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
Promoters

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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
CTTs  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
Gb3  globotriosylceramide
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
<table>
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<tr>
<td>Stx</td>
<td>Shiga toxins</td>
</tr>
<tr>
<td>T3SS</td>
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<tr>
<td>Th</td>
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<tr>
<td>Tir</td>
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<tr>
<td>TNF</td>
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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). Hereeto, antigen loaded DCs secrete in PPs and MLNs a subset of cytokines and stimulatory molecules, such as TGF\(\beta\), 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-\(\beta\) 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 CD8α+ 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, CD8αβ+ TCRαβ+ 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 scavenge 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,
Oral vaccines: immunological basis and strategies for vaccine development

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

<table>
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<th>Immunological characteristics</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Clinical testing</th>
<th>References</th>
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<td>Live-attenuated pathogens</td>
<td>Bacteria: e.g. <em>Salmonella</em> Typhimurium and <em>Listeria</em> Viruses: e.g. Adenovirus, Poxvirus and Vacinia virus</td>
<td>Epithelial and antigen-presenting cells</td>
<td>T lymphocyte response</td>
<td>+</td>
<td>- Ability to survive GI conditions</td>
<td>- Pathogenicity reversion</td>
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### Oral vaccines: immunological basis and strategies for vaccine development

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<th>Characteristics</th>
<th>Immune response</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chitosan</strong></td>
<td>Natural mucoadhesive polymer</td>
<td>Epithelial tight junctions</td>
<td>Th2 ++</td>
<td>- Long-term depot and pulsatile release of antigens&lt;br&gt;- Can mimic prime-boost effects of conventional vaccines</td>
<td>- Issues on antigen stability in microcapsules&lt;br&gt;- Issues antigen dose optimization</td>
</tr>
<tr>
<td><strong>Particles:</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>PLG</strong></td>
<td>Poly-lactic coglycolic acid micro and nanoparticles</td>
<td>ND</td>
<td>Th1, CTLs +</td>
<td>- Controlled, sustainable antigen release&lt;br&gt;- Mimic prime-boost effects of vaccines</td>
<td>Issues on antigen loading capacity and stability</td>
</tr>
<tr>
<td><strong>Liposomes</strong></td>
<td>Phospholipids microparticles</td>
<td>ND</td>
<td>Th1, Th2 +</td>
<td>Strong immune stimulants</td>
<td>- Issues on formulation stability&lt;br&gt;- Costly production</td>
</tr>
<tr>
<td><strong>ISCOMs or ISCOMATRIX</strong></td>
<td>Quillaia saponines: Quil A or QS-21</td>
<td>DCs, Th1, Th2, CTLs ++</td>
<td>- Strong CTLs responses&lt;br&gt;- Adjuvant effects</td>
<td>Toxicity due to saponines-induced hemolysis</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Virus-like particles</strong></td>
<td>Virus envelopes (capsids)</td>
<td>ND</td>
<td>Th1, CTLs, Th2 +</td>
<td>- Easy production&lt;br&gt;- Safer than live viruses</td>
<td>Non-invasive</td>
</tr>
<tr>
<td>Emulsions:</td>
<td>Th, T helper; CTLs, cytotoxic T lymphocytes; GI, gastrointestinal; DCs, dendritic cells; DDA, dimethyldioctadecylammonium; ND, not determine</td>
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<tr>
<td><strong>Cationic</strong></td>
<td>+ Slow antigen release</td>
<td></td>
<td></td>
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<tr>
<td><strong>DDA or liposomes</strong></td>
<td>Moderate immunostimulatory effect</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>DCs</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thi, CTLs</strong></td>
<td>Christensen et al., 2009</td>
<td></td>
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<th>Cationic DDA or liposomes</th>
<th>DCs</th>
<th>Th, CTLs</th>
<th>+</th>
<th>Slow antigen release</th>
<th>Moderate immunostimulatory effect</th>
<th>yes</th>
<th>Christensen et al., 2009</th>
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<tr>
<td>Cationic DDA or liposomes</td>
<td>DCs</td>
<td>Th, CTLs</td>
<td>+</td>
<td>Slow antigen release</td>
<td>Moderate immunostimulatory effect</td>
<td>yes</td>
<td>Christensen et al., 2009</td>
</tr>
</tbody>
</table>

