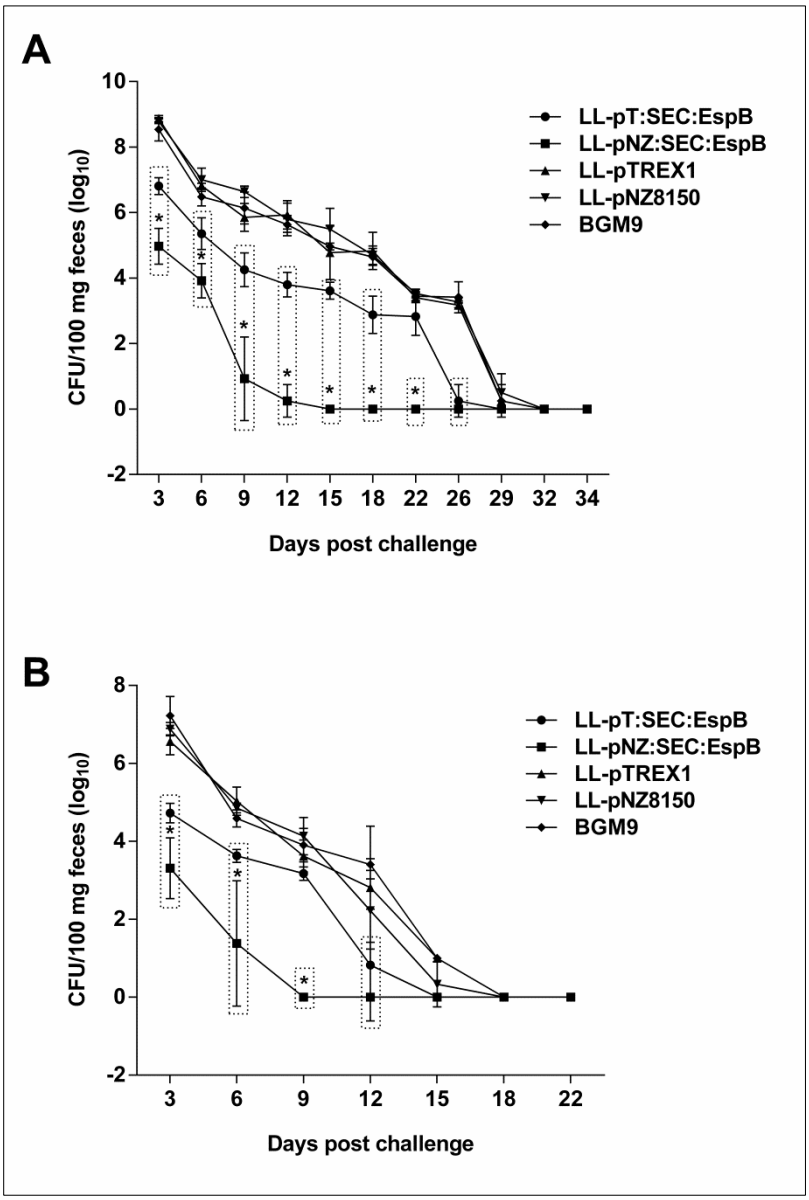

Figure 3.4. Kinetics of fecal shedding of *E. coli* O157:H7 in mice immunized orally with EspB secreting lactococci. Ten days after the booster immunization, groups of immunized mice were orally challenged with 10^{10} cfu of *E. coli* NCTC12900 (a non-toxigenic mutant of *E. coli* O157:H7), either under oral streptomycin treatment condition (n=4) (A) or without streptomycin treatment (n=3) (B). Data present the mean ± SD. Data points in rectangles indicate a significant difference between the LL-pT:SEC:EspB and pNZ:SEC:EspB immunization groups compared to the respective control groups LL-pTREX1 and LL-pNZ8150 or to the BGM9 control group ($P< 0.05$). Asterisks indicate statistical differences between the LL-pT:SEC:EspB and LL-pNZ:SEC:EspB immunization groups ($P< 0.05$). Data present the mean ± SD. Statistical differences were determined using two-way ANOVA with Bonferroni post-hoc test ($P< 0.05$).

Additionally, we compared a nisin-inducible and a constitutive expression system for the highest protein expression. In accordance with our hypothesis, secretion of EspB either under constitutive (LL-pT:SEC:EspB) or inducible (LL-pNZ:SEC:EspB) conditions resulted in a great enhancement of the total antigen yield compared to the cytoplasmic expression by LL-pT:CYT:EspB (56- and 25-fold increase, respectively). A similar enhancement of the total protein yield by secretion in comparison to cytoplasmic expression has been reported for the expression of the staphylococcal nuclease (Bermudez-Humaran *et al.*, 2003) and the *Brucella abortus* ribosomal protein L7/L12 (Ribeiro *et al.*, 2002) in *L. lactis*.

The immune response induced by the EspB-secreting lactococci is expected to be influenced by EspB expression levels, which are at least two times higher for the nisin-inducible strain, LL-pNZ:SEC:EspB compared to the constitutive strain, LL-pT:SEC:EspB. On the other hand, LL-pT:SEC:EspB is capable of constant secretion of EspB during the intestinal passage, which may compensate for the higher expression levels of LL-pNZ:SEC:EspB, whereas LL-pNZ:SEC:EspB may not continue EspB secretion along the entire duration of its intestinal passage as nisin is absent *in vivo*. However, the highest immune responses were obtained by the inducible LL-pNZ:SEC:EspB strain. In this regard, previous reports have demonstrated the ability of nisin-inducible expression systems to continue protein expression for at least ten hours after removal of nisin from the culture medium (Bermudez-Humaran *et al.*, 2003). Additionally, considering that the reported transit time of *L. lactis* throughout the mouse gastrointestinal tract is approximately 12 hours (Kimoto *et al.*, 2003), it is highly likely that both strains had a comparable persistence of EspB secretion *in vivo*, while the higher responses induced by LL-pNZ:SEC:EspB are a direct result of its higher capacity for EspB expression.

Unlike the mice immunized with the EspB-expressing *L. lactis* strains, those immunized with purified EspB did not show specific immune responses, even though EspB was given at a dose approximately equal to that present in the inoculum of LL-pT:SEC:EspB (5 μg). This finding could be explained by the ability of *L. lactis* to maintain metabolic activity (Drouault *et al.*, 1999; Huibregtse *et al.*, 2007; Steidler *et al.*, 2000) and de novo secretion of recombinant proteins (Huibregtse *et al.*, 2007; Steidler *et al.*, 2000) during its gastrointestinal passage, leading to the delivery of higher amounts of EspB than those measured in the administered inocula (Hou *et al.*,

2011). Additionally, purified EspB could have been influenced by the degrading effects of the gastrointestinal acidity and enzymes, a bottleneck that can be overcome by using *L. lactis* as an antigen delivery vector. Moreover, *L. lactis* exhibits adjuvant effects (Yam *et al.*, 2008), which can enhance the immune response to recombinant EspB in comparison to the purified protein (Adel-Patient *et al.*, 2005; Chatel *et al.*, 2001; Marelli *et al.*, 2011). These arguments support the pharmacological and the immunological value of *L. lactis* as an oral antigen delivery system (Cho *et al.*, 2007; Robinson *et al.*, 1997; Saez *et al.*, 2012).

To characterize the cellular immune responses induced by oral immunization of mice with the EspB-secreting strains, we isolated lymphocytes from PP and MLN, the main inductive sites of the gut mucosal immune system (Pabst & Rothkotter, 2006) and measured the production of INF-γ (Th1 cytokine) and IL-4 (Th2 cytokine) (Neurath *et al.*, 2002) after stimulating the cells with EspB *in vitro*. Both cytokines were produced by lymphocytes of immunized mice, indicating the induction of a mixed Th1/Th2 cell response. Previous reports have similarly described a mixed Th1/Th2 cell response to antigens orally delivered by *L. lactis* (Cauchard *et al.*, 2011; Robinson *et al.*, 1997; Villena *et al.*, 2008). Additionally, analysis of the IL-4:INF-γ ratio's indicated a dominance of the Th2 response. The Th2 dominance corresponds well with the induced mucosal IgA responses, since Th2 cells and cytokines are known to contribute to mucosal IgA class switching in PP and MLN (Okahashi *et al.*, 1996; Yamamoto *et al.*, 1996).

Reduced intestinal colonization is considered an important criterion for protection against EHEC virulence (Judge *et al.*, 2004a; Oliveira *et al.*, 2012). Our findings show that oral immunization of mice with the EspB-secreting *L. lactis* strains resulted in a reduction of *E. coli* O157:H7 fecal shedding. Moreover, the reduced bacterial shedding was consistent in two infection models of varying colonization intensities indicating the efficacy and the reproducibility of our vaccination approach. This pattern of protection could be correlated with an intestinal EspB-specific IgA response, which is consistent with the protective role of secretory IgA in mucosal infections (Pavot *et al.*, 2012). Previous mucosal immunization studies involving T3SS proteins demonstrated a reduction in the fecal shedding of EHEC after oral challenge (Amani *et al.*, 2011; Babiuk *et al.*, 2008; Gu *et al.*, 2011; Judge *et al.*, 2004b; Fan *et al.*, 2012; Wan *et al.*, 2011). Indeed, in these studies, the protection against intestinal

colonization was accompanied by the detection of specific IgA antibodies in the feces of immunized mice. In fact, IgA antibodies seem to be important for protection, since in some of these studies, protection could not be afforded by parenteral immunization, which elicited systemic antibody responses, but not intestinal IgA responses (Babiuk *et al.*, 2008; Fan *et al.*, 2012; Wan *et al.*, 2011). With regard to EspB, previous reports have described the protective effect of parenteral vaccination with formulations containing EspB (Vilte *et al.*, 2011; Yekta *et al.*, 2011). However, these studies were performed in cattle, a species in which IgG responses contribute largely to intestinal mucosal protection. The present study, however, is the first to describe the protective potential of EspB upon oral vaccination.

In conclusion, oral immunization of mice with recombinant *L. lactis* secreting EspB induces specific mucosal and systemic immune responses and confers protection against an *E. coli* O157:H7 challenge infection. These findings demonstrate the feasibility of an oral vaccine based on the recombinant expression of EspB in *L. lactis* to control EHEC infection. The safety profile of *L. lactis* as a vaccine delivery vector can particularly be beneficial to children and elderly, the high-risk groups for HUS complications (Griffin & Tauxe, 1991)

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3.6. References

A-C

Adel-Patient, K., Ah-Leung, S., Creminon, C., Nouaille, S., Chatel, J. M., Langella, P. & Wal, J. M. (2005). Oral administration of recombinant *Lactococcus lactis* expressing bovine beta-lactoglobulin partially prevents mice from sensitization. *Clin Exp Allergy* **35**, 539-546.

Amani, J., Mousavi, S. L., Rafati, S. & Salmanian, A. H. (2011). Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of *Escherichia coli* O157:H7 in mice. *Plant Sci* **180**, 620-627.

Babiuk, S., Asper, D. J., Rogan, D., Mutwiri, G. K. & Potter, A. A. (2008). Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. *Microbial pathogenesis* **45**, 7-11.

Bermudez-Humaran, L. G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R. & Corthier, G. (2003). Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. *Fems Microbiol Lett* **224**, 307-313.

Cauchard, S., Bermudez-Humaran, L. G., Blugeon, S., Laugier, C., Langella, P. & Cauchard, J. (2011). Mucosal co-immunization of mice with recombinant lactococci secreting VapA antigen and leptin elicits a protective immune response against *Rhodococcus equi* infection. *Vaccine* **30**, 95-102.

Chatel, J. M., Langella, P., Adel-Patient, K., Commissaire, J., Wal, J. M. & Corthier, G. (2001). Induction of mucosal immune response after intranasal or oral inoculation of mice with *Lactococcus lactis* producing bovine beta-lactoglobulin. *Clin Diagn Lab Immun* **8**, 545-551.

Cho, H. J., Shin, H. J., Han, I. K., Jung, W. W., Kim, Y. B., Sul, D. & Oh, Y. K. (2007). Induction of mucosal and systemic immune responses following oral immunization of mice with *Lactococcus lactis* expressing human papillomavirus type 16 L1. *Vaccine* **25**, 8049-8057.

D-H

Drouault, S., Corthier, G., Ehrlich, S. D. & Renault, P. (1999). Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. *Appl Environ Microb* **65**, 4881-4886.

Fan, H. Y., Wang, L., Luo, J. & Long, B. G. (2012). Protection against *Escherichia coli* O157:H7 challenge by immunization of mice with purified Tir proteins. *Molecular biology reports* **39**, 989-997.

Frees, D. & Ingmer, H. (1999). ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Molecular microbiology* **31**, 79-87.

Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *Journal of bacteriology* **154**, 1-9.

Griffin, P. M. & Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic reviews* **13**, 60-98.

Gu, J., Ning, Y., Wang, H. & other authors (2011). Vaccination of attenuated EIS-producing *Salmonella* induces protective immunity against enterohemorrhagic *Escherichia coli* in mice. *Vaccine* **29**, 7395-7403.

Hou, J., Lu, Y., Liu, H. Y. & other authors (2011). Oral administration of *Lactococcus lactis* delivered heat shock protein 65 attenuates atherosclerosis in low-density lipoprotein receptor-deficient mice. *Vaccine* **29**, 4102-4109.

Huibregtse, I. L., Snoeck, V., de Creus, A., Braat, H., De Jong, E. C., Van Deventer, S. J. & Rottiers, P. (2007). Induction of ovalbumin-specific tolerance by oral administration of *Lactococcus lactis* secreting ovalbumin. *Gastroenterology* **133**, 517-528.

J-N

Judge, N. A., Mason, H. S. & O'Brien, A. D. (2004a). Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of *Escherichia coli* O157 : H7 shedding in feces. *Infection and immunity* **72**, 168-175.

Judge, N. A., Mason, H. S. & O'Brien, A. D. (2004b). Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of *Escherichia coli* O157:H7 shedding in feces. *Infection and immunity* **72**, 168-175.

Kimoto, H., Nomura, M., Kobayashi, M., Mizumachi, K. & Okamoto, T. (2003). Survival of lactococci during passage through mouse digestive tract. *Can J Microbiol* **49**, 707-711.

Koch, B., Kilstrup, M., Vogensen, F. K. & Hammer, K. (1998). Induced levels of heat shock proteins in a dnaK mutant of *Lactococcus lactis*. *J Bacteriol* **180**, 3873-3881.

Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. & de Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol* **64**, 15-21.

Marelli, B., Perez, A. R., Banchio, C., de Mendoza, D. & Magni, C. (2011). Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8*subunit induces specific immune response in mice. *J Virol Methods* **175**, 28-37.

Mierau, I. & Kleerebezem, M. (2005). 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied microbiology and biotechnology* **68**, 705-717.

Neurath, M. F., Finotto, S. & Glimcher, L. H. (2002). The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* **8**, 567-573.

Nilsson, D., Lauridsen, A. A., Tomoyasu, T. & Ogura, T. (1994). A *Lactococcus lactis* Gene Encodes a Membrane-Protein with Putative Atpase Activity That Is Homologous to the Essential *Escherichia coli* Ftsh Gene-Product. *Microbiol-Uk* **140**, 2601-2610.

