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# LACTOBACILLI MEDIATED TARGETING OF GASTROINTESTINAL PATHOGENS

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Karolinska Institutet

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*To my family*

Lactobacilli Mediated Targeting of Gastrointestinal Pathogens

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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# ABSTRACT

The mucosal membranes of the gastrointestinal, genitourinary and respiratory tract are the main targets for infection in the human body and are repeatedly challenged by pathogens. Passive immunization using delivery of antibodies at the mucosal membrane may thus provide a new first line of defense against these pathogens.

The objective of this work was to genetically modify *Lactobacillus*, a natural inhabitant of the gastrointestinal tract, for *in situ* delivery of passive immunity against gastrointestinal infections.

With the aim of identifying colonizing strains of *Lactobacillus* that could be used as vehicle for delivery of passive immunity in the gastrointestinal tract, a collection of ninety-three *Lactobacillus* strains, derived from human fecal samples, was screened for markers for survival and persistence in the gastrointestinal tract (paper I). Five strains showed promising results and will be taken forward for testing of survivability in human gut and confirm their safety in human volunteers.

A novel expression system for production and delivery of therapeutic molecules in *Lactobacillus* was constructed based on the framework of the *apf* gene from *L. crispatus*. This expression system was tested for delivery of antibody fragments both in a secreted form and cell wall anchored on the surface of the *Lactobacillus*. The expression cassettes were stably integrated on the chromosome using the integrase gene from the bacteriophage A2 to create "food grade" strains of *Lactobacillus*, devoid of antibiotic markers, for delivery of antibody fragments (paper II).

The feasibility of delivering passive immunity against bacterial toxins in the gastrointestinal tract was tested with *L. paracasei* BL23 engineered to express a single chain antibody binding the anthrax protective antigen. The engineered *Lactobacillus* was able to provide protection in mice orally challenged with the anthrax edema toxin, validating the concept of *in situ* toxin neutralization in the gastrointestinal tract (paper III).

Members of the family of *Camilidae*, to which llamas belong, express a subset of their IgG antibodies as heavy chain only antibodies. Their antigen binding domain is encoded in the single variable domain (VHH) that can be produced as a single polypeptide. The VHH has higher acid and proteolytic stability compared to conventional IgG making them ideally suitable for therapeutic use in the gastrointestinal tract. Llamas were immunized with the toxins A and B, the two causative elements of *C. difficile* associated diarrhea. A range of VHH neutralizing the dominant virulence factor, toxin B, were isolated and cloned for expression in *L. paracasei*. When expressed either in a secreted or cell wall anchored form, the *Lactobacillus* produced VHH were able to provide protection against the cytotoxic effects of toxin B. Prophylactic treatment with a combination of two strains of engineered *L. paracasei* expressing two toxin B neutralizing VHH could delay and provide partial protection against the effect of a toxin B producing strain of *C. difficile* in an *in vivo* hamster model (paper IV).

In summary this work has shown the potential of using *Lactobacillus* for the delivery of passive immunity against gastrointestinal infections. *Lactobacillus* strains colonizing the gastrointestinal tract for delivery of antibody fragments could represent a potential new approach to management of the *C. difficile* associated diarrhea that could be used both prophylactically or for the prevention of recurrent infections.

# LIST OF PUBLUCATIONS

I. **Screening and evaluation of human intestinal lactobacilli for the development of novel gastrointestinal probiotics.**
Kõll P, Mändar R, Smidt I, Hütt P, Truusalu K, Mikelsaar RH, Shchepetova J, **Krogh-Andersen K**, Marcotte H, Hammarström L, Mikelsaar M.
*Current Microbiology. 2010 Dec;61(6):560-566.*

II. **Integrative expression system for delivery of antibody fragments by lactobacilli.**
Martín MC, Pant N, Ladero V, Günaydin G, **Andersen KK**, Alvarez B, Martínez N, Alvarez MA, Hammarström L, Marcotte H.
*Applied and Environmental Microbiology. 2011 Mar;77(6):2174-2179.*

III. ***In situ* gastrointestinal protection against anthrax edema toxin by single-chain antibody fragment producing lactobacilli.**
**Andersen KK**, Marcotte H, Álvarez B, Boyaka PN, Hammarström L.
*BMC Biotechnology. 2011 Dec 20;11:126.*

IV. **Neutralization of *Clostridium difficile* toxin B mediated by engineered lactobacilli producing single domain antibodies**.
**Andersen KK**, Strokape NM, Hultberg A, Kai Truusalu, Imbi Smidt, Raik-Hiio Mikelsaar, Mikelsaar M, Verrips T, Hammarström L, Marcotte H.
*Submitted 2014*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| a.a. | Amino acids |
| AMP | Adenosine monophosphate |
| ARP1 | Anti rotavirus protein 1 |
| BIC | Bovine immunoglobulin concentrate |
| CDAD | Clostridium difficile associated disease |
| CDI | Clostridium difficile infection |
| CDR | Complementarity determining region |
| DNA | Deoxyribonucleic acid |
| EF | Edema factor |
| ET | Edema toxin |
| EHEC | Enterohaemorrhagic *Escherichia coli* |
| EPEC | Enteropathogenic *Escherichia coli* |
| ETEC | Enterotoxigenic *Escherichia coli* |
| FDA | Food and drug administration |
| GRAS | Generally regarded as safe |
| HBC | Hyperimmune bovine colostrum |
| kDa | Kilo dalton |
| LAB | Lactic acid bacteria |
| LF | Lethal factor |
| LSR | Lipolysis-stimulated receptor |
| LT | Lethal toxin |
| mAb | Monoclonal antibody |
| MIC | Minimum inhibitory concentration |

| | |
|---|---|
| PA | Protective antigen |
| PaLoc | Pathogenicity locus |
| PCR | Polymerase chain reaction |
| ScFv | Single chain fragment variable |
| VHH | Variable fragment of heavy chain antibody |
| $V_L$-domain | Variable light domain |
| $V_H$-domain | Variable heavy domain |
| VP4 | Rotavirus virus protein 4 |
| VP7 | Rotavirus virus protein 7 |

# 1 INTRODUCTION

## 1.1 *LACTOBACILLUS*

*Lactobacillus* belong to the diverse group of Gram-positive lactic acid bacteria (LAB) defined by their ability to produce lactic acid as dominant metabolic end product of carbohydrate fermentation. Fermentation by LAB have for thousands of years been applied for the preservation of food components such as milk, meat and vegetables. Having not been associated with any pathogenic effects during this prolonged period of use, *Lactobacillus* have earned the status "generally regarded as safe" (GRAS) by the US Food and Drug Administration. Several species of *Lactobacillus* are natural residents of the gastrointestinal and vaginal tracts of vertebras. Due to their natural ability to thrive in the gastrointestinal tract and their close contact with the mucosal surfaces, *Lactobacillus* has been an obvious choice for direct *in situ* delivery of therapeutic molecules to the mucosa of both the gastrointestinal and vaginal tracts.

### 1.1.1 Probiotic bacteria

The human gastrointestinal microbiota constitutes a complex ecosystem that interacts symbiotically with its host. It is estimated that that it may consist of as many as 1000 – 1200 species and contain up to $10^{14}$ microorganisms (1, 2). The greatest diversity and density is found in the distal part of the small intestine and in the colon where, for the colon, concentrations of up to $10^{12}$ CFU/ml of bacteria can be reached. The gastrointestinal microbiome has been attributed with several beneficial functions affecting the health of the host. This includes modulation of the immune response, antagonistic effects towards pathogens and interactions with host metabolism. The exact mechanisms and molecular basis for these effects are currently under investigation and slowly starting to be unraveled.

Certain species of bacteria, isolated from the human microbiome or food products have been linked with the potential to excise these beneficial properties when applied therapeutically. They have been grouped under the term probiotic bacteria, defined as "live organisms which when administered in adequate amounts confer a health benefit on the host". Three mechanisms of actions have been suggested for probiotics benefiting the health of the host (3, 4): (I) Improve the epithelial barrier function: by strengthening

the tight junction, induction of mucus or defensin production and by preventing apoptosis, (II) an antagonistic effect on pathogens: by production of antimicrobial compounds, competition for mucosal binding sites or nutrients, and by contributing to the gastrointestinal homeostasis, (III) modulation of the immune response and stimulation of the production of anti-inflammatory compounds. In the group of LAB, lactobacilli and bifidobacteria are the two species that have most frequently been associated with probiotic effects. Members of both groups are found in the human gastrointestinal microbiota and have the potential to transiently colonize the intestine when given orally (5, 6). Bioengineering of probiotic lactobacilli or bifidobacteria would potentially permit the combination of a probiotic effect and ability to colonize the gastrointestinal tract with an engineered therapeutic function.