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; Leenhousts *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, pTREXi is an expression system that has been developed by replacing the PlacA promoter of pTREX by the strong constitutive lactococcal promoter P1 (Wells and Schofield, 1996). Since pTREXi combines the stability of the theta replicons with a strong promoter, a variety of heterologous proteins have been expressed in *L. lactis* using pTREXi, 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.*, 1995), and others.
Lactococcus lactis as a live-vector for oral vaccine delivery

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 heterologous 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 (PnисA). 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 drawbacks 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 heterologous 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; Nouaillle 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 \textit{L. lactis} strain with the intestinal mucosa of mice, the transcellular uptake of the bacterium was described as a rare event (Waeytens \textit{et al.}, 2008). Moreover, although \textit{L. lactis} was found adjacent to M cells, no bacteria were found on the cell membrane or in the cytoplasm. (Waeytens \textit{et al.}, 2008). Thus, an evidence of the cellular uptake of viable \textit{L. lactis} in the gut is currently lacking.

\textbf{2.4.2. Influence of the antigen cellular location on immunogenicity}

The final cellular location of antigens expressed in \textit{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 \textit{et al.}, 2005; Pontes \textit{et al.}, 2004; Rodriguez-Diaz \textit{et al.}, 2011). As discussed above, antigens targeted to the cytoplasmic compartment of \textit{L. lactis} can be degraded by the housekeeping proteases, which can result in low antigen-expression levels (Le Loir \textit{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 \textit{L. lactis} (Pei \textit{et al.}, 2005; Perez \textit{et al.}, 2005; Rodriguez-Diaz \textit{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 \textit{L. lactis} cells are metabolically active in the gastrointestinal tract and thus, antigen synthesis and secretion continues in situ (Drouault \textit{et al.}, 1999; Huibregtse \textit{et al.}, 2007; Steidler \textit{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 \textit{et al.}, 2003; Ribeiro \textit{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 \textit{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-\( \alpha \) upon incubation with a macrophage-like cell line (Suzuki \textit{et al.}, 2008). A second study showed the ability of \( L. \) lactis MG1363 to induce the secretion of TNF-\( \alpha \), when incubated with murine macrophages (Audouy \textit{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 \textit{et al.}, 2001) CCL1, CCL2, CCL3, CCL4, CXCL2 and CXCL10 mRNA (Yam \textit{et al.}, 2008). The latter are known to mediate the recruitment of the leukocytes (Mantovani \textit{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-1\( \beta \) (Yam \textit{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 \textit{et al.}, 2011; Lee \textit{et al.}, 2009; Robinson \textit{et al.}, 2004; Villena \textit{et al.}, 2008; Villena \textit{et al.}, 2010). The type of T-helper cell induced, is largely determined by the function of DCs (Rescigno, 2010). In this context, \textit{in vitro} studies have shown the ability of \( L. \) lactis ssp. cremoris to induce the maturation of DCs (Audouy \textit{et al.}, 2007; Yam \textit{et al.}, 2008), as well as the secretion of cytokines known to direct the differentiation of both Th1 (IFN-\( \gamma \) and IL-12) and Th2 (IL-6 and IL-10) cell types (Latvala \textit{et al.}, 2008; Yam \textit{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 \textit{et al.}, 1999; Kimoto \textit{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 \textit{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