O-S

Okahashi, N., Yamamoto, M., Vancott, J. L., Chatfield, S. N., Roberts, M., Bluethmann, H., Hiroi, T., Kiyono, H. & McGhee, J. R. (1996). Oral Immunization of interleukin-4 (IL-4) knockout mice with a recombinant *Salmonella* strain or cholera toxin reveals that CD4(+) Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin a responses. *Infection and immunity* **64**, 1516-1525.

Oliveira, A. F., Cardoso, S. A., Almeida, F. B. D., de Oliveira, L. L., Pitondo-Silva, A., Soares, S. G. & Hanna, E. S. (2012). Oral immunization with attenuated *Salmonella* vaccine expressing *Escherichia coli* O157:H7 intimin gamma triggers both systemic and mucosal humoral immunity in mice. *Microbiol Immunol* **56**, 513-522.

Pabst, R. & Rothkotter, H. J. (2006). Structure and function of the gut mucosal immune system. *Advances in experimental medicine and biology* **579**, 1-14.

Pavot, V., Rochereau, N., Genin, C., Verrier, B. & Paul, S. (2012). New insights in mucosal vaccine development. *Vaccine* **30**, 142-154.

Ribeiro, L. A., Azevedo, V., Le Loir, Y., Oliveira, S. C., Dieye, Y., Piard, J. C., Gruss, A. & Langella, P. (2002). Production and targeting of the Brucella abortus antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microb* **68**, 910-916.

Robinson, K., Chamberlain, L. M., Schofield, K. M., Wells, J. M. & Le Page, R. W. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature biotechnology* **15**, 653-657.

Saez, D., Fernandez, P., Rivera, A., Andrews, E. & Onate, A. (2012). Oral immunization of mice with recombinant *Lactococcus lactis* expressing Cu,Zn superoxide dismutase of Brucella abortus triggers protective immunity. *Vaccine* **30**, 1283-1290.

Saraiva, M. & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature reviews Immunology* **10**, 170-181.

Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme Microb Tech* **27**, 761-765.

Sebastiani, S., Allavena, P., Albanesi, C., Nasorri, F., Bianchi, G., Traidl, C., Sozzani, S., Girolomoni, G. & Cavani, A. (2001). Chemokine receptor expression and function in CD4(+) T lymphocytes with regulatory activity. *Journal of immunology* **166**, 996-1002.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352-1355.

V-Y

Villena, J., Medina, M., Raya, R. & Alvarez, S. (2008). Oral immunization with recombinant *Lactococcus lactis* confers protection against respiratory pneumococcal infection. *Can J Microbiol* **54**, 845-853.

Vilte, D. A., Larzabal, M., Garbaccio, S. & other authors (2011). Reduced faecal shedding of *Escherichia coli* O157:H7 in cattle following systemic vaccination with gamma-intimin C-280 and EspB proteins. *Vaccine* **29**, 3962-3968.

Wadolkowski, E. A., Burris, J. A. & Obrien, A. D. (1990). Mouse Model for Colonization and Disease Caused by Enterohemorrhagic *Escherichia coli* O157-H7. *Infection and immunity* **58**, 2438-2445.

Walle, K. V., Yekta, M. A., Verdonck, F., De Zutter, L. & Cox, E. (2011). Rectal inoculation of sheep with *E. coli* O157:H7 results in persistent infection in the absence of a protective immune response. *Vet Microbiol* **147**, 376-382.

Wan, C. S., Zhou, Y., Yu, Y., Peng, L. J., Zhao, W. & Zheng, X. L. (2011). B-cell epitope KT-12 of enterohemorrhagic *Escherichia coli* O157:H7: a novel peptide vaccine candidate. *Microbiol Immunol* **55**, 247-253.

Wells, J.M., and Schofiled, K.M. (1996). Cloning and expression vectors for lactococci. NATO ASI Series H 98, 37-62.

Wells, J. M., Wilson, P. W. & Lepage, R. W. F. (1993). Improved Cloning Vectors and Transformation Procedure for *Lactococcus lactis*. *J Appl Bacteriol* **74**, 629-636.

Woodward, M. J., Best, A., Sprigings, K. A. & other authors (2003). Non-toxigenic *Escherichia coli* O157 : H7 strain NCTC12900 causes attaching-effacing lesions and eae-dependent persistence in weaned sheep. *Int J Med Microbiol* **293**, 299-308.

Yam, K. K., Pouliot, P., N'Diaye M, M., Fournier, S., Olivier, M. & Cousineau, B. (2008). Innate inflammatory responses to the Gram-positive bacterium *Lactococcus lactis*. *Vaccine* **26**, 2689-2699.

Yamamoto, M., Vancott, J. L., Okahashi, N. & other authors (1996). The role of Th1 and Th2 cells for mucosal IgA responses. *Ann Ny Acad Sci* **778**, 64-71.

Yekta, M. A., Goddeeris, B. M., Vanrompay, D. & Cox, E. (2011). Immunization of sheep with a combination of intimin gamma, EspA and EspB decreases *Escherichia coli* O157:H7 shedding. *Veterinary immunology and immunopathology* **140**, 42-46.

CHAPTER 4

Survival and protection of *Lactococcus lactis* in the porcine gastrointestinal tract: a step towards the validation of the *L. lactis*-based EHEC vaccine in pig models

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(Submitted)

4.1. Abstract

Lactococcus lactis has recently emerged as an efficient system for the oral delivery of various prophylactic and therapeutic molecules. However, previous studies described a moderate capacity of *L. lactis* to survive the gastrointestinal (GI) passage. Improving the GI survival of *L. lactis* can directly enhance its efficiency as a live delivery vector. Therefore, we first evaluated the sensitivity of *L. lactis* to some potentially harmful digestive factors, including bile and the pancreatic enzymes, trypsin, lipase and amylase. Secondly, we evaluated the capacity of aluminium hydroxide (a bile acid binder) and camostat mesylate (a trypsin inhibitor) to protect *L. lactis* against bile toxicity and trypsin hydrolysis, respectively. The experiments were designed to mimic the physiological conditions described in the small intestine of pigs, a model species with a particular resemblance to human in regard to GI physiology. Incubation of *L. lactis* with intestinal concentrations of porcine bile resulted in a marked decrease in its survival, compared to the non-bile-treated controls ($P< 0.0001$). Moreover, the physiological pH ranges in the small intestine were found to influence the survival of *L. lactis* in bile since a significantly lower survival of the bacterium was observed at pH 5.5 compared to pH 6.8 ($P< 0.0001$). This finding indicates a positive correlation between bile toxicity and acidity. Similar to bile, incubation of *L. lactis* with trypsin, amylase and lipase at their physiological concentrations was found to significantly decrease the survival of *L. lactis* ($P< 0.0001$). The effect of trypsin was most pronounced with a 80% decrease in *L. lactis* survival versus 51% and 40% for amylase and lipase, respectively ($P<0.001$). The presence of aluminium hydroxide and camostat mesylate could significantly improve the survival of *L. lactis* in bile and trypsin solutions, respectively ($P<0.05$). We finally demonstrated that oral co-administration of aluminium hydroxide and camostat mesylate with *L. lactis* can improve the survival of the bacterium in the GI tract of pigs. This was indicated by a 38- and 24-fold increase in the *L. lactis* counts retrieved from jejunal and ileal contents, respectively, of treated pigs compared to untreated ($P<0.001$). Our results represent an early proof of concept for the feasible use of aluminium hydroxide and camostat mesylate as ingredients of oral *L. lactis* formulations, in order to improve the GI survival of the bacterium and subsequently enhance its efficacy in the intestinal delivery of antigens.

5.1. Introduction

Lactococcus lactis is a Gram-positive bacterium that offers a potential platform for the oral delivery of various therapeutic and prophylactic molecules (Berlec *et al.*, 2012). The bacterium has been widely used in food industry and is generally recognized as safe (GRAS) for human consumption (Casalta & Montel, 2008). This safety profile has particularly encouraged the utilization of *L. lactis* for the development of a new generation of safe oral delivery vectors. Additionally, *L. lactis* is an efficient cell factory, capable of recombinant expression and mucosal delivery of a wide variety of target proteins (Bahey-El-Din *et al.*, 2010). Today, there is a vast collection of reports describing the use of live recombinant *L. lactis* for the intestinal delivery of therapeutic proteins (Steidler *et al.*, 2000; Vandenbroucke *et al.*, 2004; Zhuang *et al.*, 2008), allergens (Adel-Patient *et al.*, 2005; Huibregtse *et al.*, 2007) and vaccine antigens (Cauchard *et al.*, 2011; Cho *et al.*, 2007; Robinson *et al.*, 2004), with prominent therapeutic and protective outcomes in murine models. Furthermore, the therapeutic use of recombinant *L. lactis* for the intestinal delivery of human IL-10 has recently been the subject of clinical trials in patients with Crohn's disease (Braat *et al.*, 2006) and moderate ulcerative colitis (ActoGenix press release, 2009). Besides the efficient delivery of protein antigens, the ability of *L. lactis* to deliver DNA vaccines to enterocytes has recently been demonstrated in mice models (de Azevedo *et al.*, 2012; Guimaraes *et al.*, 2005; Innocentin *et al.*, 2009).

One of the most important factors for the efficient oral delivery of protein and DNA molecules by bacteria is their ability to survive the GI conditions (Bahey-El-Din & Gahan, 2011). Previous studies in humans have demonstrated a low recovery of *L. lactis* from ileum (Vesa *et al.*, 2000) or stool (Klijn *et al.*, 1995) in comparison to *Bacillus stearothermophilus* (Klijn *et al.*, 1995) and *Lactobacillus fermentum* (Klijn *et al.*, 1995; Vesa *et al.*, 2000), which are known for their tolerance to GI transit. Also studies in mouse (Drouault *et al.*, 1999) and pig (Drouault *et al.*, 2002; Termonts, 2005) models demonstrated a severe reduction in the recovery of orally administered *L. lactis*, particularly from stomach, duodenum and jejunum in comparison to ileum and the large intestine. This low survival rate in the posterior GI tract indicates a detrimental influence of the physiological conditions encountered in these gastrointestinal compartments on the survival of *L. lactis* during its GI passage. Enteric-coating is a well-established technology that overcomes the detrimental effect of gastric acidity on

orally delivered drug molecules, has been explored for the protection of various live probiotic bacteria (Klayraung *et al.*, 2009; Stadler & Viernstein, 2003) and bacterial vectors, including *L. lactis* (Edwards *et al.*, 2010; Huyghebaert *et al.*, 2005; Poelvoorde *et al.*, 2008). Nevertheless, exploring additional strategies for the protection of *L. lactis* against the deleterious intestinal conditions is necessary for a more efficient delivery of protein and DNA molecules to intestinal targets. The successful application of such protective strategies requires the identification of these digestive factors, which influence the viability of *L. lactis* in the small intestine most.

In the present study we performed a series of *in vitro* experiments to evaluate the individual effects of some potentially harmful intestinal digestive factors, including bile and the pancreatic enzymes, trypsin, amylase and lipase, on *L. lactis* survival. Additionally, we demonstrated the capacity of aluminium hydroxide (an oral bile acid binder) and camostat mesylate (a synthetic ester with oral bioavailability as a trypsin inhibitor) to protect *L. lactis in vitro* against bile and trypsin toxicity, respectively. All the *in vitro* experiments were performed with respect to the physiological conditions described in the small intestine of pigs. This allowed us to validate *in vivo* in piglets the capacity of aluminium hydroxide and camostat mesylate to protect *L. lactis* against bile toxicity and trypsin hydrolysis, respectively. Interestingly, the pig is considered a good experimental model for human, particularly in biomedical research (Kristy N. Kuzmuk, 2011; Litten-Brown *et al.*, 2010) and more specifically in gastrointestinal research. Several anatomical and physiological features of the digestive system are highly similar between pigs and humans (Darragh & Moughan, 1995; Eubanks *et al.*, 2006; Moughan PJ, 1992; Rowan *et al.*, 1994). Therefore, findings of our study can have important implications for the development of oral formulations that enable an increased survival of *L. lactis* in the intestinal tract and thus a more efficient intestinal delivery of target molecules using this GRAS bacterium.

5.2. Materials and Methods

5.2.1. Bacterial strain, growth conditions and survival determination

L. lactis subspecies *cremoris* MG1363 is a plasmid free derivative of the dairy starter strain NCDO712 (Gasson, 1983) and is the particular lactococcal strain used for mucosal delivery of biomolecules (Bermudez-Humaran *et al.*, 2011). In the present study, we used a *L. lactis* MG1363 strain that carries an erythromycin resistance

cassette on the pTREX1 plasmid and that was selected for rifampicin resistance based on a spontaneous mutation. The strain was routinely grown overnight at 30°C in liquid M17 medium (Difco, Michigan, USA), supplemented with 0.5% glucose, 5 µg/ml erythromycin and 100 µg/ml rifampicin (GM17EMR). A saturated ON culture contains approximately $2x10^9$ CFU/ml. *L. lactis* survival was assessed by conventional plating; ten-fold dilution series were plated in duplicates or triplicates on GM17EMR plates.

5.2.2. Animals and sampling procedures

Five to six week-old pigs (Belgian Landrace x Piétrain) were used for collection of bile and intestinal contents as well as for the *in vivo* experiment. All the experimental procedures were undertaken in accordance with the regulations of the animal welfare and the ethics committee of the University of Ghent, Belgium. Euthanasia was performed by intravenous injection of pentobarbital (24mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium). For isolation of intestinal contents, ligations were applied immediately after euthanasia to prevent mixing of duodenal, jejunal and ileal contents, which thereafter, were separately collected. Hepatic bile was collected from the gallbladder under aseptic conditions.

5.2.3. Total bile acids measurement

The total molar concentration of bile acids was measured in hepatic bile or in intestinal contents using a colorimetric enzymatic cycling assay kit (Total Bile Acids Assay Kit, Bioquant, California, USA). Briefly, in the presence of Thio-NAD, the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD) converts bile acids to 3-keto steroids and Thio-NADH. The reaction is reversible and 3α-HSD can convert 3-keto steroids and Thio-NADH to bile acids and Thio-NAD. In the presence of excess NADH, the enzyme cycling occurs efficiently and the rate of formation of Thio-NADH is determined by measuring the specific change of absorbance at 405 nm.

5.2.4. Survival and protection of *L. lactis* in porcine bile and pancreatic enzymes in vitro

For evaluation of survival in bile, bacterial cells of one ml of a saturated overnight *L. lactis* culture (starting culture) were resuspended in physiological saline (0.9% NaCl), complemented with different molar concentrations of bile acids, that approximately correspond to those measured in the different compartments of the small intestine (duodenum, jejunum and ileum). Non-bile-treated control culture was

prepared by resuspending cells of one ml of the starting culture in physiological saline in absence of bile. For evaluation of survival in pancreatic enzymes, cells of three independent starting cultures were resuspended in PBS individually containing porcine trypsin, amylase or lipase (Sigma, Missouri, USA), at concentrations of 2.2, 70 and 10 IU/ml, respectively. These concentrations approximately correspond to the physiological concentrations of these enzymes in the small intestine of pigs (Hedemann & Jensen, 2004). Non-enzyme-treated control cultures were prepared for each enzyme from its respective starting culture. For amylase and lipase, PBS was supplemented with 0.9 mM Ca^{2+} and 0.49 mM Mg^{2+} to ensure functionality of the enzymes.