### 1.1.2 Expression systems

Production of therapeutic molecules in *Lactobacillus* is generally directed towards either of three domains; the cytoplasm, anchored to the cell wall or secreted into the surrounding media. Which domain to target depends both on the produced molecule and therapeutic aim, but with proteins delivered to the cytoplasmic domain requiring bacterial lysis for their release to be effective. Comparative studies of the efficacy of the method of delivery have been carried out only in a few cases. One study, on the mucosal vaccination with L. plantarum producing a non-toxic fragment of tetanus toxin, surprisingly found that the cytoplasmic production yielded the strongest immunogenic response (7) but otherwise either cell wall anchored or secreted production are most frequently used (8). Secretion of recombinant proteins in *Lactobacillus* has been achieved using both homologous and heterologous signal peptides. Numerous signal peptides have been explored in attempts to optimize secretion of the therapeutic molecules but the efficacy of the signal peptides are highly variable and seems to dependent on the protein it is fused to (9-12), paralleling observations for secretion in *B. subtilis* (13, 14). Cell wall anchoring can be achieved through several different methods with the two most frequently applied being covalent binding or non-covalently binding using a domain interacting strongly with cell wall components. Covalent binding is mediated trough the sortase partway where a C-terminal anchoring domain containing the LPXTG binding motif is covalently bound to a cell wall displayed pentaglycine (15). For non-covalent, binding, several binding domains of cell wall displayed proteins have been used for the display of heterologous proteins on the cell wall including *LysM* (16), *PsgA* (17) and *CbsA*.

Despite the wider application of *Lactobacillus* for the delivery of therapeutic molecules, several challenges still exists to improve the delivery system. Currently, most delivery systems apply plasmid based expression vectors containing antibiotic markers. For therapeutic expression, a stable food-grade chromosomal integrated expression system devoid of antibiotic markers would be required. A few integrative vectors for the construction of food grade expression systems have been developed to address this (18-20). But often, a reduction in production of the therapeutic molecule is seen when going from expression from a multi-copy plasmid to a single copy of the gene chromosomally integrated. Optimization of the promoters would be one way to address this problem and increase the levels of production. Two approaches to this end have been the development of constitutive active promoters derived from ribosomal genes (21) as well as promoters that are transcriptional active in the gastrointestinal tract (22-24). In addition, the therapeutic use of engineered strains of lactobacilli will require a biological containment system to avoid release of the bacteria into the environment. For the clinical trial on the delivery of IL10 by *L. lactis*, a containment system was developed based on the insertion of the IL-10 gene into, and thereby deleting, the *thyA* gene, generating a thymidine autotroph. This strain relies on the supply of thymidine from the contents of the gastrointestinal tract but upon release into a less thymidine rich environment starts fragmenting its DNA using it as a thymidine source and eventually lyses and dies. This containment system was both validated in a pig model and in a subsequent human trial (25, 26).

### 1.1.3 Therapeutic delivery

Much of the initial work on the use of lactic acid bacteria for delivery of therapeutic molecules were focused on mucosal vaccination. This was driven by several advantageous properties of LAB for mucosal delivery; by residing on the mucosal surface they can induce both a mucosal and systemic immune response; they constitute a safe alternative to attenuated pathogens; their natural resistance to the gastrointestinal environment and *in situ* production of the antigen overcomes the problem with vaccine degradation when given orally; and lastly they can be engineered to express multiple antigens. The first study on the use of recombinant LAB for mucosal vaccination was carried out in 1990 with a strain of *L. lactis* producing a Streptococcus mutants surface antigen cell-walled anchored (27). Since then, numerous publications on the use of LAB for mucosal vaccination have been published, for review (28), with *L. lactis* based vaccines being developed against among others: *Helicobacter pylori* (29), *Streptococcus*

*agalactiae* (30) and *Salmonella enterica* (31). One of the most seminal studies on the use of LAB for therapeutic delivery was the engineering of *L. lactis* for delivery of IL-10 for treatment of inflammatory bowel disease (IBD). Here the authors showed that delivery of murine IL-10, prevented the onset of colitis in IL10-/- mice and provided a 50% reduction in colitis in mice treated with dextran sulfate (32). A subsequent phase I trial showed that *L. lactis* mediated delivery of IL-10 was safe in patients suffering from Crohn's disease (CD) (26). The following phase II clinical trial though was a major setback, failing to show a statistical significant effect in patients with CD compared with the group receiving a placebo. Subsequently, several other studies on delivery of IL-10 by *L. lactis* has shown effects in mouse models for airway inflammation (33), food allergy (34) and murine colitis (35) validating the strategy despite the negative results in the human clinical trial. Initially, due to the ease of engineering, much of the work on therapeutic delivery of biomolecules was carried out in *L. lactis*. Recently however additional genetic tools for the engineering of lactobacilli has become available and aided its use as vector for mucosal delivery. Lactobacilli, contrary to *L. lactis*, are natural habitants of the gastrointestinal tract and have the additional advantage of being able to temporarily colonize the small intestine and colon enabling prolonged delivery of therapeutic molecules. Limited studies have been carried out on the relative merits of the different bacteria as delivery systems and the choice of vector would likely depend on the therapeutic molecule in question. One study compared the induced immune response to a human papillomavirus antigen expressed cell wall anchored in both *L. lactis* and *L. plantarum* and found that immunogenicity was higher when expressed in *L. plantarum* (36). In this case the improved effect was suggested to arise from the ability of *L. plantarum* to persist at mucosal surfaces for several days. to date, *Lactobacillus* have been used to deliver a range of therapeutic molecules, including: antigens (37-39), antibody fragments (40, 41), anti-oxidants (42), cytokines (43), peptides (44), enzymes (45) and DNA molecules (46).

### 1.1.4 *Lactobacillus* delivering passive immunity

The ability of *Lactobacillus* to thrive and colonize the gastrointestinal tract suggests that they could be ideally suited for production of antibodies for passive immunity protecting the intestinal mucosa. The continuous *in situ* production of the antibody fragment would circumvent the need for the antibody preparation to pass the acidic environment of the stomach and a prolonged exposure to the proteolytic enzymes in the gastrointestinal tract. To date, lactobacilli have been applied for the delivery of

passive immunity in the oro-gastrointestinal tract against rotavirus, *Streptococcus mutants*, *Porphyromonas gingivalis* and *Bacillus anthracis* (40, 41, 47-50). Both scFv's and VHH was used for passive immunization in these studies, but with the VHH fragment expressed much more efficiently than the scFv's tested. The first study on delivery of passive immunity by engineered lactobacilli was conducted with a strain of *L. zeae* engineered for cell wall anchored expression of a scFv against the SAI/II adhesion molecule of *Streptococcus mutants.* Tested orally in a desalivated rat model, this strain could reduce the adherence of S. mutants leading to a significant reduction in caries. This verifying the potential approach, that has since been confirmed in several studies with *L. paracasei* expressing VHH directed against rotavirus in a mouse pup model of gastrointestinal infection (41, 51).

## 1.2 ANTIBODIES FOR PASSIVE IMMUNITY

Polyclonal antibody preparations have historically been used for oral delivery of passive immunity. With preparations of hyperimmune bovine colostrum (HBC) and chicken IgY used in most studies due to the high amount needed and the ease of purifying antibodies from these sources. Hyperimmune bovine colostrum is generated by immunizing cows during late pregnancy and will result in a more than a 100 fold increase in antibody titre. With the colostrum yield being between 30 and 200 mg/ml of immunoglobulins of which approximately 75% is IgG1 (52). Chicken IgY is produced in the yolk of eggs, and the immunization of hens with only a small amount of antigen give high and long lasting IgY titres (up to 160 mg/egg) (53, 54). Despite both methods giving high yields of antigen specific antibodies, the cost of production is still high and antibody preparations varies in specificity from batch to batch.

### 1.2.1 Monoclonal antibodies

Antibodies as a modular defense system constitutes a central part of the immune system protecting the human body against infections by foreign objects like viruses and bacteria. They carry the ability to recognize a specific antigen unique to its target through their antigen binding sites, located on each tip of the "Y-shaped" structure. Immunoglobulin G is the most frequently used immunoglobulin for therapeutic applications and is also the principal component of immune globulin preparations used for intravenous delivered passive immunity (55). Human IgG is a heterotetramer consisting of two identical γ heavy chains and two identical light chains joined by disulfide bonds. Each light chain consists of one variable domain (VL) and one constant

domain (CL); while the heavy chains contain one variable domain (VH) and three constant domains (CH1, CH2, CH3). The N-terminal variable domains determine the specificity, affinity and the diversity of the antibody. Within each variable domain there are three hyper-variable regions (complementary determining regions, CDRs) responsible for antigen specificity and recognition by forming a tertiary structure complementing and binding the antigen. The role of the constant domains is to provide structural support for the antibody; determines the serum half-life of the antibody; and recruitment and activation of immune cells. Today approximately 30 mAb's have been approved by the FDA therapeutic use in the United States (56, 57).

Early attempts to produce full-length mAbs in prokaryotes were complicated by improper folding and aggregation of the polypeptides in the cytoplasm (58, 59). This was due to the complex structure of mAb's with four separate peptide chains and multiple disulfide bonds needed to be formed to get the correct conformational folding. To overcome this, much attention has been directed towards expression of antibody fragments that consists of a single polypeptide chain but retains a binding diversity comparable to that of conventional antibodies. The most frequently used are the single chain fragment variable (scFv) antibodies and the variable domain (VHH) derived from camelid heavy chain only antibodies.

### 1.2.2 Single chain antibody fragments

Single chain antibody fragments are derived from mAb by fusing the two variable domains, variable light (VL) and variable heavy (VH) domains, with a flexible linker. This provides a single polypeptide that retains the antigen specificity of the mAb from which it was derived and potentially being capable of binding the antigen with an affinity similar to that of the parent mAb (60). The peptide linker joining the two fragments usually vary in length from 10 to 25 amino acids (aa), with 15 aa most frequently used. To avoid intercalation with the variable domains during folding, the linker should predominantly consists of hydrophilic aa (61). The decapentapeptide $(Gly_4Ser)_3$ linker is most frequently used but insertions of charged aa can be used to improve solubility of aggregating scFvs (62). The optimal linker length has been estimated to span at least 35 Å to permit free folding of the scFv (63) but by alternatively using short linkers of 10-12 aa, the scFv can be forced into a dimeric or trimeric conformation, increasing the valency of the scFv for improved functional affinity (64).