<table>
<thead>
<tr>
<th>Infectious agent</th>
<th>Antigen</th>
<th>Bacterial location</th>
<th>Immune responses</th>
<th>Protection pattern</th>
<th>Animal model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria:</strong></td>
<td></td>
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<tr>
<td><em>Clostridium tetani</em> (Tetanus)</td>
<td>Tetanus toxin fragment C (TTFC)</td>
<td>CYT</td>
<td>-Serum IgG and fecal IgA&lt;br&gt;-Mixed Th1/Th2 cytokine response in PP and MLN</td>
<td>Survival after a lethal challenge with tetanus toxin</td>
<td>Mouse</td>
<td>Grangette <em>et al.</em>, 2002; Grangette <em>et al.</em>, 2004; Robinson <em>et al.</em>, 1997; Robinson <em>et al.</em>, 2004</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>L7/L12</td>
<td>CYT</td>
<td>Fecal IgA</td>
<td>-Partial protection against intraperitoneal challenge with <em>B. abortus</em></td>
<td>Mouse</td>
<td>Pontes <em>et al.</em>, 2004</td>
</tr>
<tr>
<td></td>
<td>Cu-Zn superoxide dismutase (SOD)</td>
<td>SEC</td>
<td>-Serum IgM and IgG, fecal IgA&lt;br&gt;-Lymphocyte proliferation&lt;br&gt;-Responses improved by co-administration of <em>L. lactis</em> secreting IL-12</td>
<td>-Reduced <em>B. abortus</em> counts in spleen after challenge</td>
<td>Mouse</td>
<td>Saez <em>et al.</em>, 2012</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Urease B (UreB)</td>
<td>CYT</td>
<td>Serum IgG</td>
<td>-</td>
<td>Mouse</td>
<td>Lee <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>Urease B (UreB)</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>Reduced (H.) pylori gastric counts and urease activity</td>
<td>Mouse</td>
<td>Gu et al., 2009</td>
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<tr>
<td>Cag12</td>
<td>CYT</td>
<td>Serum IgG</td>
<td></td>
<td>Mouse</td>
<td>Kim et al., 2006</td>
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</tr>
<tr>
<td>Cholera toxin B subunit plus T and B cell epitopes of UreB</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>Reduced (H.) pylori gastric counts</td>
<td>Mouse</td>
<td>Li et al., 2014</td>
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</tr>
<tr>
<td>UreB and IL-2</td>
<td>CYT</td>
<td>Serum IgG and fecal IgA IFN-γ and IL-4 in serum</td>
<td>Reduced (H.) pylori gastric colonization and urease activity</td>
<td>Mouse</td>
<td>Zhang et al., 2014</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clostridium difficile</th>
<th>nontoxic tetanus toxin fragment C (TETC), and 14 C-terminal repeats (14CDTA) of nontoxic C. difficile toxin A (TcdA)</th>
<th>SEC, CWA</th>
<th>IgG in serum and intestinal fluid</th>
<th>-Increased survival upon lethal challenge with TcdA</th>
<th>Hamster</th>
<th>Yang et al., 2013</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced cytotoxicity and enterocyte apoptosis \textit{in vitro}</td>
<td>-Reduced expression of mucosal inflammatory cytokines, ICAM-1, MCP-1, IL-6, and Gro-1</td>
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</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>SEB</td>
<td>CYT, SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>Increased survival after intraperitoneal challenge with SEB-producing <em>S. aureus</em> strain</td>
<td>Mouse</td>
<td>Asensi <em>et al.</em>, 2013</td>
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<tr>
<td><strong>Enterotoxigenic</strong></td>
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<tr>
<td><em>Escherichia coli</em> (ETEC)</td>