For protection of *L. lactis* against bile toxicity, cells of one ml of a saturated overnight culture were resuspended in saline solution containing the highest molar concentration of bile detected in the small intestine, together with a dose-gradient (0 – 80 mg/ml) of aluminium hydroxide (Sigma, Missouri, USA). For protection against trypsin hydrolysis, *L. lactis* cells were suspended in PBS containing 2.2 IU/ml of porcine trypsin (Sigma, Missouri, USA), together with a dose-gradient (0 – 250 µg/ml) of camostat mesylate (Santa Cruz, California, USA).

In the experiments involving bile, pH of the experimental solutions was adjusted either to 5.5 or 6.8, using HCl or NaOH solutions, in order to respectively mimic the physiological pH of duodenum or jejunum and ileum, as described for pigs (Snoeck *et al.*, 2004). In the experiments involving pancreatic enzymes, pH was adjusted to 6.8. Since the effect of pH on the activity of pancreatic enzymes is well studied (Beck, 1973), we considered applying the intestinal pH at which these enzymes can be most active *in vivo* (Beck, 1973). All mixtures were allowed to incubate for two hours at 37°C with mild agitation. The incubation time chosen corresponds to the average retention time of feed in the small intestinal compartments of growing pigs (Wilfart *et al.*, 2007). Plating to evaluate the survival as described above followed the incubation.

5.2.5. In vivo validation of the protective effect of aluminium hydroxide and camostat mesylate on *L. lactis* survival

To validate the protective effect of aluminium hydroxide and camostat mesylate on the survival *of L. lactis* in the GI tract, pigs of two experimental groups (n=3 per group), received a 20 mg tablet of the proton pump inhibitor, rabeprazolum (Pariet®, Janssen-

cilag, Berchem, Belgium), 12 hours before oral administration of *L. lactis*. This procedure was performed to neutralize the effect of gastric acidity in animals of both experimental groups. Pigs were then deprived of food until the administration of the bacteria. *L. lactis* inocula were prepared by resuspending the pellet of 500 ml of a saturated overnight culture (approximately 6 x 10^{11} CFU) in 20 ml of buffered GM9 medium (Schotte *et al.*, 2000). One group of pigs received a lactococcal inoculum supplemented with aluminium hydroxide and camostat mesylate at the clinical doses recommended for human (1200 mg and 50 mg, respectively). The second group received a lactococcal inoculum that contained no protectants. Eight hours following the oral administration of *L. lactis*, pigs were euthanized and samples of duodenal, jejunal and ileal contents were collected and kept on ice until plating.

5.2.6. Statistical analysis

All data are expressed as mean ± SD. The *in vitro* experiments were all performed in triplicates, on three independent occasions. Data of the *in vitro* experiments are presented as survival percentages, which were calculated separately for each replicate based on CFU counts as follows: (CFU of the test culture ÷ CFU of the starting culture) × 100. Two-way analysis of variance (ANOVA) was used to analyse the toxic effect of bile on *L. lactis* survival and the protective effect of aluminium hydroxide against bile toxicity, in relation to the intestinal pH. One-way ANOVA was used to analyse the protective effect of camostat mesylate on trypsin hydrolysis. ANOVA was followed by Tukey's post hoc tests to perform multiple comparisons among pre-selected groups. An unpaired t-test was used to compare the survival percentages of enzyme-treated and non-enzyme-treated *L. lactis* cultures and to compare the intestinal counts of *L. lactis* in orally treated and non-treated pigs.

5.3. Results

5.3.1. Influence of bile on the viability of *L. lactis* in porcine small intestine

We first measured the physiological concentration of bile acids in the different parts of the small intestine of pigs (n=3). The highest concentration of bile acids was measured in duodenal contents (14.4 ± 0.5 mM), followed by jejunal (9.5 ± 0.7 mM) and ileal contents (4.5 ± 0.4 mM). Accordingly, to evaluate the effect of bile on the survival of *L. lactis*, bile freshly collected from gallbladder of pigs was added to *L. lactis* suspensions at final concentrations of 15 mM, 10 mM and 5 mM of bile acids (Figure

4.1). Each of these intestinal concentrations of bile has resulted in a significant decrease in *L. lactis* survival in comparison to the non-bile-treated (0 mM) control culture ($P < 0.0001$). The survival percentages were significantly lower for every higher concentration of bile acids, indicating a concentration-dependent pattern of bile toxicity ($P < 0.0001$). Interestingly, the survival of *L. lactis* in bile was significantly lower at pH 5.5 compared to pH 6.8 for all bile concentrations ($P<0.0001$), indicating a positive correlation between bile toxicity and acidity (Figure 4.1).

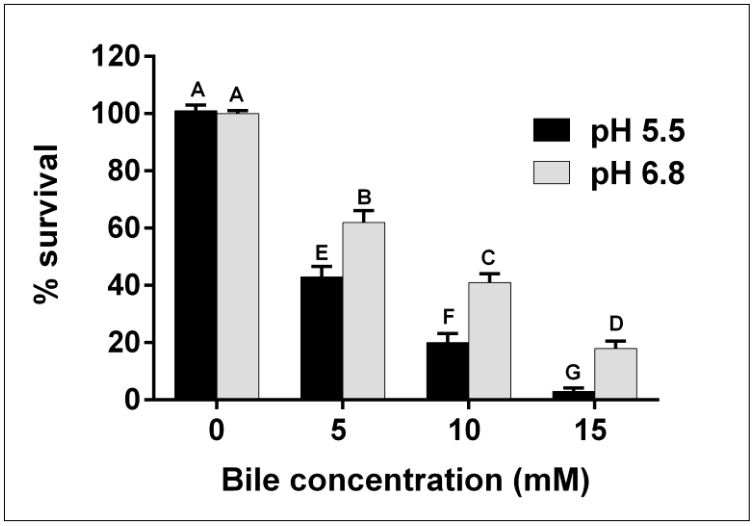

Figure 4.1. Survival of *L. lactis* in prevalent intestinal concentrations of bile acids under intestinal pH range *in vitro*. *L. lactis* was incubated for 2 h with 15, 10 or 5 mM of porcine bile under pH of 5.5 or 6.8. Survival is calculated based on CFU counts, as a percentage of the starting culture. Data represent the mean ± SD of three independent experiments. Significant differences among the conditions are indicated by a different capital letter ($P < 0.0001$).

5.3.2. Influence of the pancreatic enzymes on the viability of *L. lactis* in porcine small intestine

Exposure of *L. lactis* to the porcine pancreatic enzymes, trypsin, amylase or lipase, at activity levels mimicking those described in the small intestine of pigs, has resulted in a significant decrease in *L. lactis* survival in comparison to the non-enzyme-treated control cultures ($P < 0.0001$) (Figure 4.2). The highest enzymatic influence on *L. lactis* survival was observed for trypsin in comparison to the other enzymes (20% ± 2.52% versus 49% ± 5.86% and 60% ± 7.51% survival for lipase and amylase, respectively; $P < 0.0001$).

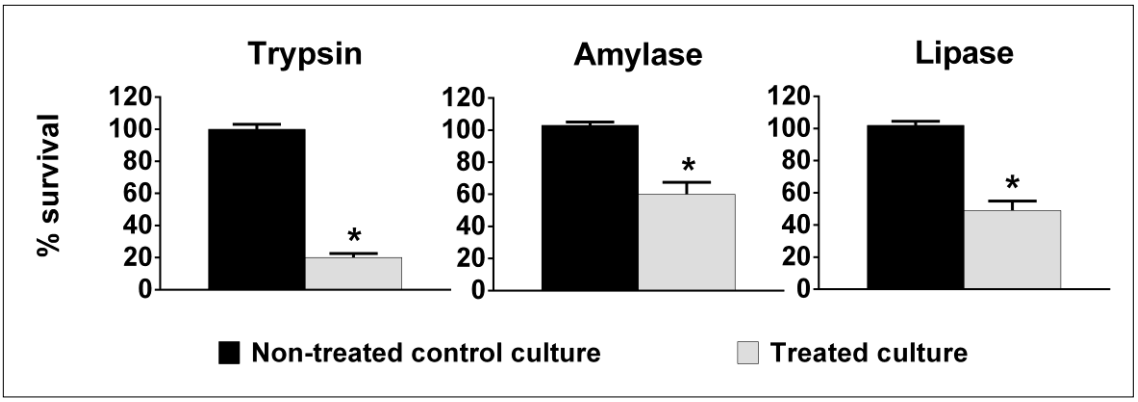

Figure 4.2. *In vitro* survival of *L. lactis* in pancreatic enzymes. *L. lactis* was incubated for 2 hours with trypsin (2.2 IU/ml), amylase (70 U/ml) or lipase (10 U/ml). Survival is expressed as a percentage of the CFUs in the starting culture. Data represent the mean ± SD of three independent experiments. *indicates a statistical difference between enzyme-treated and non-enzyme-treated control cultures (P< 0.0001).

5.3.3. Protection of *L. lactis* from bile toxicity using aluminium hydroxide

Aluminium hydroxide was capable of protecting *L. lactis* from bile toxicity in a concentration-dependent manner (Figure 4.3). Each concentration of aluminium hydroxide resulted in a significant increase in *L. lactis* viability compared to the non-protected control or to lower concentrations of the protectant (*P*< 0.0001).

At the concentrations of 20 and 40 mg/ml of aluminium hydroxide, *L. lactis* survival was significantly higher at pH 5.5 compared to 6.8 (*P*< 0.0001). This finding indicates a positive correlation between the protective effect of aluminium hydroxide at these concentrations and the acidic pH of 5.5. At pH 5.5, full protection of *L. lactis* viability is achieved at a concentration of 40 mg/ml aluminium hydroxide, whereas at pH 6.8 this was only achieved at a concentration of 80 mg/ml.

5.3.4. Protection of *L. lactis* from trypsin toxicity using camostat mesylate

As shown in Figure 4.4, the oral trypsin inhibitor camostat mesylate protected *L. lactis* from the hydrolytic effect of porcine trypsin in a concentration-dependent manner. Each concentration of camostat mesylate resulted in a significant increase of *L. lactis* viability compared to the non-protected control or to lower concentrations of the protectant (*P*< 0.05). Complete viability of *L. lactis* was retained in the presence 200 µg/ml camostat mesylate.

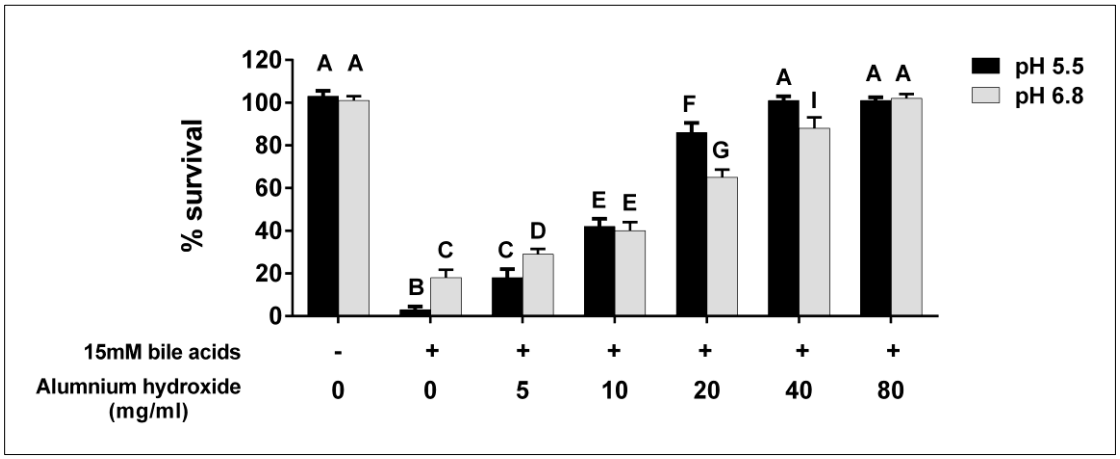

Figure 4.2. *In vitro* protection of *L. lactis* from bile toxicity using aluminium hydroxide. *L. lactis* cells were incubated for 2 h with bile containing 15 mM of bile acids and different concentrations of aluminium hydroxide either at pH 5.5 or 6.8. Survival is calculated based on CFU counts, as a percentage of the starting culture. Data represent the mean ± SD of three independent experiments. Significant differences (P<0.01) between the conditions are indicated by a different capital letter.

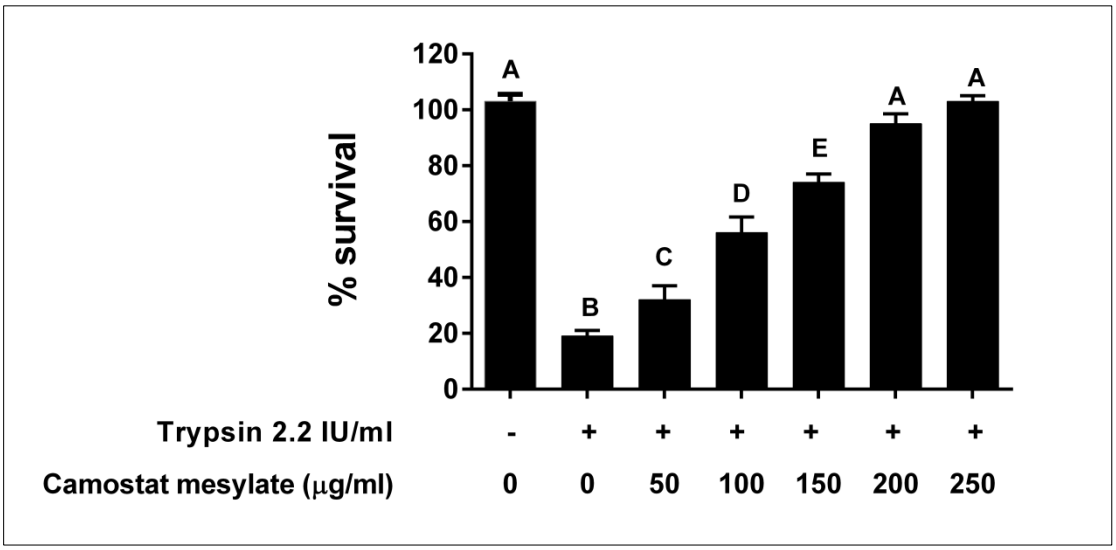

Figure 4.3. *In vitro* protection of *L. lactis* from trypsin digestion using camostat mesylate. *L. lactis* was incubated for 2 h with 2.2 IU/ml of trypsin and different concentrations of camostat mesylate. Survival is calculated based on CFU counts, as a percentage of the starting culture. Data represent the mean ± SD of three independent experiments. Significant differences (P< 0.05) are indicated with a different capital letter.