ScFvs exhibit a low degree of immunogenicity and their smaller size permits penetration into tissues inaccessible to full-size mAb (65). They are also easily amenable to engineering and can be cost effectively expressed both in prokaryotic and yeast expression systems. On the down side, the scFv lacks the multivalency of the mAb and have a short circulating half-life due to their small size as they are subjected to kidney clearance. Some scFvs also show reduced solubility as the detachment and refolding of the variable domains often exposes hydrophobic residues otherwise buried in the mAb, but this can be amended by engineering of the scFv. As a consequence of the scFv lacking the constant domains, both immune recruitment and effector functions are absent when used therapeutically. At the present stage, numerable scFvs have been expressed for a range of therapeutic and diagnostic applications (for review (66)).

### 1.2.3 Heavy chain only antibody fragments

One exception to the otherwise conserved structure of mammalian IgG is found in the species of *Camelidae*, to which camels, dromedary and llamas belong. In addition to the conventional IgG, the sera of *Camelidae* also contain two additional IgG subtypes, IgG2 and IgG3, devoid of the CH1 domain and light chain, but still retaining binding activity (67) (Figure 1). These heavy chain only antibodies are produced from a subset of immunoglobulin G heavy chain genes which due to a point mutation in a splice site, leading to a deletion of the CH1 domain (68, 69). The missing CH1 domain results in a failure to associate with the light chain and the heavy chain is secreted as a heavy chain only antibody. The binding specificity of these heavy chain antibodies is determined by the N-terminal variable domain, referred to as VHH (the variable domain of heavy chain antibodies). Within the VHH domain are four frame work regions (Fr) providing the core structure and three hypervariable regions, CDRs, involved in antigen binding. While the paratope of conventional IgG antibodies forms a flat surface or slight concave groove, highly suitable for binding to larger surfaces or linear peptides (70), the VHH domain on the contrary have a more pointing ellipsoid shape permitting it to insert into and bind to cavities on

the surface of antigens (71), sites not accessible by conventional antibodies. Expressed on its own, the VHH domain of heavy chain antibodies is the smallest naturally occurring antigen-binding molecule known to date, with a molecular weight of only 12-15 kDa.

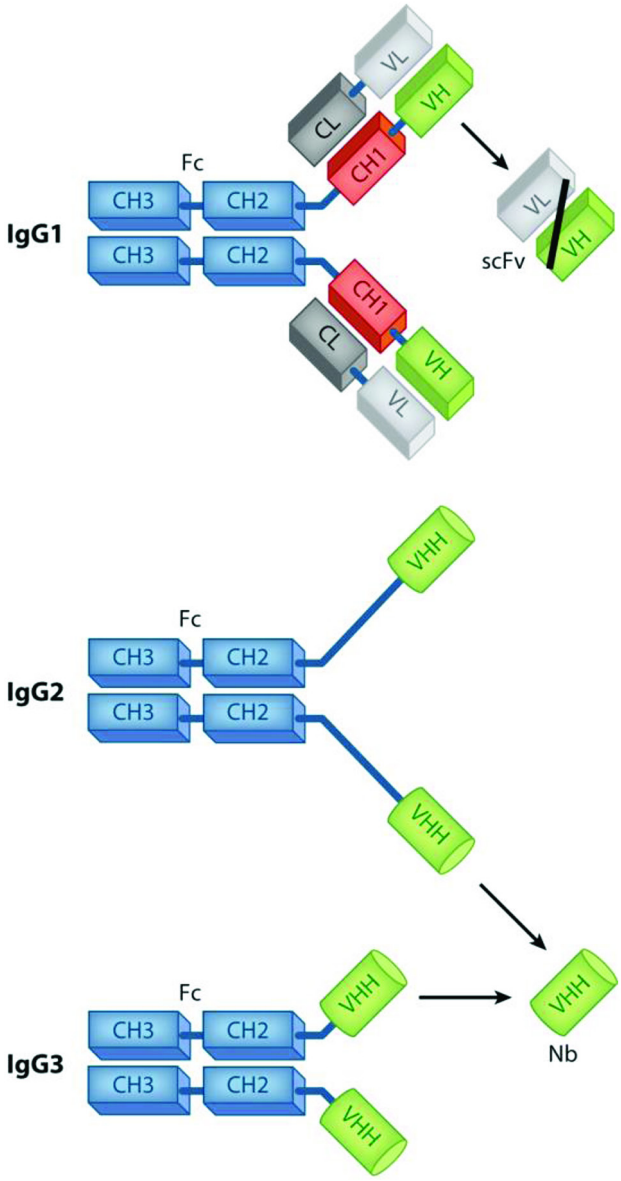


Figure 1. Schematic representation of naturally occurring antibodies in sera of camelids: Conventional antibody (IgG1) and two types of homodimeric heavy-chain antibodies , IgG2 and IgG3. Also illustrated is the single chain fragment variable (scFv) derived from a conventional IgG1 and the variable fragment of heavy chain antibody (VHH). Reprinted with permission from (233).

VHH has, in many cases, been able to replace conventional antibodies both within therapeutic, diagnostic and biotechnological applications (57, 72). Several characteristics set the VHH apart from conventional antibodies and make them ideal candidates for therapies in the gastrointestinal tract. They are markedly more resistant to acidic conditions (and heat) due to their ability to refold after denaturation (73-75) and survives passage through the acidic environment of the stomach. In addition they can be easily engineered for increased resistance to both pepsin and chymotrypsin for extended half-life in the gastrointestinal tract (76, 77). Their size, stability, rapid clearance from blood and high degree of sequence identity with the human variable heavy domain gives them a low level of immunogenicity, a prediction that has been confirmed by the lack of immune response against intravenous injected VHH in both mice and humans (78-80). Lastly, they have proven to be highly suitable for production in bacterial expression systems due to their solubility, small size and single domain structure.

## 1.3 PASSIVE IMMUNITY

Active immunity is the process where the body reacts to exposure of an antigen and generates an adaptive immune response. The process constitutes the backbone against infections in vertebrates and contains a memory function so once an adaptive immune response is achieved, an antibody response can rapidly be mounted upon subsequent infections. The drawback is that the development of adaptive immune response can take days to weeks and in some cases, a protective adaptive immune response cannot be achieved.

Passive immunity builds on the transfer of antibodies generated through the adaptive immune response, from one individual to another. Contrary to the adaptive immune response, passive immunity gives immediate protection, but the protection is short-lived and contains no “memory” of the infective agent to provide protection against future exposures. Passive immunity is classified either as natural or acquired depending on the method of antibody transfer. The transfer of maternal antibodies through the placenta to the unborn child generates a natural passive immunity that can protect the child for weeks to months after birth, giving the infant time to develop its own immune response. Acquired passive immunity on the other hand utilizes antibody preparations from immunized individuals (or animals) that are injected into a non-immune person to provide protection against a specific challenge. In both cases, the circulating antibodies only provide a transient protection for a couple of weeks to three or four months at most, as the antibodies successively are degraded. In addition to the systemic application,

passive immunity can also be applied directly to the mucosal surfaces to provide protection against infections. This occurs naturally in the transfer of maternal immunoglobulin from the naturally immunized mothers to the child through the colostrum and the milk during breastfeeding (81). Human breast milk contains secretory IgA against a range of enteric pathogens such as *E. coli* (ETEC), *Salmonella*, *Vibrio cholerae*, *Campylobacter jejuni*, *Shigella* and rotavirus (82-84). For therapeutic applications against enteric pathogens, a similar effect can be achieved by oral delivery of heterologous antibodies from immunized animals.

### 1.3.1 Mucosal delivery of passive immunity in the gastrointestinal tract

A large number of infectious agents enter the human body by breaching the epithelial barrier of the respiratory, gastrointestinal and genitourinary tracts. The mucosal immune system reacts by producing and secreting immunoglobulins as a first line of defense of the body against these infectious agents. Individuals with primary immunodeficiencies affecting immunoglobulin A are generally highly prone to both gastrointestinal and respiratory tract infections, illustrating the crucial role of antibodies in protecting the mucosal surfaces against infections. These observations support the concept of mucosal delivery of passive immunity for the protection against mucosal infections (85).

Passive immunity against enteric pathogens has been explored for the last couple of decades. Despite this, no large scale application of oral delivered passive immunity has taken place. However, several different aspects of gastrointestinal infections and their treatment have kept it as an attractive approach, (I) an increasing emergence of strains of gastrointestinal pathogens with resistance to conventional antibiotics, (II) an observed reduced efficacy of vaccinations in the developing world and (III) a growing group of elderly with a decreased immune responsiveness suffering from gastrointestinal infections.

For several gastrointestinal pathogens, passive immunization has been successfully explored (like *Escherichia coli*, *Helicobacter pylori*, Rotavirus and *Clostridium difficile*) using primarily bovine colostrum and chicken IgY derived antibody preparations.