<td>F4 (K88)</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>-Antibody secreting cells in spleen, MLN and PP</td>
<td>Mouse</td>
<td>Liu <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>(Neonatal porcine diarrhea)</td>
<td>FaeG</td>
<td>SEC</td>
<td>Serum IgG</td>
<td>Reduced mortality after challenge</td>
<td>Mouse</td>
<td>Hu <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYT</td>
<td>Serum IgG and fecal IgA</td>
<td>-Antibody secreting cells in spleen, MLN and PP</td>
<td>Mouse</td>
<td>Liu <em>et al.</em>, 2009b</td>
</tr>
<tr>
<td><strong>Vibrio cholerae</strong></td>
<td>Wzm</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>Absence of diarrhea after challenge</td>
<td>Rabbit</td>
<td>Zamri <em>et al.</em>, 2012</td>
</tr>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td>PppA</td>
<td>CWA</td>
<td>-IgM, IgG and IgA in serum and intestinal and bronchial fluids</td>
<td>Reduced pulmonary colonization and bacteremia</td>
<td>Mouse</td>
<td>Villena <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Intestinal IgA⁺ cells</td>
<td></td>
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<td>Villena <em>et al.</em>, 2008</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-Mixed Th1/Th2 response</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(spleenocyte cytokines and IgG subtypes analysis)</td>
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<tr>
<td>Pathogen</td>
<td>Protein</td>
<td>Secretion</td>
<td>Immune Response</td>
<td>Effect</td>
<td>Species</td>
<td>Reference</td>
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<tr>
<td><strong>Enterohemorrhagic Escherichia coli (EHEC)</strong></td>
<td>EspA</td>
<td>SEC</td>
<td>Serum IgG</td>
<td>Reduced cell cytoskeletal changes <em>in vitro</em></td>
<td>Mouse</td>
<td>Luan <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><strong>Rhodococcus equi</strong></td>
<td>VapA</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA, Splenocytes mixed Th1/Th2 cytokine responses</td>
<td>Reduced liver and spleen counts of <em>R. equi</em></td>
<td>Mouse</td>
<td>Cauchard <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><strong>Virus:</strong></td>
<td></td>
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<tr>
<td>Human immunodeficiency virus (HIV)</td>
<td>V2-V4 loop of gp120</td>
<td>CWA</td>
<td>Serum IgG and fecal IgA, IFN-γ producing CD8+ T cells in peritoneal lymph node and spleen</td>
<td>Decreased virus load after a challenge with HIV Env-expressing vaccinia virus</td>
<td>Mouse</td>
<td>Xin <em>et al.</em>, 2003</td>
</tr>
<tr>
<td></td>
<td>Gag</td>
<td>CWA</td>
<td>IgG and IgA in serum, feces and vaginal fluid, Rectal IL-2, IFN-γ, TNF-α and IL-17 producing CD4+ cells</td>
<td></td>
<td>Mouse</td>
<td>Chamcha <em>et al.</em>, 2012</td>
</tr>
<tr>
<td><strong>Rotavirus</strong></td>
<td>VP7</td>
<td>CYT, SEC, CWA</td>
<td>Serum antibodies</td>
<td>Virus neutralization in an <em>in vitro</em> assay</td>
<td>Mouse</td>
<td>Perez <em>et al.</em>, 2005</td>
</tr>
<tr>
<td></td>
<td>VP8</td>
<td>CYT, SEC, CWA</td>
<td>Serum IgG and fecal IgA</td>
<td>Block infection in an <em>in vitro</em> cell line-based assay</td>
<td>Mouse</td>
<td>Marelli <em>et al.</em>, 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYT, SEC</td>
<td>Serum IgG and fecal IgA</td>
<td></td>
<td>Mouse</td>
<td>Rodriguez-Diaz <em>et al.</em>, 2011</td>
</tr>
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</table>
**Lactococcus lactis** as a live-vector for oral vaccine delivery