5.3.5. Oral co-administration of aluminium hydroxide and camostat mesylate with *L. lactis* improves its GI survival in pigs

We finally validated the capacity of aluminium hydroxide and camostat mesylate to improve the survival of *L. lactis in vivo*. Pigs receiving *L. lactis* in combination with camostat mesylate and aluminium hydroxide, showed higher counts of *L. lactis* in their intestinal contents, compared to those receiving *L. lactis* only (figure 4.5). The counts were 38 and 24 times higher (P< 0.0001) in jejunal and ileal contents of camostat mesylate- and aluminium hydroxide-treated pigs respectively. These results indicate the validity of these protectants for increasing *L. lactis* survival in the GI tract pigs.

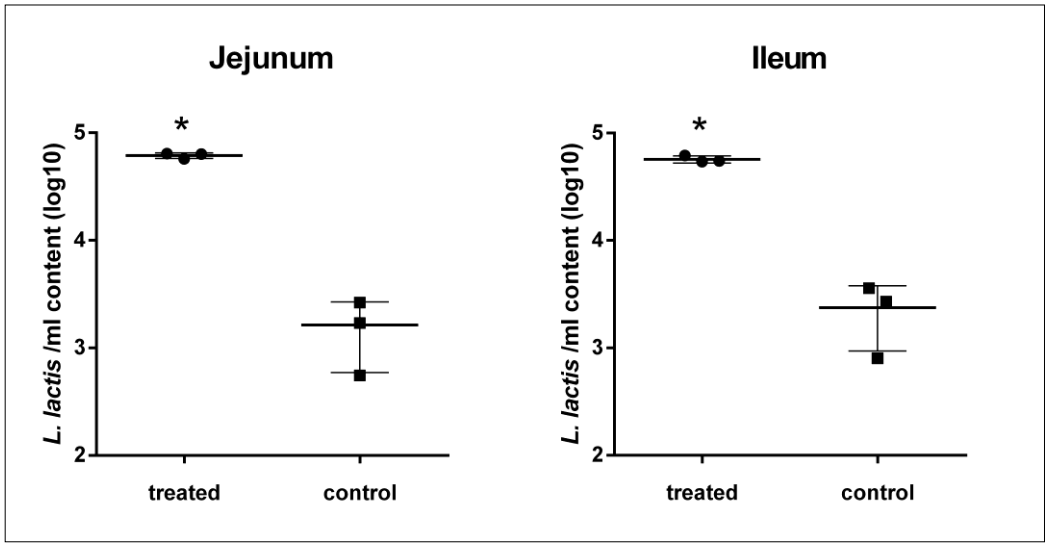

Figure 4.5. Protection of *L. lactis* in the gastrointestinal tract of pigs by co-administering aluminium hydroxide and camostat mesylate. Pigs received an oral dose of $6x10^{11}$ CFU of *L. lactis* in the presence (treated group, n=3) or absence of aluminium hydroxide and camostat mesylate (control group, n=3). Data represent mean bacterial counts (log 10) per millilitre intestinal content of each individual animal 8 hrs after inoculation (presented by dots) and the group mean $\pm$ SD (presented by horizontal lines). * indicates a statistical difference between control and treated (P< 0.0001).

5.4. Discussion

L. lactis is a GRAS bacterium with a clinical potential for the intestinal delivery of therapeutic and prophylactic molecules (Berlec *et al.*, 2012). Nevertheless, a relatively low survival of *L. lactis* has been observed in the small intestine (Drouault *et al.*, 1999; Drouault *et al.*, 2002; Termonts, 2005), suggesting a detrimental effect of the intestinal physiological conditions on its viability. In order to investigate this issue, we

performed a series of *in vitro* experiments to determine the survival of *L. lactis* in bile and the major pancreatic enzymes. Additionally we explored the possibility of protecting *L. lactis* against the highly toxic effects of bile and trypsin. Notably, our experimental conditions were chosen to mimic the physiological conditions encountered in the small intestine of pigs, a species with a known relevance to human in regard to GI anatomy and physiology (Darragh & Moughan, 1995; Eubanks *et al.*, 2006; Moughan PJ, 1992; Rowan *et al.*, 1994).

Bile is a digestive secretion that represents a serious challenge for the survival of bacteria in the small intestine (Begley *et al.*, 2005a). First, we determined the prevalent physiological concentrations of bile acids in the different parts of the pig small intestine. Concentrations of 14.4 ± 0.5 mM, 9.5 ± 0.7 mM and 4.5 ± 0.4 mM were measured for duodenum, jejunum and ileum, respectively. Interestingly, these concentrations corresponds well with those described in literature for human GI tract (15 mM, 10 mM and 4 mM, respectively) (Marteau *et al.*, 1997). Furthermore, it has been shown that the chemical composition of bile is highly conserved among humans and pigs (Begley *et al.*, 2005b). Together, these findings further support the relevance of the pig as a model species for studying the survival of *L. lactis* in the GI tract. Thereafter, we evaluated the survival of *L. lactis* following the incubation with freshly isolated porcine bile with the bile acid concentrations adjusted to mimic those measured in the small intestine of pigs under physiological conditions. The exposure of *L. lactis* to these concentrations of bile acids resulted in a marked decrease of its survival, indicating toxicity. Previous studies have reported the susceptibility of *L. lactis* subspecies *cremoris* to the presence of bile salts in the growth medium (Kim *et al.*, 1999; Kimoto-Nira *et al.*, 2009; Kimoto *et al.*, 2003). However, it is rather difficult to compare our findings to those described in these reports due to the significant variations in the applied experimental conditions. Nevertheless, to our knowledge, the present study is the first to evaluate bile toxicity to *L. lactis* under physiological conditions. Additionally, we observed a positive correlation between acidity and bile toxicity, since the viability of *L. lactis* was significantly lower at pH 5.5 compared to pH 6.8. A similar finding has also been reported for *Lactobacillus*, where a higher toxicity of glyco-conjugated bile acids was observed at acidic pH (Desmet *et al.*, 1995). These findings indicate a role of acidic pH in augmenting bile toxicity to these bacterial species. Although several mechanisms have been described for the bactericidal effect of bile, including direct dissociation of membrane lipids and proteins (Coleman *et al.*,

1980), interrupted permeability and fluidity across the bacterial cell membrane or altered activity of critical membrane-bound enzymes and increased trans-membrane flux of divalent cations, leading to cell death (Fujisawa & Mori, 1996; Noh & Gilliland, 1993), the specific mechanism by which acidic pH augments bile toxicity to bacteria remains to be elucidated.

Another group of factors, which could potentially influence the survival of *L. lactis* in the small intestine, are the pancreatic digestive enzymes. Indeed, incubation of *L. lactis* with trypsin, amylase or lipase, at activities described for these enzymes in the small intestine of pigs under normal physiological conditions, resulted in a reduced survival of *L. lactis*. This observation can be explained by the chemical composition of the Gram-positive cell wall, which reveals several potential substrates for the specific hydrolytic actions of these enzymes (Shockman & Barrett, 1983). For instance, lipase can hydrolyse phospholipids of the cytoplasmic membrane, amylase can hydrolyse the glycosidic bounds between N-acetylmuramide and N-acetylglucosamine in the cell wall, while trypsin can hydrolyse the integral membrane protein. Subsequently, degradation of these structural components can result in loss of cell wall integrity and eventually cell death. Among the tested pancreatic enzymes, we found that trypsin exerts the most detrimental effect on *L. lactis* survival. Previously, Drouault and co-workers have reported the susceptibility of *L. lactis* to trypsin hydrolysis (Drouault *et al.*, 1999). However, they described a dramatic decrease in *L. lactis* viability after 30 minutes of incubation with trypsin (0.0012 – 0.012 %), whereas our results show a 20% survival after two hours of incubation. This difference is probably a result of a high concentration/activity of trypsin in their study in comparison with the physiological levels respected in our study.

Thereafter, and in light of our findings on the deleterious effects of bile and trypsin on *L. lactis* survival, we performed a second set of *in vitro* experiments to evaluate the capacity of aluminium hydroxide and camostat mesylate to overcome bile toxicity and trypsin hydrolysis, respectively. Aluminium hydroxide is known for its capacity to bind bile acids (Clain *et al.*, 1977) and has been clinically used for the treatment of bile salt-mediated diarrhea and bile-reflux gastritis (Maton & Burton, 1999). Our findings show that aluminium hydroxide could protect *L. lactis* from bile toxicity. Additionally, we observed a higher protective effect for aluminium hydroxide at pH 5.5 compared to pH 6.8, at the concentrations of 20 and 40 mg/ml. Aluminium hydroxide typically

shows an enhanced bile acid binding capacity at acidic pH (Mangnall *et al.*, 1986). This feature can be particularly useful for protection of *L. lactis* in the duodenum and jejunum, where a low pH and a high concentration of bile are simultaneously encountered. Edwards and Slater have previously reported the use of cholestyramine, a bile acid binding resin, to protect a live *Salmonella typhimurium* vector against bile toxicity *in vitro* (Edwards & Slater, 2009; Edwards *et al.*, 2010). However, our preliminary experiments showed a negative effect of cholestyramine on *L. lactis* viability (data not shown), which on the contrary, has not been observed for aluminium hydroxide. Camostat mesylate is a synthetic ester that can inhibit trypsin activity *in vivo* following oral administration. Currently, it is clinically used for the treatment of pancreatitis (Kanoh *et al.*, 1989) and reflux esophagitis (Sasaki *et al.*, 1989) in Japan. Thus, we considered investigating the efficiency of camostat mesylate to counteract the detrimental effect of trypsin on *L. lactis* survival. Our results show a protective effect of camostat mesylate on *L. lactis* against trypsin hydrolysis. In regard to amylase and lipase, unfortunately, the approach of using enzyme inhibitors to protect *L. lactis* against their hydrolytic effects could not be evaluated. Although numerous inhibitors have been identified to pancreatic amylase, none of them has been proven efficacious *in vivo* (de Sales *et al.*, 2012), while orlistat, the only clinically approved oral lipase inhibitor (Heck *et al.*, 2000), is not commercially available for research use.

After demonstrating the protective effects of aluminium hydroxide and camostat mesylate on *L. lactis* survival against bile toxicity and trypsin hydrolysis, respectively *in vitro*, we next validated the capacity of these protectants to improve the GI survival of *L. lactis in vivo*. Oral co-administration of aluminium hydroxide and camostat mesylate with *L. lactis* has increased the viability of the bacterium in the small intestine of pigs (38 and 24 fold-increased *L. lactis* counts in jejunal and ileal contents, respectively). These findings have direct implications for the development of oral formulations that can be used clinically to improve the GI survival of *L. lactis* and subsequently improve its efficiency in the intestinal delivery of biomolecules. The clinical application of live oral delivery vectors is generally performed either in a liquid formulation containing acid-neutralizing buffers or in enteric-coated capsules. The former is particularly favoured in preliminary clinical trials that are performed to establish efficacy and safety before pursuing the procedures to select and optimize an enteric-coating strategy (Kirkpatrick *et al.*, 2005; Launay *et al.*, 2009; McKenzie *et al.*,

2008; Sack *et al.*, 1997). Our findings support the use of aluminium hydroxide and camostat mesylate in oral liquid formulations of *L. lactis* in order to improve its efficiency as a live delivery vector. Furthermore, they provide a basis for further development of enteric-coated capsules that can simultaneously release aluminium hydroxide and camostat mesylate with *L. lactis* so as to improve its survival in the small intestine. A similar approach has been described for the use of cholestyramine as a composite of enteric-coated tablets to protect a live *Salmonella Typhimurium* vaccine vector against bile toxicity upon enteric release (Edwards & Slater, 2009; Edwards *et al.*, 2010).

5.5. Conclusion

The present study shows that the *in vitro* exposure of *L. lactis* to the physiological concentrations of bile, trypsin, amylase and lipase, as described the small intestine of pigs, can significantly reduce the survival of *L. lactis*. Given the high similarity of human and porcine GI physiology, these findings could explain the low survival of *L. lactis* following the passage through the human GI tract. Furthermore, we demonstrated *in vitro* the capacity of the bile acid binder, aluminium hydroxide and the oral trypsin inhibitor, camostat mesylate to protect *L. lactis* against bile toxicity and trypsin hydrolysis respectively. Finally, we proved the concept of using camostat mesylate and aluminium hydroxide in oral *L. lactis* formulation to improve its GI survival. These findings have significant implications for the design of oral liquid or enteric-coated formulations that ensure a higher survival of *L. lactis* in the GI tract and to subsequently enhance its efficiency in the intestinal delivery of prophylactic and therapeutic molecules.

5.6. Acknowledgement

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5.7. References

A-B

ActoGenix (2009). ActoGeniX completes Phase 2A clinical trial of AG011. *ActoGeniX NV Press release*.

Adel-Patient, K., Ah-Leung, S., Creminon, C., Nouaille, S., Chatel, J. M., Langella, P. & Wal, J. M. (2005). Oral administration of recombinant *Lactococcus lactis* expressing bovine beta-lactoglobulin partially prevents mice from sensitization. *Clin Exp Allergy* **35**, 539-546.

Bahey-El-Din, M., Gahan, C. G. M. & Griffin, B. T. (2010). *Lactococcus lactis* as a Cell Factory for Delivery of Therapeutic Proteins. *Curr Gene Ther* **10**, 34-45.

Bahey-El-Din, M. & Gahan, C. G. (2011). *Lactococcus lactis*-based vaccines: current status and future perspectives. *Hum Vaccin* **7**, 106-109.

Beck, I. T. (1973). The role of pancreatic enzymes in digestion. *The American journal of clinical nutrition* **26**, 311-325.

Begley, M., Gahan, C. G. M. & Hill, C. (2005a). The interaction between bacteria and bile. *Fems Microbiol Rev* **29**, 625-651.

Begley, M., Sleator, R. D., Gahan, C. G. & Hill, C. (2005b). Contribution of three bile-associated loci, bsh, pva, and btlB, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infection and immunity* **73**, 894-904.

Berlec, A., Ravnikar, M. & Strukelj, B. (2012). Lactic acid bacteria as oral delivery systems for biomolecules. *Pharmazie* **67**, 891-898.

Bermudez-Humaran, L. G., Kharrat, P., Chatel, J. M. & Langella, P. (2011). Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. *Microb Cell Fact* **10**.

Braat, H., Rottiers, P., Hommes, D. W. & other authors (2006). A phase I trial with Transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol H* **4**, 754-759.

C-D

Casalta, E. & Montel, M. C. (2008). Safety assessment of dairy microorganisms: The *Lactococcus* genus. *International journal of food microbiology* **126**, 271-273.

Cauchard, S., Bermudez-Humaran, L. G., Blugeon, S., Laugier, C., Langella, P. & Cauchard, J. (2011). Mucosal co-immunization of mice with recombinant lactococci secreting VapA antigen and leptin elicits a protective immune response against *Rhodococcus equi* infection. *Vaccine* **30**, 95-102.

Cho, H. J., Shin, H. J., Han, I. K., Jung, W. W., Kim, Y. B., Sul, D. & Oh, Y. K. (2007). Induction of mucosal and systemic immune responses following oral immunization of mice with *Lactococcus lactis* expressing human papillomavirus type 16 L1. *Vaccine* **25**, 8049-8057.