#### *1.3.1.1 Escherichia coli*

*Escherichia coli* is a Gram-negative facultative anaerobic bacteria belonging to the normal gastrointestinal microbiota of both humans and animals. The majority of *E. coli* strains are commensals and colonize the lower intestine. Several pathogenic strains causing diarrheal diseases exist and are divided into six different pathotypes based on their virulence properties (86); with three of the non-invasive pathotypes being the enteropathogenic *E. coli* (EPEC), enterohaemorrhgic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC). Second to rotavirus, ETEC is regarded as one of the major diarrheal pathogens causing up to 400 million diarrheal episodes and more than 300,000 deaths annually among preschool children in the developing world (87). ETEC is non-invasive but causes a profuse watery diarrhea by producing one or both of two enterotoxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST).

The traditional use of antibiotic for management of diarrhoeagenic *E. coli* is becoming increasingly difficult with strains of ETEC and EPEC progressively gaining resistance to commonly used antibiotics. For EHEC, the use of antibiotics is not recommended for treatment of the infection as it induces the production of Shiga toxins (Stx-1 and Stx-2), aggravating the infection (88) and potentially triggering hemolytic-uremic syndrome (HUS).

One of the main indicators for a role of passive immunity in the management of diarrhoeagenic *E. coli* infections, arise from the fact that secretory immunoglobulins in breast milk protects newborns against ETEC and EPEC (89). Studies on the use of immunoglobulin preparations from cows and chickens immunized with EHEC, ETEC and EPEC have been shown to be an effective prophylactic treatment against infection in farm animals (90-92). In humans, infant feeding formulas supplemented with milk immunoglobulin concentrate (MIC) containing antibodies to EPEC has been shown to be efficient in treating EPEC infections in infants (93). In another study, hyperimmune bovine milk antibody preparations raised against purified colonization factor antigens was shown to provide protection in volunteers challenged orally with ETEC (94). on the contrary one randomized, placebo controlled study, on children infected with diarrhoeagenic *E. coli*, using a preparation of bovine immunoglobulin concentrate (BIC) from cows hyperimmunized with EPEC and ETEC, showed no significant therapeutic effect (95). A difference that could arise from variations in the properties of the antibody preparations. Further studies, using intraperitoneal injections of monoclonal antibodies neutralizing the cytotoxic effect of Shiga toxins, have also been shown to be effective in

reducing diarrhoeagenic EHEC infection both in mice and in a gnotobiotic piglet model (96-98).

#### *1.3.1.2 Rotavirus*

Rotavirus is the most common cause of severe diarrhea in infants and young children worldwide. With gastroenteritis caused by rotavirus leading to between 55,000 to 70,000 hospitalizations per year in the United States (99) and an estimated 500,000 deaths per year, primarily in the developing world (100). Two vaccines against rotavirus are currently licensed (RotateqTM and RotarixTM) and have showed high efficacies (>85%) in reducing episodes of severe diarrhea when used in developed countries (101, 102). Recent trials conducted in the developing world with the most pressing need for protection have had been less efficient with an efficacy ranging from 39.3 % (RotateqTM tested in Sub-Saharan Africa) and to 61.2% (RotarixTM tested in South Africa and Malawi) (103, 104).

Studies using monoclonal antibodies against the viral coat proteins, VP4 and VP7, have shown that virus infection can be inhibited by blocking the cellular attachment of the virus (105) and by inhibiting decapsidation of the virus (106). Passive immunization has been explored in several studies using either HBC or hyperimmunized chicken yolk immunoglobulins and have shown protection in both animal models and human clinical trials. (85, 107-111) Recently a number of anti-rotavirus VHH fragments with protective effects have been developed through immunizations of llamas (112, 113), with the aim of bringing down the cost of treatment. One of these VHH fragments (ARP1) has, as a further development of the passive immunization strategy against rotavirus, been cloned both for expression in *Lactobacillus* (41) and rice (114) for direct delivery in the gastrointestinal tract.

#### *1.3.1.3 Clostridium difficile*

*Clostridium difficile* is a gram positive, sporeforming , anarobic bacterium and the primary cause of antibiotic associated diarrhea in the hospital setting (for details see section 5). The primary virulence factors and the causative elements of the diarrhea are the two endotoxins toxin A and toxin B and for passive immunity, much of the focus has been directed towards neutralization of these toxins. Passive immunization by either subcutaneous or intraperitonal injection of neutralising antibodies against these two toxins have been shown to prevent the mortality caused by *C. difficile* infection in a hamster model (115, 116). Despite recent evidence suggesting that toxin B is the

dominant virulence factor (117) and the one causing the disease in this model, other studies have shown that antibodies against both toxins are usually needed for complete protection (116, 118). In humans, similar studies with antibodies directed against both toxins have been shown to be able to resolve the symptoms in patients with *C. difficile* infections (119, 120). Recently, two toxin neutralizing human MAbs directed against the receptor binding domain of toxin A and toxin B have been developed (116). Tested in a phase II clinical trial, a single infusion (10 mg/kg bw) of these two antibodies given in combination with metronidazole or vancomycin could significantly reduce the rate of recurrent infection with *C. difficile* (121). Currently, these antibodies have been taken forward and are currently undergoing phase III clinical trials for their use for passive immunization against recurrent *C. difficile* infections. Less work has been carried out on the use of oral delivery of passive immunity against *C. difficile*. This is possibly due to the large doses of antibody fragments required and the prolonged period of administration needed to prevent relapse of infection. However, oral delivery of passive immunity has though been tested using bovine-enriched whey from cows immunized with either whole *C. difficile* cells or the toxins only and were shown to be able to prevent the relapse of *C. difficile* infections following antibiotic therapy (121-123). The knowledge that the *C. difficile* toxins rely on attachment to a cellular receptor displayed on the epithelial cells for cellular uptake suggests that blocking of toxin binding to the receptor could effectively prevent the cytotoxicity of the toxins. Experiments with oral delivery of both chicken IgY and bovine antibodies against toxin A and toxin B have been shown to be protective in a hamster model and that this strategy is possible (118, 124-126).

### 1.3.2 Summary

In summary the delivery of passive immunity to the mucosa of the gastrointestinal for the treatment of infections has shown promising results for a range of pathogens. For several of the pathogens, the data is indicative and better characterized antibody preparations targeting specific virulence factors could be needed to show the real potential of the approach. Large-scale studies on the therapeutic effects also still need to be carried out to evaluate the efficacy of the strategy. For oral delivery, this is likely hampered by the large doses needed for these studies and the costs associated with the production of these but by addressing these issues, the potential of passive immunization of the gastrointestinal mucosa could be realized.

## 1.4 *BACILLUS ANTHRACIS*

Anthrax is caused by the bacterium *Bacillus anthracis*, a Gram-negative spore forming bacteria. The disease is still endemic in some parts of the world, primarily among herbivores in less-developed countries, but can affect a range of species, including humans. Anthrax and the management of anthrax infections though mostly remain in focus due to its status as one of the most prominent bioterrorism threats. The spores of *Bacillus anthracis* are the transmissible factor in the spread of the disease and is ranked as one of the leading bioweapons due to their stability, high lethality and ease of dispersion (127). Immunization, either passive or active, is central in countering the effects of the anthrax toxins and safe guarding against the development of strains resistant to antibiotics.

### 1.4.1 Anthrax infections

Anthrax infections fall into three different categories, reflecting the route of entry; inhalational, gastrointestinal or cutaneous in order of severity of the infection. With regard to bioterrorism, the most realistic mode of mass exposure includes inhalational or gastrointestinal infections. Conceptually, the idea of targeting the food supply is not new (128) and a few records of planned use of anthrax spores for deliberately targeting the oral route exist (129, 130). Relatively little is however known about the physiopathology of gastrointestinal anthrax, despite it being prevalent in ruminant livestock. Initial infection is established in the Peyer's Patches throughout the small intestine, eventually leading to systemic infection by spreading to the draining jejunal lymph nodes, the spleen and, finally, the lungs. Gastrointestinal infection by B. anthracis preferentially occurs after abrasions in the mucosa but can also occur in the absence of damage in which case infection propagation is slower (131). Natural occurrence of human gastrointestinal anthrax in the western world is rare due to the high standard of the food supply chain but is more common than inhalational anthrax in the developing world (132).