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Antigen</th>
<th>Location</th>
<th>Immune Response</th>
<th>In Vitro Effect</th>
<th>Animal Model</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Rotavirus</strong></td>
<td>VP4</td>
<td>CYT</td>
<td>IgA and IgG were found in feces, ophthalmic and vaginal washes and in serum</td>
<td>Virus <em>in vitro</em> neutralizing effect</td>
<td>Mouse</td>
<td>Li <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><strong>Human papilloma virus-16</strong> (HPV-16)</td>
<td>L1 capsid protein</td>
<td>CYT, SEC</td>
<td>Serum IgG and vaginal IgA</td>
<td>-</td>
<td>Mouse</td>
<td>Cho <em>et al.</em>, 2007</td>
</tr>
<tr>
<td><strong>Sever Acute Respiratory Syndrome (SARS)-coronavirus</strong></td>
<td>Nucleocapsid (N) protein</td>
<td>CYT, SEC</td>
<td>Serum IgG</td>
<td>-</td>
<td>Mouse</td>
<td>Pei <em>et al.</em>, 2005</td>
</tr>
<tr>
<td><strong>Dengue virus</strong></td>
<td>Envelope protein E domain III (EDIII)</td>
<td>CYT</td>
<td>Serum IgG</td>
<td>Virus neutralization in an <em>in vitro</em> assay</td>
<td>Mouse</td>
<td>Sim <em>et al.</em>, 2008</td>
</tr>
<tr>
<td><strong>Chicken infectious bronchitis virus</strong> (IBV)</td>
<td>Multiple epitope antigen EpiC</td>
<td>CWA</td>
<td>Serum IgG and intestinal IgA</td>
<td>Absence of clinical signs</td>
<td>Chicken</td>
<td>Cao <em>et al.</em>, 2013</td>
</tr>
<tr>
<td><strong>Hepatitis A virus</strong></td>
<td>VP1-P2a</td>
<td>CYT, CWA</td>
<td>Serum IgG and fecal IgA</td>
<td>No protection in <em>in vitro</em> neutralization assay</td>
<td>Mouse</td>
<td>Berlec <em>et al.</em>, 2013</td>
</tr>
<tr>
<td><strong>Hepatitis B virus</strong></td>
<td>PreSa</td>
<td>SEC</td>
<td>Serum IgG and fecal IgA</td>
<td>-</td>
<td>Mouse</td>
<td>Zhang <em>et al.</em>, 2011a</td>
</tr>
<tr>
<td><strong>Avian influenza virus</strong></td>
<td>Hemagglutinin 1 (HA-1)</td>
<td>CYT</td>
<td>Serum IgG and fecal IgA, Splenocyte secretion of IFN-γ and IL-4</td>
<td>Protection against lethal H5N1 challenge</td>
<td>Mouse</td>
<td>Wang <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Vector</td>
<td>Response</td>
<td>Protection Result</td>
<td>Model</td>
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<tr>
<td>Avian influenza virus</td>
<td>Hemagglutinin 1 (HA-1)</td>
<td>Serum IgG and fecal IgA, Splenocyte cell proliferation and IFN-γ secreting cells</td>
<td>Protection against lethal H5N1 challenge</td>
<td>Mouse</td>
<td></td>
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</tr>
<tr>
<td>Porcine transmissible gastroenteritis virus</td>
<td>SN protein</td>
<td>Serum IgG and fecal IgA</td>
<td>Virus neutralization <em>in vitro</em></td>
<td>Mouse</td>
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<tr>
<td>Protozoa:</td>
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<tr>
<td><em>Plasmodium yoelii</em> (Malaria)</td>
<td>MSP-1</td>
<td>-</td>
<td>Reduced parasitemia after challenge with <em>Plasmodium yoelii</em></td>
<td>Mouse</td>
<td></td>
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<tr>
<td></td>
<td>MSA2cP</td>
<td>Serum IgG and fecal IgA</td>
<td></td>
<td>Mouse</td>
<td></td>
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<tr>
<td></td>
<td>Cyst wall protein-2 (CWP-2)</td>
<td>Mixed Th1/Th2 cytokine response in MLN and PP</td>
<td>Reduced cyst fecal output after oral <em>Giardia lamblia</em> challenge</td>
<td>Mouse</td>
<td></td>
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<tr>
<td><em>Giardia lamblia</em></td>
<td>3-1E</td>
<td>-</td>
<td>Reduced fecal oocyst release and cecal lesion score</td>
<td>Chicken</td>
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<tr>
<td><em>Eimeria tenella</em></td>
<td>LACK plus IL-12</td>
<td>Intestinal IgA</td>
<td>Reduced food-pad swelling and parasitic burden</td>
<td>Mouse</td>
<td></td>
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</tr>
<tr>
<td><em>Leishmania major</em></td>
<td></td>
<td>Thy response in spleen and MLN</td>
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</table>