Clain, J. E., Malagelada, J. R., Chadwick, V. S. & Hofmann, A. F. (1977). Binding properties in vitro of antacids for conjugated bile acids. *Gastroenterology* **73**, 556-559.

Coleman, R., Lowe, P. J. & Billington, D. (1980). Membrane Lipid-Composition and Susceptibility to Bile-Salt Damage. *Biochim Biophys Acta* **599**, 294-300.

Darragh, A. J. & Moughan, P. J. (1995). The 3-Week-Old Piglet as a Model Animal for Studying Protein Digestion in Human Infants. *J Pediatr Gastr Nutr* **21**, 387-393.

de Azevedo, M., Karczewski, J., Lefevre, F., Azevedo, V., Miyoshi, A., Wells, J. M., Langella, P. & Chatel, J. M. (2012). *In vitro* and *in vivo* characterization of DNA delivery using

recombinant *Lactococcus lactis* expressing a mutated form of *L. monocytogenes* Internalin A. *Bmc Microbiol* **12**.

de Sales, P. M., de Souza, P. M., Simeoni, L. A., Magalhaes, P. D. & Silveira, D. (2012). alpha-Amylase Inhibitors: A Review of Raw Material and Isolated Compounds from Plant Source. *J Pharm Pharm Sci* **15**, 141-183.

Desmet, I., Vanhoorde, L., Woestyne, M. V., Christiaens, H. & Verstraete, W. (1995). Significance of Bile-Salt Hydrolytic Activities of Lactobacilli. *J Appl Bacteriol* **79**, 292-301.

Drouault, S., Corthier, G., Ehrlich, S. D. & Renault, P. (1999). Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. *Appl Environ Microb* **65**, 4881-4886.

Drouault, S., Juste, C., Marteau, P., Renault, P. & Corthier, G. (2002). Oral treatment with *Lactococcus lactis* expressing *Staphylococcus hyicus* lipase enhances lipid digestion in pigs with induced pancreatic insufficiency. *Appl Environ Microbiol* **68**, 3166-3168.

E-H

Edwards, A. D. & Slater, N. K. H. (2009). Protection of live bacteria from bile acid toxicity using bile acid adsorbing resins. *Vaccine* **27**, 3897-3903.

Edwards, A. D., Chatterjee, P., Mahbubani, K. T., Reis, C. M. & Slater, N. K. H. (2010). Optimal protection of stabilised dry live bacteria from bile toxicity in oral dosage forms by bile acid adsorbent resins. *Chem Eng Sci* **65**, 4844-4854.

Eubanks, D. L., Cooper, R. & Boring, J. G. (2006). Surgical technique for long-term cecal cannulation in the yucatan minipig (Sus scrofa domestica). *J Am Assoc Lab Anim* **45**, 52-56.

Fujisawa, T. & Mori, M. (1996). Influence of bile salts on beta-glucuronidase activity of intestinal bacteria. *Lett Appl Microbiol* **22**, 271-274.

Gasson, M. J. (1983). Plasmid complements of Streptococcus *lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**, 1-9.

Guimaraes, V. D., Gabriel, J. E., Lefevre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V. & Langella, P. (2005). Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and infection / Institut Pasteur* **7**, 836-844.

Heck, A. M., Yanovski, J. A. & Calis, K. A. (2000). Orlistat, a new lipase inhibitor for the management of obesity. *Pharmacotherapy* **20**, 270-279.

Hedemann, M. S. & Jensen, B. B. (2004). Variations in enzyme activity in stomach and pancreatic tissue and digesta in piglets around weaning. *Archives of animal nutrition* **58**, 47-59.

Huibregtse, I. L., Snoeck, V., De Creus, A., Braat, H., De Jong, E. C., Van Deventer, S. J. H. & Rottiers, P. (2007). Induction of ovalbumin-specific tolerance by oral administration of *Lactococcus lactis* secreting ovalbumin. *Gastroenterology* **133**, 517-528.

Huyghebaert, N., Vermeire, A., Rottiers, P., Remaut, E. & Remon, J. P. (2005). Development of an enteric-coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*. *Eur J Pharm Biopharm* **61**, 134-141.

I-K

Innocentin, S., Guimaraes, V., Miyoshi, A., Azevedo, V., Langella, P., Chatel, J. M. & Lefevre, F. (2009). *Lactococcus lactis* Expressing either *Staphylococcus aureus* Fibronectin-Binding Protein A or Listeria monocytogenes Internalin A Can Efficiently Internalize and Deliver DNA in Human Epithelial Cells. *Appl Environ Microb* **75**, 4870-4878.

Kanoh, M., Ibata, H., Miyagawa, M. & Matsuo, Y. (1989). Clinical Effects of Camostat in Chronic-Pancreatitis. *Biomed Res-Tokyo* **10**, 145-150.

Kim, W. S., Ren, J. & Dunn, N. W. (1999). Differentiation of *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains by their adaptive response to stresses. *Fems Microbiol Lett* **171**, 57-65.

Kimoto-Nira, H., Kobayashi, M., Nomura, M., Sasaki, K. & Suzuki, C. (2009). Bile resistance in *Lactococcus lactis* strains varies with cellular fatty acid composition: analysis by using different growth media. *International journal of food microbiology* **131**, 183-188.

Kimoto, H., Nomura, M., Kobayashi, M., Mizumachi, K. & Okamoto, T. (2003). Survival of lactococci during passage through mouse digestive tract. *Can J Microbiol* **49**, 707-711.

Kirkpatrick, B. D., Tenney, K. M., Larsson, C. J. & other authors (2005). The novel oral typhoid vaccine M01ZH09 is well tolerated and highly immunogenic in 2 vaccine presentations. *The Journal of infectious diseases* **192**, 360-366.

Klayraung, S., Viernstein, H. & Okonogi, S. (2009). Development of tablets containing probiotics: Effects of formulation and processing parameters on bacterial viability. *Int J Pharm* **370**, 54-60.

Klijn, N., Weerkamp, A. H. & Devos, W. M. (1995). Genetic Marking of Lactococcus *lactis* Shows Its Survival in the Human Gastrointestinal Tract. *Appl Environ Microb* **61**, 2771-2774.

Kristy N. Kuzmuk, L. B. S. (2011). Pigs as a model for biomedical sciences. *MF Rothchild, A Ruvinsky (Eds), The genetics of the pig, CAB International, Wallingford*, 426–444.

L-P

Launay, O., Sadorge, C., Jolly, N. & other authors (2009). Safety and immunogenicity of SC599, an oral live attenuated *Shigella dysenteriae* type-1 vaccine in healthy volunteers: results of a Phase 2, randomized, double-blind placebo-controlled trial. *Vaccine* **27**, 1184-1191.

Litten-Brown, J. C., Corson, A. M. & Clarke, L. (2010). Porcine models for the metabolic syndrome, digestive and bone disorders: a general overview. *Animal* **4**, 899-920.

Mangnall, Y. F., Smythe, A. & Johnson, A. G. (1986). The Ability of Antacids and Cholestyramine to Bind Bile Acids - Effect of Ph. *Scand J Gastroentero* **21**, 789-794.

Marteau, P., Minekus, M., Havenaar, R. & Veld, J. H. J. H. I. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: Validation and the effects of bile. *J Dairy Sci* **80**, 1031-1037.

Maton, P. N. & Burton, M. E. (1999). Antacids revisited - A review of their clinical pharmacology and recommended therapeutic use. *Drugs* **57**, 855-870.

McKenzie, R., Darsley, M., Thomas, N. & other authors (2008). A double-blind, placebo-controlled trial to evaluate the efficacy of PTL-003, an attenuated enterotoxigenic *E. coli* (ETEC) vaccine strain, in protecting against challenge with virulent ETEC. *Vaccine* **26**, 4731-4739.

Moughan PJ, B. M., Cranwell PD, Smith WC, Pedraza M. (1992). The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World review of nutrition and dietetics* 40-113.

Noh, D. O. & Gilliland, S. E. (1993). Influence of Bile on Cellular Integrity and Beta-Galactosidase Activity of *Lactobacillus acidophilus*. *J Dairy Sci* **76**, 1253-1259.

Poelvoorde, N., Huyghebaert, N., Vervaet, C. & Remon, J. P. (2008). Optimisation of an enteric coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*. *Eur J Pharm Biopharm* **69**, 969-976.

Robinson, K., Chamberlain, L. M., Lopez, M. C., Rush, C. M., Marcotte, H., Le Page, R. W. F. & Wells, J. M. (2004). Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. *Infection and immunity* **72**, 2753-2761.

R-T

Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K. & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *The British journal of nutrition* **71**, 29-42.

Sack, D. A., Shimko, J., Sack, R. B., Gomes, J. G., MacLeod, K., O'Sullivan, D. & Spriggs, D. (1997). Comparison of alternative buffers for use with a new live oral cholera vaccine, Peru-15, in outpatient volunteers. *Infection and immunity* **65**, 2107-2111.

Sasaki, I., Suzuki, Y., Naito, H. & other authors (1989). Effect of Camostat in the Treatment of Reflux Esophagitis after Gastrectomy- an Experimental-Study in Rats and a Pilot Clinical-Study. *Biomed Res-Tokyo* **10**, 167-173.

Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme Microb Tech* **27**, 761-765.

Shockman, G. D. & Barrett, J. F. (1983). Structure, Function, and Assembly of Cell-Walls of Gram-Positive Bacteria. *Annu Rev Microbiol* **37**, 501-527.

Snoeck, V., Cox, E., Verdonck, F., Joensuu, J. J. & Goddeeris, B. M. (2004). Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. *Vet Microbiol* **98**, 45-53.

Stadler, M. & Viernstein, H. (2003). Optimization of a formulation containing viable lactic acid bacteria. *Int J Pharm* **256**, 117-122.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352-1355.

Termonts, S. (2005). Optimization of *Lactococcus lactis* mediated delivery of cytokines. *PhD dissertation.*

V-Z

Vandenbroucke, K., Hans, W., Van Huysse, J., Neirynck, S., Demetter, P., Remaut, E., Rottiers, P. & Steidler, L. (2004). Active delivery of trefoil factors by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice. *Gastroenterology* **127**, 502-513.

Vesa, T., Pochart, P. & Marteau, P. (2000). Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment Pharm Therap* **14**, 823-828.

Wilfart, A., Montagne, L., Simmins, H., Noblet, J. & van Milgen, J. (2007). Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. *Livest Sci* **109**, 27-29.

Zhuang, Z., Wu, Z. G., Chen, M. & Wang, P. G. (2008). Secretion of human interferon-beta 1b by recombinant *Lactococcus lactis*. *Biotechnol Lett* **30**, 1819-1823.

5.1. General discussion

5.1.1. Oral vaccination to control enterohemorrhagic *Escherichia coli* (EHEC) infection:

Currently, there is not a single vaccine approved for use against EHEC infection in humans (Garcia-Angulo *et al.*, 2013). However, two parenteral vaccines were recently licensed to control EHEC infection in cattle, the main reservoir for the bacteria (*Escherichia coli* Bacterial Extract SPR® in the US, based on bacterial outer membrane siderophore receptor and porin (SRP) proteins and Econiche™ in Canada, based on T3SS proteins). Econiche was the result of several studies demonstrating that parenteral vaccination with multiple T3SS proteins reduced faecal excretion of EHEC. Either a combination of EspA, EspB and Tir or, one of those former proteins combined with intimin were effective (Allen *et al.*, 2011; McNeilly *et al.*, 2010; Peterson *et al.*, 2007; Potter *et al.*, 2004; Smith *et al.*, 2009; Vilte *et al.*, 2011). However, at least three subsequent intramuscular immunizations were needed to significantly reduce the number of bacteria shed in the faeces, the number of animals excreting bacteria as well as the duration of shedding. Two vaccinations could not significantly reduce the number of shedders (Van Donkersgoed *et al.*, 2005). This showed the need for improvement of vaccines and vaccination strategy.

T3SS proteins are in particular promising candidates, since the induction of specific immune responses against them can interfere with the process of intestinal colonization (Garcia-Angulo *et al.*, 2013). A vast collection of preclinical vaccination studies have been conducted, mainly in mice models, to target EHEC infection in humans (Garcia-Angulo *et al.*, 2013). These studies demonstrated that also mucosal immunizations (nasal and oral) using T3SS proteins such as Intimin, Tir, EspA or their combinations reduced the fecal shedding of EHEC (Amani *et al.*, 2011; Babiuk *et al.*, 2008; Fan *et al.*, 2012; Gu *et al.*, 2011; Judge *et al.*, 2004; Wan *et al.*, 2011). The protection was accompanied by the appearance of specific IgA in the feces of immunized mice. In fact, IgA seem to be important for protection, since in some of these studies, protection could not be afforded by parenteral immunization, which elicited serum IgG, but not intestinal IgA responses (Babiuk *et al.*, 2008; Fan *et al.*, 2012; Wan *et al.*, 2011).

Our immunization studies (**Chapters 2 and 3**), were the first to target the induction of a specific systemic and intestinal immune response against the T3SS

protein EspB via oral immunization. In agreement with other immunization studies involving T3SS antigens (intimin, Tir and EspA), mice orally immunized with EspB showed protection against colonization by *E. coli* O157:H7. This protection could be correlated with the detection of EspB-specific IgA in their feces (**Chapter 3**). Although EspB exhibits marked immunogenicity in the course of natural human and animal infections as well as in experimental infections (Li, Frey et al. 2000, Karpman, Bekassy et al. 2002, Cataldi, Yevsa et al. 2008, Asper, Karmali et al. 2011), the potential of the antigen for mucosal immunization has been evaluated in only one other study. In that study nasal immunization of mice using EspB resulted in the induction of specific IgG and IgA responses in serum and lung lavage, respectively (Cataldi *et al.*, 2008). However, our studies are the first to describe the potential of EspB as an oral vaccine candidate.