### 1.4.2 Anthrax toxins

The pathogenesis of B. anthracis is due to three plasmid encoded (pXO1) toxicity genes; pagA (PA), lef (LF) and cya (EF) forming a tripartite protein complex, causing the lethal symptoms associated with anthrax. The protective antigen (PA) combines with the lethal factor (LF) and edema factor (EF) to form the lethal toxin (LT) and edema toxin (ET) respectively (133). PA is the component affording binding to

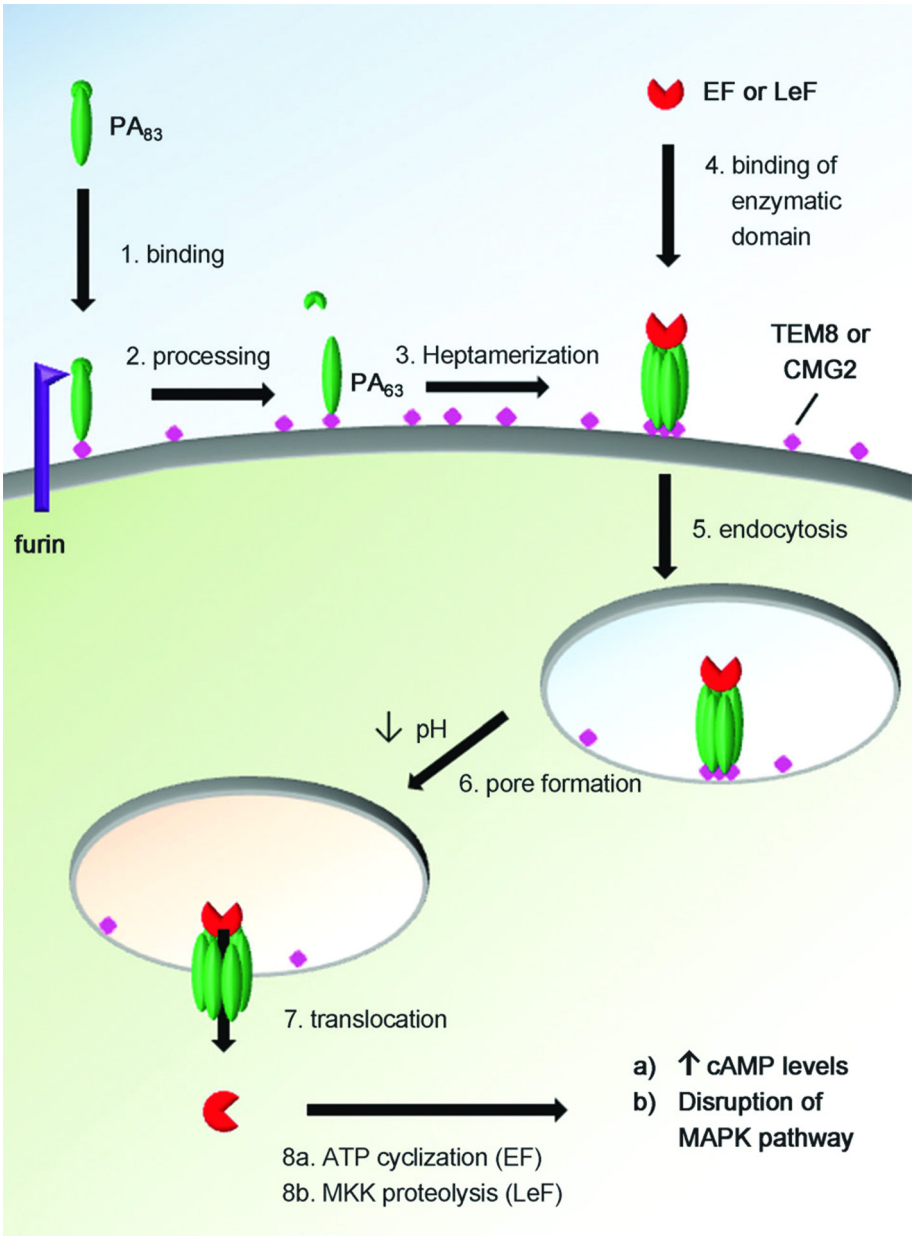


Figure 2. Molecular mechanism of action of anthrax toxin. Protective antigen 83 kDa in size ($PA_{83}$) binds either TEM8 or CMG2 at the cell surface and is processed to $PA_{63}$ by PCs such as furin. $PA_{63}$ heptamerizes, allowing it to bind EF and/or LeF. The complex formed is endocytosed and subsequent acidification of the endosome leads to translocation of the bound EF and/or LeF. Once in the cytosol, EF catalyzes the formation of cAMP from ATP and LeF mediates the proteolytic cleavage of MKKs, which disrupts the mitogen-activated protein kinase (MAPK) pathway. Reprinted with permission from (253).

either of two receptors, the tumor endothelial marker 8 (TEM8) and the capillary morphogenesis 2 (CMG2) (134) (figure 2). The receptor bound PA is proteolytically activated, facilitating oligomerization of PA into a heptameric prepore structure, forming the binding sites for LF and EF. The complete toxin complex is endocytosed and, upon acidification of the early endosome, the prepore undergoes conformational change whereby LF and EF are translocated into the cytosol (for review see (135)). LF is a metalloprotease cleaving MAPK (mitogen-activated protein kinase) kinases (136), inactivating MAPK signaling pathways and inducing an atypical vascular collapse in mice (137). EF is a calmodulin-dependent adenylate cyclase which increases cyclic AMP levels in cells and induces extensive intestinal fluid accumulation and hemorrhaging lesions (138, 139). Both active and passive vaccination strategies against anthrax have previously been attempted and directed primarily towards inactivation of the toxin components, where PA is the dominant immunogen, and several neutralizing antibodies binding to epitopes blocking the binding to its receptors have previously been developed (140, 141).

### 1.4.3 Treatment

*B. anthracis* is sensitive to a range of antibiotics including amoxicillin, ciprofloxacin, doxycycline, levofloxacin and penicillin (142). However, the window of opportunity for treatment is limited as antibiotics are only effective on the bacteria and not blocking the continuous action of the anthrax toxins. Once symptoms of toxemia have started, antibiotics will have a limited effect as stand-alone treatment (143, 144). Currently, there is one vaccine available for the treatment of anthrax in the US, BioThrax, which is a recent improved version of the Anthrax Vaccine Adsorbed (AVA). Current indications suggest that only three priming doses will generate immunity after 6 months, compared to the 6 doses and 12- 18 months for immunity with the previous version of AVA. Due to the time frame, costs and possible side effects, therapeutic treatment is generally considered most cost effective (145, 146).

Therapeutic treatment for anthrax infection is based on antibiotic use, post exposure vaccination and anti-toxin antibodies, with a combinatorial approach of rapid post exposure vaccination combined with antibiotics treatment being the most promising (147).

### 1.4.4 Anti-toxin antibodies

Antibodies against the anthrax toxins is one of the most promising areas for increasing the treatment window post infection as well as counteracing the toxemia from strains resistant to antibiotics. Currently, a range of therapeutic antibodies are under clinical development with ABthraxTM (148, 149), an IgG1 mAb against PA, having been approved for treatment of inhalation anthrax. Delivery of passive immunity by monoclonal antibodies direct targeting the toxin components is a feasible strategy, as results from the AVA vaccine have indicated that anti-PA antibodies are critical for the immunity to anthrax (150-152). Monoclonal antibodies against the anthrax toxins are also under development, targeting each of the three toxin components; PA, EF and LF (153, 154).

## 1.5 *CLOSTRIDIUM DIFFICILE*, A GASTROINTESTINAL PATHOGEN

*Clostridium difficile* is an anaerobic Gram-positive endospore forming bacteria belonging to the phylum Firmicutes. It is the primary cause of nosocomial infections in the western world (155) and the main cause of antibiotic-associated pseudomembranous colitis (156). The spectrum of disease can range from antibiotic associated diarrhea to severe pseudomembranous colitis that can be fatal (157, 158). The bacterium was identified in 1935 as part of the gut flora of neonates (159) and first described as a cause of antibiotic associated diarrhea in 1978 (160-162). Initially the infections were regarded as mild side effects of antibiotic use (157) but over the years both the frequencies and the severity of the infections have increased to the state where CDI is regarded as a serious threat to the health care settings with high rates of morbidity and mortality (163), reaching an estimated treatment costs in excess of three billion USD and causing more than 14,000 deaths in the USA alone in 2012, according to the Center of Disease Control and Prevention. This rising challenge has been attributed to a more widespread use of antibiotics and an increased virulence of strains of *C. difficile* encountered in hospitals.

### 1.5.1 Infection and disease progression

The spores of *C. difficile* are shed by infected individuals and found throughout the environment with a wide distribution in healthcare settings. Spores are taken up by ingestion through contact with contaminated surfaces. During the gastrointestinal passage, germination receptors are activated and the dormant spore begins its vegetative growth cycle and commence colonization when they reach the anaerobic environment of

the cecum and colon (164). Of the patients colonized with *C. difficile* in hospitals, only between one third to half of the individuals develops a *C. difficile* infection (CDI), with the remaining becoming asymptomatic carriers (165, 166). Asymptomatic carriage is found in up to 7 - 17 percent of hospitalized patients (167, 168) and has been attributed to the spread of *C. difficile*, by functioning as a reservoir for infection. Previously, *C. difficile* was predominantly considered a hospital-acquired infection but recently, community-acquired CDI is also on the rise with infection now commonly being identified in populations previously considered low risk, including children, young adults and people with no health care exposure (169, 170).

The predominant risk factor for acquisition of CDI is the use of antibiotics, with up to 20 percent of antibiotic associated diarrhea being caused by CDI (171, 172). The highest rate of infection is found concurrent or during the first month following treatment, but an elevated risk of acquiring CDI exists for up to three months after cessation of antibiotic therapy (173). CDI is directly linked with the use of broad spectrum antibiotics which destabilizes the gastrointestinal flora and permits the colonization of *C. difficile*. Other risk factors for acquiring CDI include long-term hospitalization, advanced age and immune suppression (172).

Recurrent infections are common and one of the major challenges in treatment of CDI. With studies showing that 33% of patients get re-infected after the first CDI episode and of these, 45% will experience even a third or multiple rounds of infection (174). It has been suggested that it may relate to an inability of the host to restore an optimal gut flora (175, 176) or that strains of *C. difficile* have developed mechanisms to persist in the gastrointestinal tract despite antibiotic treatment (177).