CYT, cytoplasmic expression; SEC, secretion; CWA, cell-wall anchoring; MLN, mesenteric lymph nodes; PP, Peyer’s Patches

* Not performed
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, Shigatoxin (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
Enterohemorrhagic *Escherichia coli*

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 (Lpfi) (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
Enterohemorrhagic Escherichia coli

(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 1-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, globotriosylceramide (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 LpfI, 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
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 < 15×10⁹ 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).

<table>
<thead>
<tr>
<th>Model</th>
<th>Mouse strains</th>
<th>Inoculum (CFU)</th>
<th>Inoculation method</th>
<th>Outcomes</th>
<th>Pathology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>C3H/HeN, C3H/HeJ</td>
<td>10^7, 10^8</td>
<td>Intragastric</td>
<td>Morbidity, mortality</td>
<td>Kidney, intestine</td>
<td>Karpman et al., 1997</td>
</tr>
<tr>
<td></td>
<td>CD1, BALB/c, C57BL/6</td>
<td>~ 2 x 10^{10}</td>
<td>Intragastric</td>
<td>Colonization</td>
<td>-</td>
<td>Conlan &amp; Perry, 1998a</td>
</tr>
<tr>
<td></td>
<td>ICR (CD-1)</td>
<td>10^{11}/kg body weight</td>
<td>Intragastric</td>
<td>Colonization</td>
<td>-</td>
<td>Nagano et al., 2003b</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>6 x 10^9/kg</td>
<td>Intragastric</td>
<td>Morbidity and mortality</td>
<td>Kidney, intestine</td>
<td>Brando et al., 2008</td>
</tr>
<tr>
<td>Streptomycin-treated</td>
<td>CD-1</td>
<td>10^{10}</td>
<td>Feeding</td>
<td>Colonization, morbidity,</td>
<td>Kidney</td>
<td>Wadolkowski et al., 1990a</td>
</tr>
<tr>
<td></td>
<td>CD-1</td>
<td>\leq 10^1</td>
<td>Feeding</td>
<td>Colonization, morbidity,</td>
<td>Kidney</td>
<td>Lindgren et al., 1993</td>
</tr>
<tr>
<td>Mitomycin C and streptomycin</td>
<td>ICR</td>
<td>&gt; 10^9</td>
<td>Intragastric</td>
<td>Colonization, morbidity,</td>
<td>Kidney, brain</td>
<td>Fujii et al., 1994</td>
</tr>
<tr>
<td>Protein-calorie malnutrition</td>
<td>C57BL/6</td>
<td>$2 \times 10^3$ – $2 \times 10^7$</td>
<td>Intragastric</td>
<td>Colonization, morbidity, mortality</td>
<td>Kidney, intestine, brain</td>
<td>Kurioka <em>et al.</em>, 1998</td>
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<tr>
<td>Germ-free</td>
<td>IQI</td>
<td>$5 \times 10^7$</td>
<td>Intragastric</td>
<td>Colonization, morbidity, mortality</td>
<td>Kidney, intestine, brain</td>
<td>Taguchi <em>et al.</em>, 2002</td>
</tr>
<tr>
<td></td>
<td>Swiss-Webster</td>
<td>$10^3$–$10^6$</td>
<td>Intragastric</td>
<td>Colonization, morbidity, mortality</td>
<td>Kidney</td>
<td>Eaton <em>et al.</em>, 2008</td>
</tr>
</tbody>
</table>
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 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 CTXφ 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).
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Chapter 1

Literature review


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

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 10⁹ 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*

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.

![Diagram](image_url)  
*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.
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 10⁹ 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).
Mucosal priming of the murine immune system against enterohemorrhagic
Escherichia coli O157:H7 using Lactococcus lactis expressing the type III secretion system protein EspB

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 H₂O₂ (Roche diagnostics). The optical density was spectrophotometrically measured at 405 nm (OD₄₀₅). 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 OD₄₀₅-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 OD₄₀₅ 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: \(113 \pm 42\) ng per \(2 \times 10^9\) 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.](image)

**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

This work was supported by the Special Research Fund (BOF) of Ghent University and the FWO Flanders. We gratefully acknowledge Professor Emeritus Dr. Erik Remaut, Department of Biomedical Molecular Biology, Ghent University, for providing the strain *L. lactis* GM1363 as well as the lactococcal expression vector, pTREX1.
2.6. References

A-C


F-I


K-N


**W-Y**


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

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 \mu g/ml, respectively. \textit{E. coli} NCTC12900 was grown at 37°C in Luria-Bertani broth (Difco) supplemented with 80, 15 and 100 \mu g/ml of novobiocin, naladixic acid and streptomycin, respectively.