5.1.2. *Lactococcus lactis* for oral delivery of vaccine antigens: lessons from the case of EspB

5.1.2.1. Cytoplasmic expression versus secretion

As we describe in **Chapter 2**, the cytoplasmic expression of EspB in *L. lactis* has resulted in a low antigen yield, which is probably the main reason why a weak immune-priming was obtained by this recombinant strain upon oral immunization. Indeed, *L. lactis* possess at least two cytoplasmic proteases (ClpP and FtsH) (Frees & Ingmer, 1999; Nilsson *et al.*, 1994), in addition to DnaK, the cytoplasmic chaperon that promotes antigen proteolysis by maintaining the unfolding state (Koch *et al.*, 1998). These factors can cause degradation of heterologous antigens designated to cytoplasmic expression, resulting in low expression yields (Bermudez-Humaran *et al.*, 2003b; Ribeiro *et al.*, 2002). On the other hand, secretion of antigens allows them to escape cytoplasmic proteolysis, since researchers have previously observed that the cytoplasmic precursors of the secreted antigens are resistant to the action of cytoplasmic proteases compared to the proteins initially designated to cytoplasmic expression (Le Loir *et al.*, 2005). Thus, secretion increases antigen yield and delivery as it allows the continuous release of antigens by viable bacteria and simultaneously accumulates the antigen in the cytoplasm (precursor of the secreted antigen) (Bermudez-Humaran *et al.*, 2003a; Bermudez-Humaran *et al.*, 2003b; Ribeiro *et al.*, 2002). The latter can significantly contribute to antigen delivery, providing that a part of the *L. lactis* inoculum gets lysed in the gastrointestinal tract, leading to the release

of the antigen content of the cytoplasm. Therefore, in **Chapter 3**, we explored secretion as a strategy to enhance the expression yield and the delivery of EspB by the bacteria. Indeed, secretion of EspB in *L. lactis* has markedly enhanced the overall expression levels (cytoplasmic and secreted) compared to cytoplasmic expression (56 - 25-fold increase). Unlike what was observed with cytoplasmic expression of EspB, optimized expression of EspB in *L. lactis,* via antigen secretion, has allowed the induction of complete immune responses in mice upon oral immunization

5.1.2.2. Constitutive versus inducible expression systems

Antigen expression in *L. lactis* can be done using either constitutive or inducible expression systems (Bahey-El-Din *et al.*, 2010b). Although several inducible expression systems have been developed for *L. lactis*, the nisin-inducible expression system (NICE) is the most characterized and commonly used system for antigen delivery. Constitutive expression systems would imply the continuous synthesis and release of antigens by viable lactococci in situ. On the other hand, inducible systems require the presence of the inducer in the medium to sustain antigen synthesis. Thus, since inducers are absent *in vivo*, antigen release in situ may not be sustained. Notwithstanding this limitation, *in vitro* studies show that nisin-inducible expression systems produce larger amounts of antigen compared to constitutive counterparts (Bermudez-Humaran *et al.*, 2004; Chatel *et al.*, 2001).

The work presented in **Chapter 3,** provide the first comparison between the constitutive and nisin-inducible expression systems for their capacity for oral antigen delivery, as indicated by the strength of the resultant immune responses. Indeed, *in vitro* analysis of EspB expression using either constitutive or nisin-inducible expression systems shows that the overall expression of EspB by the latter is approximately two-folds higher than by the former. Both recombinant strains were used in oral immunization studies. Results were in agreement with the higher capacities for antigen expression, in that the nisin-inducible recombinant strain induced the higher antibody responses. In light of these findings, it seems that the sustainability of in situ antigen release by the constitutive strain does not constitute an advantage over nisin-inducible strains in mice. This could be explained by the findings of a previous study, where antigen synthesis by nisin-inducible expression systems continued *in vitro* for up to 10 hours after the removal of nisin from the culture medium (Bermudez-Humaran *et al.*, 2003b). Considering that the transit time of *L. lactis* in the mouse

gastrointestinal tract is approximately 12 hours and that the immune inductive sites are reached within one hour (Drouault *et al.*, 1999), it is likely that the immunologically important antigen release by either the constitutive or the nisin-inducible strain is comparably long in mice *in vivo*. However, further investigations are required to determine whether a similar outcome can be obtained in larger species with a longer transit time such as humans. For instance, in pigs with a normal gastrointestinal transit, an average time of 9 hours is required for the majority of the bacteria to reach the ileum, where the immune inductive sites locate (Steidler *et al.*, 2003). Thus, despite a lower antigen expression levels likely obtained by constitutive expression systems, they are probably more suitable for *L. lactis*-based vaccines in humans and pigs. In addition, constitutive systems can readily secrete antigens *in vivo* without the need for a prior nisin induction *in vitro*. The induction step can be a limiting factor for application of a vaccine in practice.

5.1.2.3. The immunomodulatory effect of *L. lactis* on mucosal immune responses

Besides the mechanical delivery of antigen to mucosal targets, a desirable advantage of vaccine delivery vectors is their ability to modulate the quality of the immune responses elicited to the antigens. In this regard, studies involving the mucosal delivery of vaccine antigens using *L. lactis* have described mixed Th1/Th2 cell responses against these antigens (Cauchard *et al.*, 2011; Robinson *et al.*, 1997; Villena *et al.*, 2008). In agreement with these reports, oral delivery of EspB using recombinant *L. lactis* strains induced mixed Th1/Th2 response in Peyer's patches and mesenteric lymph nodes of immunized mice (**Chapter 3**). Lymphocytes of these organs simultaneously secreted IFN-γ and IL-4 (Th1 and Th2 marker cytokines, respectively) in an *in vitro* antigen recall assay. It is well-established that the type of T helper response induced by a vaccine is mainly determined by dendritic cells. *In vitro* studies have shown the ability of *L. lactis* to induce dendritic cell maturation (Audouy *et al.*, 2007; Yam *et al.*, 2008), and secretion of the cytokines that potentially direct the differentiation of naive Th cells to both Th1 and Th2 cell types (IFN-γ and IL-12, and IL-6 and IL-10, respectively)(Latvala *et al.*, 2008).

5.1.3. Towards the validation of the *L. lactis*-based EHEC vaccine in porcine models of infection: the issue of gastrointestinal survival

We have demonstrated the protective potential of our vaccine strains in a mouse model of colonization. Although mice models are basically acceptable to study the pathogenicity of EHEC and to evaluate EHEC vaccines, there is not a single animal model that mimics the complete pathogenicity of EHEC (Mohawk & O'Brien, 2011). The EHEC infection models developed in gnotobiotic pigs mimics the human pathogenicity to a larger extent (Francis *et al.*, 1986; Tzipori *et al.*, 1986; Tzipori *et al.*, 1989; Tzipori *et al.*, 2004; Zhang *et al.*, 2009). More and more data support that pigs are a suitable model species to human in biomedical research (Kristy N. Kuzmuk, 2011; Litten-Brown *et al.*, 2010; Meurens *et al.*, 2012). Pigs share several physiological, anatomical and immunological similarities with human compared to murine species (Bailey *et al.*, 2013; Dawson *et al.*, 2013; Peter A . McAnulty 2011). Accordingly, the evaluation of oral vaccines that target diseases in pig models can be feasible to validate their efficacy prior the initiation of human clinical trials. However, several reports have described the low capacity of *L. lactis* to survive the passage through human and porcine gastrointestinal tracts (Drouault *et al.*, 2002; Klijn *et al.*, 1995; Termonts, 2005; Vesa *et al.*, 2000). Although this reflects the sensitivity of the bacteria to the physiological conditions encountered during the alimentary passage, limited studies have studied the survival of *L. lactis* in these conditions (Termonts, 2005).

Thus, in the present thesis (**Chapter 4),** we studied the detrimental effects of gastrointestinal conditions on *L. lactis* survival *in vitro*. Importantly, in our experimental design, we have considered mimicking the prevalent physiological levels of these factors in the small intestine, as described for healthy young pigs. We identified bile and trypsin as two detrimental factors for *L. lactis* survival. Accordingly, we explored the utilization of two protectants, aluminum hydroxide (Bile acid binder) and camostat mesylate (trypsin inhibitor) to protect *L. lactis* against bile toxicity and trypsin hydrolysis, respectively. A particularly interesting advantage of aluminium hydroxide and camostat mesylate is their bioactivity *in vivo*. The former is well-known for the treatment of bile salt-mediated diarrhea and bile-reflux gastritis (Maton & Burton, 1999), while the latter is currently used in Japan for the treatment of pancreatitis (Kanoh *et al.*, 1989) and reflux esophagitis (Sasaki *et al.*, 1989). On the basis of these effects, we evaluated for the first time the capacity of these protectants

to protect *L. lactis in vivo* in pigs against bile toxicity and trypsin hydrolysis, respectively. Interestingly, oral co-administration of aluminium hydroxide and camostat mesylate with *L. lactis* can improve the survival of the bacterium in the gastrointestinal tract of pigs. This was indicated by a 38- and 24-fold increase in the number of the bacteria retrieved from jejunal and ileal contents, respectively, of treated pigs compared to untreated controls.

5.2. Future perspectives:

In the present thesis, we describe the development and preclinical evaluation of the efficacy of an oral vaccine against EHEC infections. However, further steps either in design or in efficacy validation should be pursued to prepare the vaccine to clinical trials on human subjects.

5.2.1. Validation of vaccine efficacy in porcine models

As discussed above, pigs are generally considered as good models for humans in biomedicine. Additionally, pig models of EHEC pathogenicity are the closest to the human disease. Therefore, evaluation of efficacy of our oral EHEC vaccine in pigs is a feasible step towards the extrapolation of the vaccine to human application. An immunization study in pigs will particularly validate the protective efficacy of EspB-specific mucosal IgA antibodies against EHEC colonization. The co-administration of aluminum hydroxide and camostat mesylate can improve the delivery of EspB using the recombinant *L. lactis* strains.

5.2.2. Vaccine design: the issue of biological containment

Regulatory bodies are concerned about introducing genetically modified microorganisms in human medicine. Especially, the horizontal transfer of virulence and antibiotic resistance genes is a likely event if the modified microorganism disseminates in the environment without control. Consequently, strategies for biological containment must be applied before a live bacteria-based vaccine is approved for clinical trials. In the majority of the published studies involving *L. lactis* as a live vector for oral vaccine delivery, plasmid expression systems have been used (Wells and Mercenier 2008). Since these plasmids carry antibiotic resistance genes, they are unacceptable for clinical application. This problem can be solved by integrating the antigen gene in the chromosome of *L. lactis* (Bahey-El-Din *et al.*, 2010a). This would ensure the stability of the recombinant strain and eliminate the

need for antibiotic resistance markers. However, biological containment strategies which prevent the bacterial vaccine vector from maintaining viability *ex vivo* offer the most satisfactory solution. Active and passive biological containment strategies have been published for several microorganisms. Active containment systems involve the use of genes that are tightly regulated and are expressed only under certain conditions producing a toxin or a product that kills the host cell (Kaplan *et al.*, 1999; Knudsen *et al.*, 1995; Torres *et al.*, 2000). On the other hand, passive containment systems depend on the requirement of essential metabolites where the mutant organism cannot grow in their absence (auxotrophy). Ideally, the metabolite should be absent outside the body. (Sorensen, Larsen et al. 2000). Active containment systems usually have a bactericidal effect while passive systems are usually bacteriostatic. Thymidine auxotrophy containment is a model system that is being integrated in the ActioBiotics™ technology, which was developed by the biotechnology company ActoGenix.

5.3. References

A-B

Allen, K. J., Rogan, D., Finlay, B. B., Potter, A. A. & Asper, D. J. (2011). Vaccination with type III secreted proteins leads to decreased shedding in calves after experimental infection with Escherichia coli O157. *Can J Vet Res* **75**, 98-105.

Amani, J., Mousavi, S. L., Rafati, S. & Salmanian, A. H. (2011). Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of *Escherichia coli* O157:H7 in mice. *Plant Sci* **180**, 620-627.

Audouy, S. A. L., van Selm, S., van Roosmalen, M. L. & other authors (2007). Development of lactococcal GEM-based pneumococcal vaccines. *Vaccine* **25**, 2497-2506.

Babiuk, S., Asper, D. J., Rogan, D., Mutwiri, G. K. & Potter, A. A. (2008). Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. *Microbial pathogenesis* **45**, 7-11.

Bahey-El-Din, M., Casey, P. G., Griffin, B. T. & Gahan, C. G. (2010a). Efficacy of a *Lactococcus lactis* DeltapyrG vaccine delivery platform expressing chromosomally integrated hly from *Listeria monocytogenes*. *Bioengineered bugs* **1**, 66-74.

Bahey-El-Din, M., Gahan, C. G. M. & Griffin, B. T. (2010b). *Lactococcus lactis* as a Cell Factory for Delivery of Therapeutic Proteins. *Curr Gene Ther* **10**, 34-45.

Bailey, M., Christoforidou, Z. & Lewis, M. C. (2013). The evolutionary basis for differences between the immune systems of man, mouse, pig and ruminants. *Veterinary immunology and immunopathology* **152**, 13-19.

Bermudez-Humaran, L. G., Cortes-Perez, N. G., Le Loir, Y., Gruss, A., Rodriguez-Padilla, C., Saucedo-Cardenas, O., Langella, P. & Montes de Oca-Luna, R. (2003a). Fusion to a carrier protein and a synthetic propeptide enhances E7 HPV-16 production and secretion in *Lactococcus lactis*. *Biotechnology progress* **19**, 1101-1104.

Bermudez-Humaran, L. G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R. & Corthier, G. (2003b). Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. *Fems Microbiol Lett* **224**, 307-313.

Bermudez-Humaran, L. G., Cortes-Perez, N. G., Le Loir, Y., Alcocer-Gonzalez, J. M., Tamez-Guerra, R. S., de Oca-Luna, R. M. & Langella, P. (2004). An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. *Journal of medical microbiology* **53**, 427-433.

Cataldi, A., Yevsa, T., Vilte, D. A., Schulze, K., Castro-Parodi, M., Larzabal, M., Ibarra, C., Mercado, E. C. & Guzman, C. A. (2008). Efficient immune responses against Intimin and EspB of enterohaemorragic *Escherichia coli* after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. *Vaccine* **26**, 5662-5667.

Cauchard, S., Bermudez-Humaran, L. G., Blugeon, S., Laugier, C., Langella, P. & Cauchard, J. (2011). Mucosal co-immunization of mice with recombinant lactococci secreting VapA antigen and leptin elicits a protective immune response against Rhodococcus equi infection. *Vaccine* **30**, 95-102.

C-J

Chatel, J. M., Langella, P., Adel-Patient, K., Commissaire, J., Wal, J. M. & Corthier, G. (2001). Induction of mucosal immune response after intranasal or oral inoculation of mice with *Lactococcus lactis* producing bovine beta-lactoglobulin. *Clin Diagn Lab Immun* **8**, 545-551.

Dawson, H. D., Loveland, J. E., Pascal, G. & other authors (2013). Structural and functional annotation of the porcine immunome. *BMC genomics* **14**, 332.

Drouault, S., Corthier, G., Ehrlich, S. D. & Renault, P. (1999). Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. *Appl Environ Microb* **65**, 4881-4886.

Drouault, S., Juste, C., Marteau, P., Renault, P. & Corthier, G. (2002). Oral treatment with *Lactococcus lactis* expressing *Staphylococcus hyicus* lipase enhances lipid digestion in pigs with induced pancreatic insufficiency. *Appl Environ Microbiol* **68**, 3166-3168.

Fan, H. Y., Wang, L., Luo, J. & Long, B. G. (2012). Protection against *Escherichia coli* O157:H7 challenge by immunization of mice with purified Tir proteins. *Molecular biology reports* **39**, 989-997.

Francis, D. H., Collins, J. E. & Duimstra, J. R. (1986). Infection of Gnotobiotic Pigs with an *Escherichia coli* O157-H7 Strain Associated with an Outbreak of Hemorrhagic Colitis. *Infection and immunity* **51**, 953-956.

Frees, D. & Ingmer, H. (1999). ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Molecular microbiology* **31**, 79-87.

Garcia-Angulo, V. A., Kalita, A. & Torres, A. G. (2013). Advances in the development of enterohemorrhagic *Escherichia coli* vaccines using murine models of infection. *Vaccine* **31**, 3229-3235.