### 1.5.2 *Clostridium difficile* toxins

The two toxins, toxin A and toxin B, have though since long been established as the causative virulence factors for *Clostridium difficile* associated disease (CDAD). At least one of the two toxins is required for the pathogenicity of CDI but their individual contribution to the disease is still debated. In clinical isolates, strains giving rise to CDAD have either been toxin $A^-B^+$ or toxin $A^+B^+$. No disease causing strains have to date been isolated that were toxin $A^+B^-$, highlighting the role of toxin B in the disease. Recent studies with engineered toxin deletion strains tested in a hamster model have also suggested toxin B as the dominant virulence factor but with conflicting data on whether toxin A can induce the disease on its own (117, 178, 179). The role of a third toxin, the

binary toxin, in CDI was for long debated but recent understanding of its mode of action and role in epidemiology have highlighted its importance for the pathogenicity of *C. difficile* (180).

*Toxin A and toxin B*

The toxicity of *C. difficile* primarily arises from two main virulence factors, toxin A (*tcdA*, 308 kDa) and toxin B (*tcdB*, 269 kDa), both being large single-subunit exotoxins which share extensive homology (for review see (178)). Both have a modular domain structure with a N-terminal enzymatic domain, a central translocation domain and a C-terminal receptor binding domain. The binding domain, consisting of repetitive oligopeptides, is responsible for the initial binding to epithelial cells and induces toxin uptake through receptor mediated endocytosis (Figure 3). Upon lowering of the endosomal pH, the central domain exposes a hydrophobic membrane insertion domain that inserts and translocates the N-terminal catalytic domain from the endosome to the cytosol. The N-terminal enzymatic domain encodes a cysteine protease that, through autocatalytic cleavage, releases the domain from the endosome to the cytosol. The released N-terminal glucosyltransferase domain glycosylates the host GTPases in the cytosol, leading to alterations in the actin cytoskeleton, disruption of barrier functions and apoptosis (181). This results in an inflammatory response and degradation of the intestinal epithelial cell layer. The genes for tcdA and tcdB are both encoded within the 19.6 kb pathogenicity locus (PaLoc) together with regulatory genes for their expression; the negative regulator tcdC and the positive regulator tcdR.

*Binary toxin*

The binary toxin, CDT, is produced in approximately 10 % of *C. difficile* strains. It belongs to the family of clostridial iota-like toxins and consists of two components (182, 183). The enzymatic component, a ribosyltransferase encoded by the *cdtA* gene and the binding component encoded by the *cdtB* gene. The cellular uptake of the binary toxin follows a mechanism similar to the protective antigen (PA) mediated uptake of the anthrax toxins. With the binding component (CDTb), after binding to the cell surface receptor, LSR, undergoes proteolytic activation and forms a heptameric structure that mediates the uptake and release of CDTa in the cytosol (for review (184)). After translocation into the cytosol, CDTa ADP-ribosylates actin resulting in complete destruction of the actin cytoskeleton of the cells and uncontrolled growth of

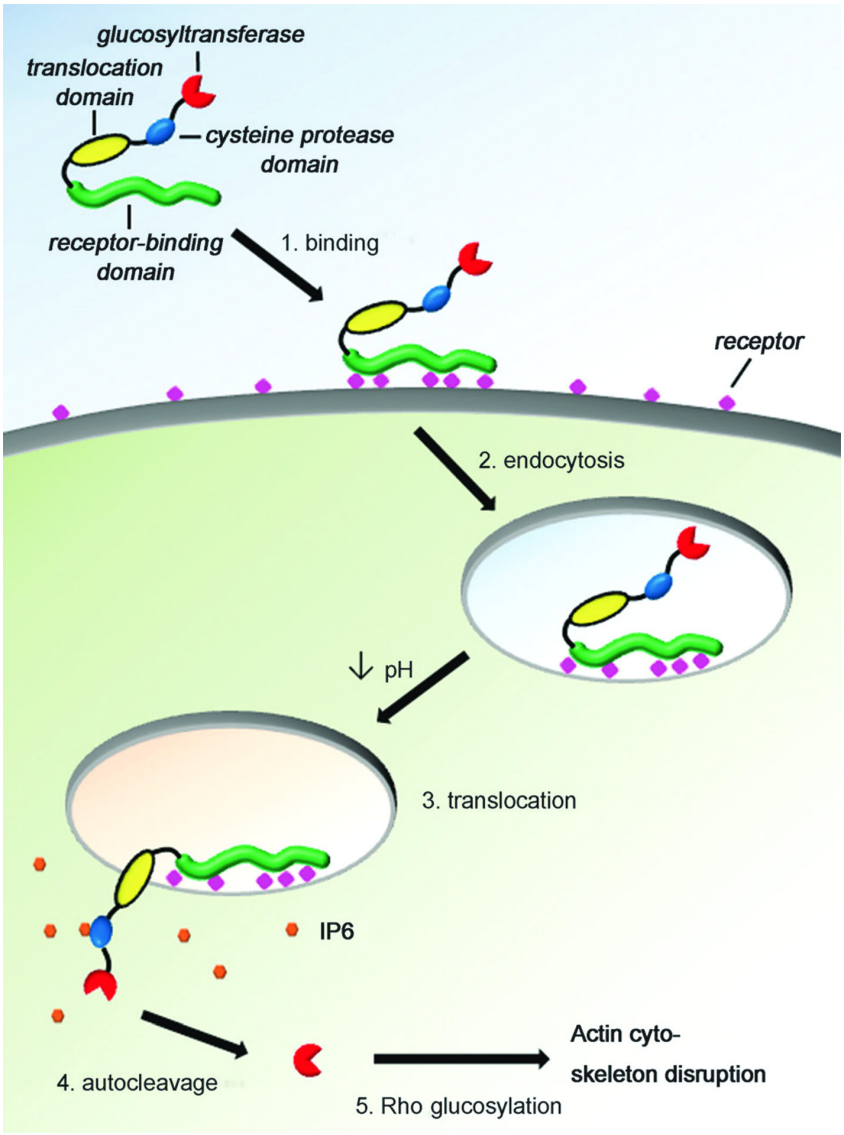


Figure 3. Molecular mechanism of action of TcdA and TcdB. The toxins bind to the surface of enterocytes through the receptor-binding domain and are endocytosed. Acidification of the endosome leads to translocation of the enzymatic domain and the CPD into the cytosol. Cytosolic IP6 then binds to the CPD, thus activating it and initiating autoprocessing. The released enzymatic domain catalyzes the transfer of a glucose moiety to a conserved threonine residue on Rho/Ras proteins, which inhibits downstream signaling events. Reprinted with permission from (253).

microtubules. This creates long membrane protrusions on the epithelial cells that increase the adherence and colonization of *C. difficile* (185, 186).

### 1.5.3 Hypervirulence

Since 2004, a dramatic increase in both the severity and number of cases of CDI has been noted. This was initially linked to a PCR ribotype 027 strain (FT027;BI/NAP01) that caused severe outbreaks with increased mortality in both North America and Europe. The RT027 strain together with RT078, were identified as examples of new hypervirulent strains posing a mounting challenge to the management of CDI. The cause of the increased virulence of these strains has since been studied in detail.

Multiple factors have been assigned as possible hypervirulent factors in attempt to understand and develop methods to contain these strains. Initially, the hypervirulence of RT027 were linked to an abrogative mutation in the repressor gene, tdcC, leading to increased levels of toxin A and B being produced (187). However subsequent studies failed to find the link between *tdcC* deletions and toxin levels (188, 189). The binary toxin has also been attributed to the increased severity of the hypervirulent strains (190-192), but its presence in non-epidemic strains complicates this conclusion (193). In addition, increased sporulation frequencies (194) and resistance to fluoroquinolones (195) have been suggested as factors increasing the virulence but again, this notion has been disputed by others (196). Overall, the picture remains complicated and it is likely that there are multiple factors contributing to the increased virulence of these strains.

### 1.5.4 Treatment of *Clostridium difficile* infections

The primary treatment for CDI is, like the causative element, antibiotics combined with rehydration therapy. The two most common used antibiotics are metronidazole and vancomycin. With the current European guidelines recommending the use metronidazole for non-severe CDI and vancomycin for severe and recurrent CDI. Cure rates of 73 - 94 percent for patients treated with metronidazole and 84 - 94 percent for those treated with vancomycin has been reported in a recent systematic review comparing the effectiveness of antimicrobials for the treatment of CDI (197). Recently, a new narrow spectrum antibiotic, Fidaxomicin, has been approved for treatment of CDI. It targets a narrow spectrum of Gram-positive bacteria and is minimally absorbed from the gastrointestinal tract. Fidaxomicin has shown comparable effectiveness as

vancomycin but patients experience significantly lower rates of recurrent infection (198, 199). This effect has been attributed to the preservation of the microflora due to its narrow spectrum of activity (200).

Fecal transplantation has over the last couple of years emerged as one of the promising new treatments against CDI. It builds on the observation that the disruption of the microbiome is central to the onset of the disease and aims at restoring the colonic flora by transplanting a liquid suspension of intestinal bacteria from a healthy donor. Fecal transplantation has proven highly effective in reducing the recurrencies of CDI that has otherwise been difficult to manage. A systematic review including data on 317 patients with recurrent CDI showed a 92 overall success rate with 89 percent of the patients responding after a single dose (201).

The use of probiotic bacteria for the treatment of CDI builds on the concept of stabilizing the gut flora with the aid of beneficial microbes. A recent meta-analysis on the use of probiotics for the treatment of CDI found that the use of probiotics can prevent CDAD but not for *C. difficile* infections in patients receiving antibiotics (202). Others have proposed that a multi-strain probiotic, resembling the human microbiota, would be most efficient for treating CDAD (203). Overall, a multitude of studies have been carried out but the evaluation of the efficacy has to some extend been hampered by the use of different probiotic strains and study design and to date the verdict is still out and the approach controversial (204).