### 3.2.2. Constructions of the EspB-expressing \textit{L. lactis} strains

Plasmids \textit{pT:SEC-EspB} and \textit{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

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<th>Plasmid or strain</th>
<th>Description</th>
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<td>A derivative of the dairy starter strain NCDO712, which was cured of all resident plasmids</td>
<td>Gasson, 1983</td>
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<td>\textit{L. lactis} NZ9000</td>
<td>\textit{L. lactis} subspecies \textit{cremoris} MG1363 carrying nisR and nisK genes on the chromosome</td>
<td>Kuipers \textit{et al.}, 1998</td>
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<td>\textit{L. lactis} subspecies \textit{cremoris} MG1363 carrying plasmid \textit{pTREX}</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>LL-\textit{pNZ8150}</td>
<td>\textit{L. lactis} NZ9000 carrying plasmid \textit{pNZ8150}</td>
<td>This study</td>
</tr>
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<td>LL-\textit{pT:CYT:EspB}</td>
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<td>Chapter 2</td>
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<td>This study</td>
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<tr>
<td>LL-\textit{pNZ:SEC:EspB}</td>
<td>\textit{L. lactis} NZ9000 carrying plasmid \textit{pNZ:SEC:EspB}</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{pTREX1}</td>
<td>Lactococcal expression plasmid containing P\textsubscript{t} promoter with downstream start codon and</td>
<td>Wells and Schofield, 1996</td>
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Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization

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<th>Plasmid</th>
<th>Description</th>
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<td>Mierau &amp; Kleerebezem, 2005</td>
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<td>pT:CYT:EspB</td>
<td>Modified pTREX&lt;sub&gt;1&lt;/sub&gt; containing P&lt;sub&gt;1&lt;/sub&gt; promoter with downstream espB gene, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>pT:SEC:EspB</td>
<td>Modified pTREX&lt;sub&gt;1&lt;/sub&gt; containing P&lt;sub&gt;1&lt;/sub&gt; promoter with downstream secretion signal (SP&lt;sub&gt;Usp45&lt;/sub&gt;) and espB. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNZ:SEC:EspB</td>
<td>Modified pNZ8150 containing P&lt;sub&gt;nisA&lt;/sub&gt; promoter with downstream secretion signal (SP&lt;sub&gt;Usp45&lt;/sub&gt;) and espB. Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Na<sup>R</sup>, Str<sup>R</sup>, Cm<sup>R</sup>, Em<sup>R</sup> 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´-GGGGGATCCAACTATCGATAACACTCAAG-3´; reverse: 5´-GGGACTAGTTTAACCAGCAAGACG-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<sub>Usp45</sub> at the BamHI/SpeI ends of pTREX<sub>1</sub> (Figure 3.1). For the construction of plasmid pNZ:SEC-EspB, *espB* along with SP<sub>Usp45</sub> (SP<sub>Usp45</sub>-*espB*) was PCR amplified from pT:SEC-EspB using sequence-specific primer pairs (forward 5´-ATGAAAAAAAGATTATCTCAGC-3´; reverse: 5´-GGGACTAGTTTAACCAGCAAGACG-3´) and was inserted at the Scal/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 (SPUsps45-espB) between Scal 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 $2 \times 10^9$ 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 $1.5 \times 10^6$ 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 \textit{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 \textit{E. coli} NCTC12900 in 100 µl of PBS containing 20% sucrose. Faecal excretion of \textit{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 \textit{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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expression features</th>
<th>EspB expression in fractions of cultures (µg/ml)†</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell lysate</td>
<td>Supernatant</td>
</tr>
<tr>
<td>LL-pT:CYT:EspB</td>
<td>Constitutive cytoplasmic</td>
<td>0.082± 0.02</td>
<td>ND*</td>
</tr>
<tr>
<td>LL-pNZ:SEC:EspB</td>
<td>Nisin-inducible secretion</td>
<td>0.83± 0.19</td>
<td>3.8± 1.1</td>
</tr>
<tr>
<td>LL-pT:SEC:EspB</td>
<td>Constitutive secretion</td>
<td>0.15± 0.05</td>
<td>1.9± 0.4</td>
</tr>
</tbody>
</table>

† Culture density approximately equal to 8 x 10⁸ cfu/ml. Data represent means ± standard deviation of three independent measurements.