Gu, J., Ning, Y. Y., Wang, H. G. & other authors (2011). Vaccination of attenuated EIS-producing Salmonella induces protective immunity against enterohemorrhagic *Escherichia coli* in mice. *Vaccine* **29**, 7395-7403.

Judge, N. A., Mason, H. S. & O'Brien, A. D. (2004). Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of *Escherichia coli* O157 : H7 shedding in feces. *Infection and immunity* **72**, 168-175.

K-N

Kanoh, M., Ibata, H., Miyagawa, M. & Matsuo, Y. (1989). Clinical Effects of Camostat in Chronic-Pancreatitis. *Biomed Res-Tokyo* **10**, 145-150.

Kaplan, D. L., Mello, C., Sano, T., Cantor, C. & Smith, C. (1999). Streptavidin-based containment systems for genetically engineered microorganisms. *Biomolecular engineering* **16**, 135-140.

Klijn, N., Weerkamp, A. H. & Devos, W. M. (1995). Genetic Marking of *Lactococcus lactis* Shows Its Survival in the Human Gastrointestinal Tract. *Appl Environ Microb* **61**, 2771-2774.

Knudsen, S., Saadbye, P., Hansen, L. H., Collier, A., Jacobsen, B. L., Schlundt, J. & Karlstrom, O. H. (1995). Development and testing of improved suicide functions for biological containment of bacteria. *Appl Environ Microbiol* **61**, 985-991.

Koch, B., Kilstrup, M., Vogensen, F. K. & Hammer, K. (1998). Induced levels of heat shock proteins in a dnaK mutant of *Lactococcus lactis*. *J Bacteriol* **180**, 3873-3881.

Kristy N. Kuzmuk, L. B. S. (2011). Pigs as a model for biomedical sciences. *MF Rothchild, A Ruvinsky (Eds), The genetics of the pig, CAB International, Wallingford*, 426–444.

Latvala, S., Pietila, T. E., Veckman, V., Kekkonen, R. A., Tynkkynen, S., Korpela, R. & Julkunen, I. (2008). Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World journal of gastroenterology : WJG* **14**, 5570-5583; discussion 5581-5572.

Le Loir, Y., Azevedo, V., Oliveira, S. C. & other authors (2005). Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb Cell Fact* **4**, 2.

Litten-Brown, J. C., Corson, A. M. & Clarke, L. (2010). Porcine models for the metabolic syndrome, digestive and bone disorders: a general overview. *Animal* **4**, 899-920.

Maton, P. N. & Burton, M. E. (1999). Antacids revisited- A review of their clinical pharmacology and recommended therapeutic use. *Drugs* **57**, 855-870.

McNeilly, T. N., Mitchell, M. C., Rosser, T., McAteer, S., Low, J. C., Smith, D. G., Huntley, J. F., Mahajan, A. & Gally, D. L. (2010). Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of *Escherichia coli* O157:H7 following oral challenge. *Vaccine* **28**, 1422-1428.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. & Gerdts, V. (2012). The pig: a model for human infectious diseases. *Trends Microbiol* **20**, 50-57.

Mohawk, K. L. & O'Brien, A. D. (2011). Mouse models of *Escherichia coli* O157:H7 infection and shiga toxin injection. *Journal of biomedicine & biotechnology* **2011**, 258185.

Nilsson, D., Lauridsen, A. A., Tomoyasu, T. & Ogura, T. (1994). A *Lactococcus lactis* Gene Encodes a Membrane-Protein with Putative Atpase Activity That Is Homologous to the Essential *Escherichia coli* Ftsh Gene-Product. *Microbiol-Uk* **140**, 2601-2610.

P-T

Peter A . McAnulty , A. D. D., Niels-Christian Ganderup , and Kenneth L . Hastings (2011). A Comparative Assessment of the Pig, Mouse and Human Genomes. *The Minipig in Biomedical Research (Book)*, 323–342.

Peterson, R. E., Klopfenstein, T. J., Moxley, R. A., Erickson, G. E., Hinkley, S., Bretschneider, G., Berberov, E. M., Rogan, D. & Smith, D. R. (2007). Effect of a vaccine product containing type III secreted proteins on the probability of *Escherichia coli* O157:H7 fecal shedding and mucosal colonization in feedlot cattle. *Journal of food protection* **70**, 2568-2577.

Potter, A. A., Klashinsky, S., Li, Y. L. & other authors (2004). Decreased shedding of *Escherichia coli* O157 : H7 by cattle following vaccination with type III secreted proteins. *Vaccine* **22**, 362-369.

Ribeiro, L. A., Azevedo, V., Le Loir, Y., Oliveira, S. C., Dieye, Y., Piard, J. C., Gruss, A. & Langella, P. (2002). Production and targeting of the Brucella abortus antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microb* **68**, 910-916.

Robinson, K., Chamberlain, L. M., Schofield, K. M., Wells, J. M. & Le Page, R. W. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature biotechnology* **15**, 653-657.

Sasaki, I., Suzuki, Y., Naito, H. & other authors (1989). Effect of Camostat in the Treatment of Reflux Esophagitis after Gastrectomy- an Experimental-Study in Rats and a Pilot Clinical-Study. *Biomed Res-Tokyo* **10**, 167-173.

Smith, D. R., Moxley, R. A., Peterson, R. E., Klopfenstein, T. J., Erickson, G. E., Bretschneider, G., Berberov, E. M. & Clowser, S. (2009). A two-dose regimen of a vaccine against type III secreted proteins reduced *Escherichia coli* O157:H7 colonization of the terminal rectum in beef cattle in commercial feedlots. *Foodborne Pathog Dis* **6**, 155-161.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. & Remaut, E. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nature biotechnology* **21**, 785-789.

Termonts, S. (2005). Optimization of *lactococcus lactis* mediated delivery of cytokines *PhD dissertation*

Torres, B., Jaenecke, S., Timmis, K. N., Garcia, J. L. & Diaz, E. (2000). A gene containment strategy based on a restriction-modification system. *Environmental microbiology* **2**, 555-563.

Tzipori, S., Wachsmuth, I. K., Chapman, C., Birner, R., Brittingham, J., Jackson, C. & Hogg, J. (1986). The Pathogenesis of Hemorrhagic Colitis Caused by *Escherichia coli* O157-H7 in Gnotobiotic Piglets. *Journal of Infectious Diseases* **154**, 712-716.

Tzipori, S., Gibson, R. & Montanaro, J. (1989). Nature and Distribution of Mucosal Lesions Associated with Enteropathogenic and Enterohemorrhagic *Escherichia coli* in Piglets and the Role of Plasmid-Mediated Factors. *Infection and immunity* **57**, 1142-1150.

Tzipori, S., Sheoran, A., Akiyoshi, D., Donohue-Rolfe, A. & Trachtman, H. (2004). Antibody therapy in the management of Shiga toxin-induced hemolytic uremic syndrome. *Clin Microbiol Rev* **17**, 926-+.

V-Z

Van Donkersgoed, J., Hancock, D., Rogan, D. & Potter, A. A. (2005). *Escherichia coli* O157:H7 vaccine field trial in 9 feedlots in Alberta and Saskatchewan. *The Canadian veterinary journal La revue veterinaire canadienne* **46**, 724-728.

Vesa, T., Pochart, P. & Marteau, P. (2000). Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment Pharm Therap* **14**, 823-828.

Villena, J., Medina, M., Raya, R. & Alvarez, S. (2008). Oral immunization with recombinant *Lactococcus lactis* confers protection against respiratory pneumococcal infection. *Can J Microbiol* **54**, 845-853.

Vilte, D. A., Larzabal, M., Garbaccio, S. & other authors (2011). Reduced faecal shedding of *Escherichia coli* O157:H7 in cattle following systemic vaccination with gamma-intimin C-280 and EspB proteins. *Vaccine* **29**, 3962-3968.

Wan, C. S., Zhou, Y., Yu, Y., Peng, L. J., Zhao, W. & Zheng, X. L. (2011). B-cell epitope KT-12 of enterohemorrhagic *Escherichia coli* O157:H7: a novel peptide vaccine candidate. *Microbiol Immunol* **55**, 247-253.

Yam, K. K., Pouliot, P., N'diaye, M. M., Fournier, S., Olivier, M. & Cousineau, B. (2008). Innate inflammatory responses to the Gram-positive bacterium *Lactococcus lactis*. *Vaccine* **26**, 2689-2699.

Zhang, Q., Donohue-Rolfe, A., Krautz-Peterson, G., Sevo, M., Parry, N., Abeijon, C. & Tzipori, S. (2009). Gnotobiotic piglet infection model for evaluating the safe use of antibiotics against *Escherichia coli* O157:H7 infection. *The Journal of infectious diseases* **199**, 486-493.

SUMMARY

Oral vaccination provides a practical and effective approach for disease prevention. Considering that the majority of infectious pathogen gain access to the body through the gut mucosa, the induction of mucosal immune responses constitutes a key strategy for protection (discussed in **Chapter 1**). However, the efficient delivery of oral vaccines is confronted with several challenges. These include the susceptibility of vaccine antigens to degradation by gastrointestinal enzymes, the difficulty in passing the epithelial barrier, the poor uptake of most soluble antigens by antigen-presenting cells with insufficient maturation of these cells, and the induction of tolerance instead of immunity. Therefore, strategies for efficient delivery of oral vaccines have been actively explored (reviewed in **Chapter 1**). *Lactococcus lactis* is a Gram-positive lactic acid bacterium that offers a valuable platform for the delivery of oral vaccines. The bacterium has been widely used in fermented and dairy food industry. However, during the last two decades, and with the advances in genetic engineering and molecular biology, scientists have explored the utilization of the bacteria as a live-vector for oral vaccine delivery. This technological trend has been particularly motivated by the well-established safety of the bacterium (classified as a generally regarded as safe "GRAS" organism by FDA) for oral consumption, as well as its amenability to genetic modification. Today, a vast collection of reports are available describing the oral delivery of vaccine antigens using *L. lactis*, an approach that successfully resulted in the induction of protective mucosal and systemic immune responses against various pathogens (reviewed in **Chapter 1**). The value of *L. lactis* as a live-vector for oral vaccine delivery particularly lies in its ability to synthesize and release antigens at the intestinal immune-inductive sites, as well as its adjuvant effects, which enhance the immune responses to vectored antigens.

In the present thesis we explored some preclinical aspects of the utilization of *L. lactis* for the development of an oral vaccine against enterohemorrhagic *Escherichia coli* (EHEC) infection. As reviewed in **Chapter 1**, EHEC constitutes a subset of enteropathogenic *E. coli* that has been responsible for frequent outbreaks of hemorrhagic colitis and hemolytic uremic syndrome worldwide. The latter represents a serious public health threat, as it the most common cause of renal failure in children and causes high fatalities among both children and elderly. Antibiotic treatment seems to exert no beneficial effects in the management of the hemolytic uremic syndrome. Even more, several reports have linked antibiotic therapy to disease exacerbation.

Thus, vaccination remains the most suitable approach to control the infection. Unfortunately, there is currently no approved vaccine against EHEC infections.

In **Chapter 2**, we describe our first attempt to express the EHEC antigen, EspB in *L. lactis* for oral vaccination purposes. EspB is a major protein of the type three secretion system employed by EHEC to colonize the small intestine. Additionally, the protein shows a remarkable immunogenicity in the course of both experimental and natural infections. Thus, the induction of mucosal immune responses, particularly IgA antibodies, against EspB can interfere with the EHEC colonization process and thus prevent the associated diseases. EspB was successfully expressed in the cytoplasmic compartment of the bacteria. However, oral immunization of BALB/C mice using the recombinant cytoplasmic expression strain did not elicit detectible levels of serum total-Igs and fecal IgA antibodies. Since weak priming of the immune system can be one of the reasons for the absence of humoral antibodies, we next applied an intraperitoneal booster immunization using purified EspB. Interestingly, ten days after the booster immunization both serum Igs and fecal IgA were detected in the mice orally immunized using EspB-expressing *L. lactis*, but not in those receiving the control strain. This finding indicates that the mucosal immune system of the orally immunized mice was primed against EspB. However, further optimization of the recombinant vaccine strain would be required to elicit complete immune responses upon oral prime-boost immunizations. In **Chapter 3,** we describe the optimization of EspB expression in *L. lactis* by constructing recombinant strains designated for EspB secretion in the extracellular medium. Unlike cytoplasmic expression, secretion allows antigens to escape cytoplasmic proteolysis, which likely resulted in low expression levels and poor delivery of EspB in our first study. Additionally, we compared constitutive and nisin-inducible expression systems for the highest antigen expression yield. Indeed, secretion of EspB in *L. lactis*, under either constitutive or inducible conditions, has markedly enhanced the expression levels compared to cytoplasmic expression (25- and 56-fold increase, respectively). Notably, the nisin-inducible secretion system showed a two-fold increase in EspB expression compared to the constitutive one. Thereafter, we evaluated the capacity of the two expression systems in inducing immune responses upon immunizing BALB/c mice orally using the corresponding recombinant strains (**Chapter 3**). Mice immunized using either strains showed a significant increase in EspB-specific serum total Igs or fecal IgA compared to control mice. The highest antibody responses were

obtained using the nisin-inducible strain. Next, the cellular immune responses induced by the oral immunization of mice using *L. lactis* secreting EspB was characterized in Peyer's patches and mesenteric lymph nodes of immunized mice. Lymphocytes isolated from these sites showed the production of both Th1 and Th2 cytokines (IFN-γ and Il-4 and IL-10, respectively) in an *in vitro* antigen-recall assay. This indicates the induction of a mixed Th/Th2 response. Nevertheless, IL-4:IFN-γ ratio analysis showed a Th2 bias. The Th2 dominance is consistent with the induction of intestinal IgA. Furthermore, the protection conferred by the oral immunization using *L. lactis* secreting EspB was evaluated during a challenge infection using *E. coli* O157:H7 strain. Indeed the immune responses induced by the vaccine strains were capable of protecting the immunized mice against intestinal colonization by EHEC. This pattern of protection can be correlated to the detected IgA antibody responses, which could interfere with the attachment of EHEC to the intestinal epithelium. Altogether, these findings demonstrate the potential of EspB as candidate for oral vaccination against EHEC infection, as well as the efficiency of *L. lactis* as a delivery vector.