# 2 AIM

## 2.1 GENERAL AIM

The aim of this work was to explore the possibilities for using *Lactobacillus* as a vehicle for delivering passive immunity against gastrointestinal bacterial infections.

## 2.2 SPECIFIC AIMS:

**Paper I:** To screen and characterize human derived *Lactobacillus* with the aim of identifying strains that can be used as vectors for delivery of therapeutic molecules in the gastrointestinal tract.

**Paper II:** The development of a food grade expression system for production and delivery of antibodyfragments in the gastrointestinal tract.

**Paper III:** To construct a strain of *Lactobacillus* expressing single chain antibody fragments against the *Bacillus anthracis* protective antigen as a model system for gastrointestinal toxin neutralization.

**Paper IV:** To engineer *L. paracasei* BL23 for the expression of toxin neutralizing single domain antibodies as a therapeutic strategy against *C. difficile* associated diarrhea.

# 3 MATERIALS AND METHODS

## 3.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

*E. coli* DH5α (Invitrogen, Carlsbad, CA) was grown in LB media at 37°C with 220 rpm orbital shaking or on LB-agar plates at 37°C. Lactobacilli were grown in lactobacilli MRS broth (Difco, Sparks, MD) at 37°C without agitation or anaerobically on MRS-agar plates (BD - GazPak EZ, Sparks, MD). Antibiotics were added at the following concentrations when indicated: ampicilin (100 μg/ml) and erythromycin (300 μg/ml *E. coli* and 5 μg/ml lactobacilli).

**Construction of recombinant *Lactobacillus* Strains**

Three expression plasmids, (pAF100, pAF400 and pAF900) were utilized for therapeutic expression of antibody fragments in the current studies. Directing the expression towards secreted, secreted and attached or cell wall anchored display respectively. For construction of plasmids please se detailed description in paper II. The DNA fragment containing the antibody fragment for cloning into the expression plasmids was excised using NcoI and NotI restriction enzymes (Promega) and ligated into the NcoI/NotI digested *Lactobacillus* expression vectors, pAF100, pAF400 or pAF900. The correct sequence of the expression cassette was verified by sequencing. The expression plasmids were transformed into *L. paracasei* BL23 (previously known as *L. casei* or *L. zeae* ATCC 393 pLZ15- (246)) by electroporation as previously described [23,33]. Thereby, generating the *Lactobacillus* strains expressing the antibody fragment either anchored, secreted or attached. For detailed description of how the respective constructs were generated please consult the respective papers where complete lists of plasmids generated also can be found.

## 3.2 EXPRESSION ANALYSIS

**Western Blot**

The transformants were grown in MRS with 5 μg/ml erythromycin until an OD600 of 1.0. The cultures were centrifuged at 3,200 × g to separate the pellet from the supernatant. The supernatant was filter sterilised, pH adjusted to 7.0, dialysed against 10 mM Tris (pH 8.0) and concentrated using Amicon Ultra-4 centrifugal filter units (10 kDa cut off, Millipore, Carrigtwohill, Co. Cork, Ireland). The concentrated supernatant was mixed with 2 × Laemmli buffer and boiled for 5 minutes (min). The cell culture pellet was washed twice with PBS, resuspended in 100 μl Laemmli buffer and boiled for 5 min. The cell extract was centrifuged at 16,000 × g to remove cell debris and the supernatant containing soluble proteins was kept. The supernatant and cell extract were run on a 10% SDS-polyacrylamide gel at 170 volts and the proteins were transferred onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare, Little Chalfont,

Buckinghamshire, UK). The membrane was blocked with PBS-T (PBS with 0.05% (v/v) Tween 20 + 5% (w/v) milk powder) and successively incubated with mouse anti-E-tag antibodies (1 µg/ml, GE-Healthcare) and HRP (horse radish peroxidase) labelled goat anti-mouse antibodies (DAKO A/S, Glostrup Denmark). The signal was detected by chemiluminescence using the ECL Plus™ Western Blotting detection system (GE Healthcare).

**Enzyme-Linked ImmunoSorbent Assay (ELISA)**

96 well microtiter plates (EIR/RIA plate, Costar, Lowell, MA) were coated with 100 µl antigenat 1 µg/ml in PBS overnight (o/n) at 4°C. Plates were subsequently blocked with 200 µl 1% BSA (in PBS containing 0.05% Tween 20, PBS-T) for two hours at 4°C. After washing with PBS-T, dilutions of *Lactobacillus* culture supernatants were added and the plates incubated at 4°C o/n. Plates were subsequently washed three times and 100 µl mouse anti-E-tag antibody (GE-healthcare) was added (1 µg/ml) in blocking solution, followed by incubation at room temperature for 2 h. Plates were then washed three times in PBS-T and incubated with 100 µl AP conjugated rabbit anti-mouse antibody at 1/1000 (Dako A/S, Glostrup Denmark) in blocking solution. Following an additional 1 hour incubation at room temperature, the plates were washed twice in PBS-T and once in PBS, resuspended in 100 µl of diethanolamine buffer (1M, pH 10.0) containing 1 mg/ml pNPP (Sigma-Aldrich, St. Louis, MO) and absorbance was read after 10-30 min at 405 nm in a Varioskan Flash (Thermo Scientific, Waltham, MA). General protocol, for variations see respective papers.

**Flow cytometry**

50 µl of *Lactobacillus* cultures grown to an OD600 of 1.0 in MRS were harvested by centrifugation (8000 rpm, 1 min) and washed three times in PBS. Bacteria were resuspended in 50 µl PBS with 1% BSA (PBS-BSA) and incubated for 30 min on ice sequentially with 50 µl anti-E-tag antibody (10 µg/ml) and 50 µl FITC conjugated anti-mouse immunoglobulins (diluted 1/100) (Jackson Immunoresearch Laboratories, West Growe, PA), all diluted in PBS-BSA. Bacteria were washed with 500 µl PBS between all both incubations. Samples were resuspended and fixed in 300 µl 2% paraformaldehyde in PBS and analysed using a FACS Calibur machine (Becton Dickinson, Franklin Lakes, NJ). Protocol for detection of antibody fragments on the bacterial surface. For other protocols please consults respective papers.

## 3.3 PHENOTYPIC TESTING

**Auto-Aggregation**

Auto-aggregation assay was performed according to Pascual et al. (247) with certain modifications. Lactobacilli were grown for 48 h at 37°C on MRS agar (Oxoid) plates in microaerobic environment (10% CO2). A loopful (10 μl) of culture was suspended on a glass microscope slide in 1 ml of 0.9% saline solution (pH 6.7) to a final concentration that corresponded to McFarland Nephelometer Standard 3. Auto-aggregation was determined as the ability to form aggregates (clearly visible sand-like particles) within 2 min at room temperature. The results were expressed as: score 0, no auto-aggregation; score 1, intermediate auto-aggregation (presence of some flakes); and score 2, strong auto-aggregation.

**Acid, Bile and Pancreatin Tolerance**

The effect of low pH, bile, and pancreatin on the survival of lactobacilli was examined in microwell plates (Costar® 96 Well Cell Culture Clusters, Myriad Industries, San Diego, CA). MRS broth (Oxoid) was adjusted to a pH range between pH 5.0 and pH 2.0 to test acid tolerance and contained oxgall (2% w/v) (Sigma, Steinheim, Germany) and pancreatin (0.5% w/v) (Sigma) to test bile and pancreatin tolerance. Each 180-μl volume of adjusted and non-adjusted MRS broth (as control; pH 6.0) was inoculated with 20 μl of suspension of lactobacilli (McFarland 1.0 turbidity standard) and incubated in microaerobic environment at 37°C for 4 h. The number of cells in the suspension of lactobacilli (CFU ml−1) and the number of surviving cells following incubation in pH-, bile- and pancreatin-adjusted media was determined by plating 100 μl of tenfold serially diluted sample onto the MRS agar (228, 247). Strains with viable cell counts equal to viable counts before incubation in pH-, bile- and pancreatin-adjusted media were considered as resistant to a particular pH, bile and pancreatin concentration.

**Antibiotic Susceptibility**

Minimum inhibitory concentrations (MICs) of 13 antibiotics were determined by E-test method. Wilkins-Chalgren (Oxoid) agar plates with 5% horse blood, E-test antibiotic strips (AB Biodisk, Solna, Sweden) and 48 h of incubation at 37°C in an anaerobic glove chamber were applied. The breakpoints were determined in accordance with the CLSI guidelines for gram-positive microorganisms as follows: ciprofloxacin and rifampicin (4 μg ml−1); erythromycin (8 μg ml−1); ampicillin, imipenem, gentamicin and tetracycline (16 μg ml−1); cefoxitin, cefuroxime, vancomycin, chloramphenicol and metronidazole (32 μg ml−1); and trimethoprim-sulfamethoxazole (4/76 μg ml−1).

**Haemolytic Activity**

A single line of lactobacilli culture (grown in MRS broth (Oxoid) for 48 h) was streaked onto blood agar plates containing either human or horse blood. Haemolysis was evaluated following 24 and 48 h of incubation in aerobic, microaerobic (10% CO2) and anaerobic (90% N2, 5% CO2, 5% H2) environment. One Staphylococcus aureus strain (ATCC 25923) and two Streptococcus pyogenes strains (ATCC 19615 and a human clinical isolate) were used as positive controls.