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

**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 \textit{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 $10^6$ cfu of \textit{E. coli} NCTC12900 (a non-toxigenic mutant of \textit{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
Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization

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)

3.5. Acknowledgement

This work was supported by the Special Research Fund (BOF) of Ghent University and the FWO Flanders. We gratefully acknowledge Professor Emeritus Dr. Erik Remaut, Department of Biomedical Molecular Biology, Ghent University, for providing the strain *L. lactis* GM1363 as well as the lactococcal expression vector, pTREX1. We also gratefully acknowledge Pieter Almgren, for his technical help.
3.6. References

A-C


D-H


O-S


V-Y


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

B. Ahmed, M. Loos, D. Vanrompay, E. Cox

(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 5% 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 2x10⁹ 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 \textit{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. \textit{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\textsuperscript{®}, Janssen-
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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 $6 \times 10^{11}$ 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 ($14.4 \pm 0.5$ mM), followed by jejunal ($9.5 \pm 0.7$ mM) and ileal contents ($4.5 \pm 0.4$ 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
Each of these intestinal concentrations of bile has resulted in a significant decrease in \textit{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 \textit{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).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure4_1}
\caption{Survival of \textit{L. lactis} in prevalent intestinal concentrations of bile acids under intestinal pH range \textit{in vitro}. \textit{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 \((P<0.0001)\).
}
\end{figure}

5.3.2. Influence of the pancreatic enzymes on the viability of \textit{L. lactis} in porcine small intestine

Exposure of \textit{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 \textit{L. lactis} survival in comparison to the non-enzyme-treated control cultures \((P<0.0001)\) (Figure 4.2). The highest enzymatic influence on \textit{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; \(P<0.0001\)).
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**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.
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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.

![Graph showing % survival of *L. lactis* in the presence of aluminium hydroxide at pH 5.5 and 6.8.](image)

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.

![Graph showing % survival of *L. lactis* in the presence of camostat mesylate at different concentrations.](image)
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](image.png)

**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 $6 \times 10^9$ 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 ± 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.,

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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 \textit{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 \textit{Salmonella typhimurium} vector against bile toxicity \textit{in vitro} (Edwards & Slater, 2009; Edwards et al., 2010). However, our preliminary experiments showed a negative effect of cholestyramine on \textit{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 \textit{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 \textit{L. lactis} survival. Our results show a protective effect of camostat mesylate on \textit{L. lactis} against trypsin hydrolysis. In regard to amylase and lipase, unfortunately, the approach of using enzyme inhibitors to protect \textit{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 \textit{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 \textit{L. lactis} survival against bile toxicity and trypsin hydrolysis, respectively \textit{in vitro}, we next validated the capacity of these protectants to improve the GI survival of \textit{L. lactis} \textit{in vivo}. Oral co-administration of aluminium hydroxide and camostat mesylate with \textit{L. lactis} has increased the viability of the bacterium in the small intestine of pigs (38 and 24 fold-increased \textit{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 \textit{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.,
Survival and protection of Lactococcus lactis in the porcine gastrointestinal tract: a step towards the validation of L. lactis-based EHEC vaccine in pig models (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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A-B


C-D


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R-T


V-Z


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 \textit{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 \textit{et al.}, 1999; Knudsen \textit{et al.}, 1995; Torres \textit{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 \textit{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\textsuperscript{TM} technology, which was developed by the biotechnology company ActoGenix.
5.3. References

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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 \textit{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-\(\gamma\) and IL-4 and IL-10, respectively) in an \textit{in vitro} antigen-recall assay. This indicates the induction of a mixed Th1/Th2 response. Nevertheless, IL-4:IFN-\(\gamma\) 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 \textit{L. lactis} secreting EspB was evaluated during a challenge infection using \textit{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 \textit{L. lactis} as a delivery vector.

\textbf{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 \textit{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 \textit{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 \textit{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 \textit{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. \textit{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 darmsmucosa, 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. Onder meer 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, onder meer 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 frequentie 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 coloniseren. 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 geïmmuniseerde 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 immunititeit 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 kolonisatietproces 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
Samenvatting

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 behandeld 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.

STUDIES ABROAD

June 2008: Department of Pathology, Infection and Immunity, Faculty of Veterinary Medicine, University of Bristol, UK. “The effect of rearing environment on the development of the mucosal immune system in neonates”

SYMPOSIA AND CONFERENCES


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**PUBLICATIONS**


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