Chapter 4 describes our first steps towards the validation of our EHEC vaccine in pigs. Although mice models of EHEC colonization are acceptable for the evaluation of EHEC vaccines, they do not fully mimic the EHEC pathogenicity in humans. Pigs offer an alternative animal model, in which EHEC pathogenicity in humans is fairly reproducible. In addition, from an immunological standpoint, pigs share a higher degree of similarity with humans than the latter do with mice. Therefore, validation of vaccines targeting human diseases in pig models can be a feasible step towards the translation of their application in humans, particularly in the case of EHEC infection. Despite these facts, there is not a single report published on the use of a L. lactis-based vaccine in pig model. A possible reason is the previously reported low survival of the bacteria in the porcine gastrointestinal tract. To address this issue, we evaluated the sensitivity of *L. lactis* to some potentially harmful digestive factors, including bile and the major pancreatic enzymes, trypsin, lipase and amylase. Thereafter, we evaluated the capacity of aluminium hydroxide (a bile acid binder) and camostat mesylate (a trypsin inhibitor) to protect *L. lactis* against bile toxicity and trypsin hydrolysis, respectively. These experiments were designed to mimic the physiological conditions described in the small intestine of pigs. Incubation of *L. lactis* with freshly isolated porcine bile resulted in a marked reduced survival of the bacteria. Moreover, a positive correlation between bile toxicity and acidity was observed. *L. lactis* survival was also

influenced by the pancreatic enzymes. Among these enzymes, trypsin reduced *L. lactis* survival the highest (20% versus 49% and 60% survival for amylase and lipase, respectively). The presence of aluminium hydroxide and camostat mesylate could significantly improve the survival of *L. lactis* in bile and trypsin solutions, respectively. Furthermore, we demonstrated that oral co-administration of aluminium hydroxide and camostat mesylate with *L. lactis* can improve the survival of the bacterium in the GI tract of pigs. This was indicated by a 38- and 24-fold increase in the *L. lactis*-counts retrieved respectively from jejunal and ileal contents of treated pigs. Our results proof the concept of using bile and pancreatic enzyme inhibitors in oral formulations of *L. lactis* to improve the survival of the bacterium in the gastrointestinal tract of pigs and to subsequently enhance its efficiency in oral vaccine delivery. In a next step the vaccine should be validated in a pig model of EHEC infection considering the physiological and the immunological similarities among pigs and humans, it would be interesting to extend our findings to humans.

SAMENVATTING

Aangezien de meerderheid van de infectieuze pathogenen het lichaam binnenkomen via het darmslijmvlies, is het induceren van een immuunrespons ter hoogte van de darmmucosa een belangrijke strategie om te beschermen tegenover deze pathogenen. Orale vaccinatie is de meest aangewezen methode om deze immuunrespons te bekomen. (besproken in **Hoofdstuk 1**). Er zijn echter meerdere uitdagingen die moeten overwonnen worden om efficiënte orale vaccins te bekomen. Ondermeer de gevoeligheid van vaccinantigenen voor afbraak door de gastrointestinale pH, enzymen en galzouten, de gebrekkige passage doorheen de darmbarrière, de gebrekkige opname van oplosbare antigenen door antigeen-presenterende cellen met onvolledige activatie van deze laatste waardoor eerder tolerantie dan immuniteit ontstaat. Daarom werden verschillende strategieën onderzocht om tot een efficiënte orale vaccinatie te komen (gereviewed in **Hoofdstuk 1**).

Lactococcus lactis is een Gram-positieve melkzuurbacterie die een interessante vector is voor orale toediening van antigenen. Inderdaad, deze bacterie wordt veel gebruikt in de gefermenteerde levensmiddelen- en zuivelindustrie. De laatste twee decennia onderzochten wetenschappers het gebruik van de bacterie als een levende vector voor orale vaccinatie, ondermeer dankzij de vooruitgang in genetische engineering en moleculaire biologie. Het onderzoek werd enerzijds gemotiveerd door de vaststelling dat de bacterie veilig is voor orale consumptie (ingedeeld als een "GRAS" organisme door de FDA) en anderzijds door haar geschiktheid voor genetische modificatie. Inmiddels bestaan er een groot aantal rapporten die beschrijven hoe de orale toediening van vaccinantigenen met behulp van *L. lactis* met succes leidde tot de inductie van beschermende mucosale en systemische immuunresponsen tegen verschillende pathogenen (gereviewed in **Hoofdstuk 1**). De waarde van *L. lactis* als levende vector voor orale toediening van een vaccin ligt vooral in haar mogelijkheid om antigenen te synthetiseren en vrij te geven ter hoogte van het mucosa-geassocieerd lymfoïd weefsel in de dunne darm, maar ook in haar adjuvanseffecten die de immuunrespons tegen antigenen versterken.

In deze thesis onderzochten we of *L. lactis* het potentieel heeft om gebruikt te worden als een oraal vectorvaccin tegen enterohemorragische *Escherichia coli* (EHEC). Zoals beschreven in **Hoofdstuk 1** zijn EHEC een groep van pathogene *E. coli* die verantwoordelijk zijn voor frequente uitbraken van hemorragische colitis en het hemolytisch uremisch syndroom. Dit laatste is een ernstige bedreiging voor de

volksgezondheid aangezien het de meest voorkomende oorzaak is voor acuut nierfalen bij kinderen en het voor een hoog sterftecijfer zorgt bij kinderen en ouderen. Antibioticabehandeling schijnt geen gunstig effect te hebben in de behandeling van EHEC infecties. Sterker nog, verschillende rapporten linken antibioticabehandeling aan verergering van de ziekte. Vaccinatie is dus de meest geschikte aanpak voor de controle van infecties. Helaas is er momenteel geen vaccin geregistreerd tegen EHEC infecties bij de mens.

In **Hoofdstuk 2** beschrijven we onze eerste poging om het EHEC antigeen, EspB, tot expressie te brengen in *L. lactis*. EspB is een belangrijk eiwit van het type drie secretiesysteem dat door EHEC gebruikt wordt om de dunne darm te koloniseren. Daarenboven vertoont dit eiwit een opmerkelijke immunogeniciteit, zowel bij experimentele als natuurlijke besmettingen. Daarom wordt EspB beschouwd als een potentiële vaccinkandidaat. De inductie van mucosale immuunresponsen, in het bijzonder IgA, tegen EspB kan interfereren met het kolonisatieproces van EHEC en kan op die manier de eraan gerelateerde ziekte voorkomen.

EspB werd succesvol tot expressie gebracht in het cytoplasma van *L. lactis*. Echter, orale immunisatie van BALB/c muizen met deze recombinante stam induceerde geen waarneembare hoeveelheden Ig's in het serum en IgA antistoffen in de faeces. Aangezien een zwakke stimulatie van het immuunsysteem één van de redenen kan zijn voor de afwezigheid van humorale antilichamen, pasten we vervolgens een intraperitoneale booster-immunisatie toe met behulp van opgezuiverd EspB. Tien dagen na de booster-immunisatie werden zowel serum Ig's als faecale IgA gedetecteerd in de muizen die geïmmuniseerd werden met de EspB-producerende *L. lactis*, maar niet in deze die de controlestam toegediend kregen. Deze bevinding wijst erop dat het mucosaal immuunsysteem van de oraal geïmmunizeerde muizen wel degelijk geprimed werd tegen EspB. Het toonde echter ook aan dat er verdere optimalisatie van de recombinante vaccinstam nodig was om een completere immuunrespons te kunnen induceren via prime-boost immunisaties.

In **Hoofdstuk 3** beschrijven we de optimalisatie van de EspB-expressie in *L. lactis* door het ontwikkelen van recombinante stammen die EspB uitscheiden in het extracellulair medium. Anders dan bij cytoplasmatische expressie laat secretie de antigenen toe te ontsnappen aan cytoplasmatische proteolyse, die vermoedelijk resulteerde in lage expressieniveaus en een te beperkte beschikbaarheid van EspB voor het immuunsysteem om tot een duidelijke immuunrespons te komen in onze eerste

studie. Bijkomend vergeleken we de expressieniveaus van antigenen in constitutieve en nisine-geïnduceerde expressiesystemen. De secretie van EspB, in constitutieve of induceerbare omstandigheden, leidde inderdaad tot opmerkelijk hogere expressieniveaus in vergelijking met de cytoplasmatische expressie, namelijk respectievelijk 25 en 56 keer hoger. De verhoging in expressieniveau was dus dubbel zo groot bij het nisine-induceerbaar secretiesysteem in vergelijking met het constitutief systeem. Nadien evalueerden we het vermogen van de twee expressiesystemen om immuunresponsen te induceren in BALB/c muizen na immunisatie door orale toediening van de recombinante stammen (**Hoofdstuk 3**). Muizen, die geïmmuniseerd werden met een van beide stammen, toonden een significante toename van EspB-specifieke totale Ig's in het serum of van IgA in de faeces in vergelijking met de controlemuizen. De hoogste antilichaamresponsen werden verkregen met de nisine-induceerbare stam. Vervolgens werd de cellulaire immuunrespons, die door middel van orale immunisatie van muizen met EspB-uitscheidende *L. lactis* geïnduceerd werden, gekarakteriseerd in Peyerse platen en mesenteriale lymfeknopen. Lymfocyten uit deze weefsels geïsoleerd produceerden na *in vitro* restimulatie met EspB zowel Th1 cytokines (IFN-γ) als Th2 cytokines (IL-4 en IL-10). Dit wijst op de inductie van een gemengde Th1/Th2 respons. Desalniettemin toont de IL-4:IFN-γ ratio een bias naar Th2, een typisch kenmerk voor immuunresponsen ter hoogte van het darmslijmvlies. De Th2-dominantie is consistent met de inductie van intestinaal IgA. Verder werd de beschermende immuniteit van de orale immunisatie met de EspB secreterende *L. lactis* tegen een challenge-infectie met de *E. coli* O157:H7 stam geëvalueerd. De immuunresponsen die geïnduceerd werden door de vaccinstammen waren in staat om de geïmmuniseerde muizen te beschermen tegen kolonisatie van de darm door EHEC. De graad van bescherming kon gecorreleerd worden met de EspB-specifieke IgA responsen. Waarschijnlijk interfereren deze antistoffen met de aanhechting van EHEC aan het darmepitheel. Al deze bevindingen samen tonen het potentieel aan van EspB als kandidaat voor orale vaccinatie tegen EHEC-infectie en van *L. lactis* als vector voor targeting van het darm-geassocieerd mucosaal immuunsysteem.

In **Hoofdstuk 4** worden de eerste stappen gezet naar het gebruik van ons EHEC vaccin in het varken. Niettegenstaande muismodellen van EHEC infectie aanvaardbaar zijn voor de evaluatie van vaccins die het kolonisatieproces als doelwit hebben, bootsen ze de pathogeniciteit van EHEC in de mens niet volledig na. Daarentegen bieden varkens een alternatief diermodel waarin EHEC pathogeniciteit in mensen

behoorlijk reproduceerbaar is. Vanuit immunologisch standpunt zijn er trouwens meer overeenkomsten tussen varkens en de mens dan tussen de mens en muizen. Daardoor kan validatie van vaccins voor menselijke ziektes in varkensmodellen een nuttige stap zijn in de omzetting van de toepassing voor mensen, in het bijzonder in het geval van een EHEC infectie.

Desondanks is er tot hiertoe geen enkel rapport gepubliceerd dat *L. lactis*-gebaseerde vaccins evalueert in een varkens infectiemodel. Een mogelijke reden is de eerder gerapporteerde lage overlevingskans van de bacterie in het spijsverteringsstelsel van varkens. In verband hiermee evalueerden we de gevoeligheid van *L. lactis* voor een aantal potentieel schadelijke spijsverteringsfactoren, zoals gal en de voornaamste pancreasenzymen: trypsine, lipase en amylase. Daarna evalueerden we het vermogen van aluminiumhydroxide (een galzuurbinder) en camostat mesylaat (een trypsine-inhibitor) om *L. lactis* te beschermen, tegen respectievelijk de toxiciteit van gal en de hydrolyse door trypsine. Voor deze experimenten werden de fysiologische condities nagebootst die beschreven zijn in de dunne darm van varkens. Incubatie van *L. lactis* met vers verzamelde varkensgal leidde tot een duidelijk verlaagde overlevingsgraad van de bacteriën. Hierbij werd een positieve correlatie gezien tussen toxiciteit van de gal en zuurtegraad. Hoe lager de pH hoe toxischer de gal was. De overleving van *L. lactis* werd ook verlaagd door alle geteste pancreasenzymen. Van deze enzymen had trypsine de grootste negatieve invloed op de overleving van *L. lactis* (20% versus 49% voor amylase en 60% voor lipase). De aanwezigheid van aluminiumhydroxide en camostat mesylaat kon de overleving van *L. lactis* in respectievelijk gal- en trypsine-oplossingen significant verbeteren. Daarenboven toonden we aan dat orale toediening van aluminiumhydroxide en camostat mesylaat samen met *L. lactis* de overleving van deze laatste in het spijsverteringsstelsel van varkens bevordert met respectievelijk 38-maal in het jejunuminhoud en 24-maal in ileuminhoud van behandelde varkens. Onze resultaten bewijzen het concept van het gebruik van aluminiumhydroxide en camostat mesylaat in orale formuleringen van *L. lactis* om de overleving van de bacterie in het spijsverteringsstelsel van varkens te verhogen en bijgevolg de efficiëntie voor orale toediening van het vectorvaccin te bevorderen. Gezien de fysiologische en immunologische overeenkomsten tussen varkens en de mens, kunnen deze bevindingen ook gebruikt worden om de doeltreffendheid van *L. lactis*-gebaseerde vaccins in het humaan spijsverteringsstelsel te verbeteren.

CURRICULUM VITAE

Bakr Ahmed is an Egyptian national, who was born in 1984 in Sharkia, Egypt. In 2005, Bakr was graduated from the Faculty of Veterinary Medicine, Zagazig University, Egypt, with honors. In 2006, Bakr was hired at the same faculty as a research and teaching assistant, at the Department of Pathology, to which he is still affiliated. Being fascinated by research and overseas learning, in 2008, Bakr has completed a short-term research fellowship, at the Department of Pathology, Infection and Immunity, Faculty of Veterinary Medicine, University of Bristol, UK. Bakr started his doctoral studies in 2009 at the Laboratory of Immunology with a two-year GOA research fund from UGent. In 2011, he was awarded a BOF Grant of two years from UGent for the completion of his PhD research. Bakr is the first author of three international peer-reviewed publications. He was a speaker in two symposia and has actively participated in several national and international scientific conferences.

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SYMPOSIA AND CONFERENCES

Ahmed B, Loos M, Vanrompay D, Cox E. Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization. Vaccines for Enteric Diseases, 7th International conference, Bangkok, 2013.

Ahmed B, Loos M, Vanrompay D, Cox E. Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization. Seminar on Inflammation and Vaccination, Ghent, 2012.

Ahmed B, Loos M, Cox E. Mucosal priming of the murine immune system against *Escherichia coli* O157:H7 using *Lactococcus lactis* expressing a virulence antigen. Verocytotoxin producing *Escherichia coli* (VTEC), Amsterdam, 2012.

Ahmed B, Loos M, Cox E. Mucosal priming of the murine immune system against enterohemorrhagic *Escherichia coli* using *Lactococcus lactis* expressing the type III secretion system protein EspB. *Escherichia coli* and the Mucosal Immune System (ECMIS), Ghent, 2011.

PUBLICATIONS

Ahmed B, Loos M, Vanrompay D, Cox E. Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization. VACCINE. 2014; 32(31): 3909–16.

Ahmed B, Loos M, Vanrompay D, Cox E. Mucosal priming of the murine immune system against enterohemorrhagic *Escherichia coli* O157:H7 using *Lactococcus lactis* expressing the type III secretion system protein EspB. Cox E, editor. VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY. 2013; 152(1-2): 141–5.

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