## 3.4 PROTEIN PURIFICATION

scFv's purifification see paper III

VHH fragment purification se paper IV

Toxin fragments purification se paper IV

## 3.5 *IN VITRO* PROTECTION ASSAYS

**Macrophage toxicity assay to assess neutralisation by scFvs**

Protection by *Lactobacillus* and *E. coli* produced scFvs were analysed by their capacity to protect the J774 MΦ cell line from killing by LT (248). Briefly, J774 MΦ were added to 96-well, flat-bottom wells ($5 \times 10^4$ MΦ/well) and incubated at 37°C in 5% CO2 in air. After 12 hours of incubation, LT (i.e., 1 μg/ml rPA and 1 μg/ml LF, (List labs)) pre-mixed with scFvs were added to the cultures and incubated for an additional 12 hours. Viable MΦ were evaluated by colorimetric assay by reading absorption at 562nm after addition of Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) (249). MTT was used at a concentration of 5 mg/ml, and a volume of 20 μl (100 μg/well) was added to individual wells.

***C. difficile In vitro* toxin neutralization assay**

Neutralization of toxin A and toxin B by anti-toxin VHH was analyzed on the MA-104 cell line that have previously been characterized for sensitivity to *C. difficile* toxin A and toxin B. Toxin A and toxin B (List Biological laboratories) were titrated on cell line before use to adjust for batch variations and used at a 2-4 fold the killing dose unless otherwise stated. MA-104 were seeded at $1x10^5$ cells per well in a 96 well microtitre plate and incubated for 24 hours at 37°C, in 5% CO2 in DMEM GlutaMAXTM (Dulbecco's Modified Eagle Medium) (Life Technologies, Grand Island, NY) with 10% BFS (bovine fetal serum) reaching 70 – 80 % confluence. Toxin A and toxin B were mixed with VHH at varying concentrations in sera free DMEM and incubated on ice for

45 min. Cells were washed with serum free DMEM and overlaid with 100 µl VHH/toxin mix and incubated at 37°C for 24 hours in 5% CO2. Cytotoxic effect of non-neutralized toxins was scored microscopically as beginning to complete cell rounding. Complete toxin neutralization was characterized as visually undamaged cells.

Toxin neutralization by llama sera were tested in fourfold sera dilutions (1/100 – 1/12800) incubated with 10 ng/ml toxin B and 50 ng/ml toxin A. The sera-toxin mixes were overlaid on washed MA-104 cells and toxin neutralization scored as presence of undamaged cells after 24 hours incubation as described above.

Adsorption of toxin B by *Lactobacillus* cell wall displayed VHH was carried out by incubating twofold serial dilutions of *Lactobacillus* in DMEM ($8x10^9$ to $1.25x10^8$ CFU/ml) with a fivefold cytotoxic dose of toxin B (50ng/ml) under mild agitation at 37°C for one hour. Prior to incubation, *Lactobacillus* were washed three times in DMEM with 25mM hepes buffer to bring pH to 7.2. The DMEM buffer was supplemented with 50 units/ml of penicillin, 50 µg/ml streptomycin and 25 µg/ml gentamicin (all from Lifetechnologies, Grand Island, NY) to avoid bacterial growth. *Lactobacillus* and adsorbed toxin B were pelleted by centrifugation at 12000 rpm for 5 minutes and 100 µl of supernatant was transferred to each well of a microtitre plate with washed MA-104 cells. Cytotoxicity of the remaining toxin B in the adsorbed supernatant was recorded as described above.

## 3.6 *IN VIVO* MODELS

**Mouse safety model (Paper I)**

The animal trial was approved by the Ethic Committee on Animal Experiments of the Ministry of Agriculture of Estonia. Ten BALB/c mice (Scanbur BK AB, Sweden) were fed a mixture of six *Lactobacillus* strains (with each freshly cultured strain being present at a concentration of 107 CFU per daily dose) in their drinking water for 5 consecutive days. Throughout the trial, the animal's activity, behaviour and general health were observed daily. Five randomly selected mice were sacrificed on Day 5, and the other five mice on Day 15. Samples for histological and microbiological analyses were collected.

For histological analysis, tissue sections of liver, spleen, kidney and lungs of the sacrificed mice were fixed in 10% of formaldehyde and embedded in paraffin. The samples were stained with haematoxylin and eosin, and by using van Gieson method. Alterative and inflammatory changes in tissues were evaluated.

For microbiological analysis, heart blood (10 µl) and homogenized tissue of liver, spleen, kidney and lungs were plated onto blood agar and MRS agar (Oxoid). After 72 h of incubation in aerobic (blood agar plates) and microaerobic environment (MRS plates), colonies were enumerated and lactobacilli identified. *Lactobacillus* strains were typed by using arbitrarily primed polymerase chain reaction (AP-PCR) with three different primer sets: ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G -3′) (6), primer 5′-ACG CGC CCT-3′ (7) and primer 5′-ATG TAA CGC C-3′ (8).

***In vivo* neutralization of anthrax toxins (Paper III)**

Female C57BL/6 mice, six-seven weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and provided food and water ad libitum. All studies were performed in accordance with both National Institutes of Health and Institutional guidelines and approved by the Ohio State University Institutional Animal Care and Use Committee (Protocol number 2009A0210).

A dose study of the oral effect of ET was carried out on groups of mice challenged with 10, 25, 50 and 100 µg of ET (equal amount of rPA plus EF (List Labs)) given in 100 µl PBS by gavage. After 16 hours, the toxic effect was measured as ET induced fluid accumulation in the small and large intestine. Mice were euthanized with CO2 and death confirmed by cervical dislocation prior to removal of small and large intestine. Fluid accumulation was measured as percent of the weight of the small and large intestine compared to total body weight.

The KKA307, KKA308, KKA317 and *L. paracasei* pAF400 (3) strains were grown in MRS to an OD600 of 1.0, harvested by centrifugation and resuspended in culture supernatant with pH adjusted to 7.0 to give $5 \times 10^9$ cfu/ml. Nine-twelve weeks old C57BL/6 mice (body weight 15-20 g) were given $2.5 \times 10^9$ cfu recombinant *Lactobacillus* by gavage. Four hours later they were challenged with a non-lethal dose of 50 µg ET (50 µg rPA plus 50 µg EF (List Labs)) together with an additional $2.5 \times 10^9$ cfu recombinant *Lactobacillus* by gavage. After 16 hours, the toxic effect of the ET was measured as fluid accumulation in the small and large intestine.

**Prophylactic hamster model (Paper IV)**

Six weeks old male Syrian Golden hamsters were obtained from Harlan laboratories UK. Hamsters were housed individually under specific pathogen free conditions with commercial diet, R-70 (Lactamin, Sweden), and water ad libitum. Studies were

conducted according to guidelines of University of Tartu and approved by the Ethic Committee on Animal Experiments of the Ministry of Agriculture of Estonia.

Hamsters were treated with a single orogastric dose of clindamycin (30 mg/kg bw, Sigma Aldrich) to destabilize the intestinal flora 24 hours before a challenge by $10^3$ spores of a toxin $A^-B^+$ strain of *C. difficile* 630 (9). Prophylactic treatment with yeast produced anti-toxin B VHH started on the same day as clindamycin treatment and was continued for a total of seven days. One group of hamsters (n=6) received a mixed dose of 125 µg af each of the three yeast produced toxin B neutralizing VHH fragments (VHH-B2, VHH-G3 and VHH-D8) twice daily by gavage. The two control groups (n=6 each) received either 375 µg of an irrelevant anti-rotavirus VHH twice daily or no VHH.

In an identical prophylactic model, hamsters received *Lactobacillus* expressing cell wall anchored anti-toxin B VHH twice daily by gavage. The *Lactobacillus* strains KKA413, KKA416 and KKA101 were grown in MRS (Oxoid, UK) to an OD600 of 1.0, harvested by centrifugation and washed twice in PBS. Three groups of hamsters (n=6) received, by gavage, either: (I) twice daily $5x10^9$ CFU of each of the two strains of *L. paracasei* BL23 (KKA413 and KKA416) expressing the VHH-B2 and VHH-G3 cell wall anchored respectively; (II) twice daily $1x10^{10}$ CFU of a non-expressing strain of *L. paracasei* BL23 (KKA101) or (III) spores only.

Hamster activity, behavior and general health, including diarrhea and mortality were evaluated for the duration of the experiments. The hamster model was terminated on day five after the spore challenge to comply with the ethical permit and surviving hamsters sacrificed with cervical dislocation. Autopsy on sacrificed hamsters was performed under sterile conditions using a class II microbiological safety cabinet (Jouan, France).

For full protocol please consult paper IV

# 4 RESULTS AND DISCUSSION

Several of the criteria that have been outlined for characterization of new probiotic strains concerns viability, persistence and safety of the bacteria in the gastrointestinal tract. These traits would also be desirable for strains of *Lactobacillus* used to deliver passive immunity, as it would ensure a stable and persisting delivery system. In paper I, ninety-three human isolates of *Lactobacillus*, isolated from the fecal samples of Swedish and Estonian children, were characterized for markers of colonization, tolerance to gastric conditions, antibiotic resistance and potential adverse effects.

Aggregation is a marker that has frequently been used as an indicator of adhesive ability (205-207) prior to running assays on epithelial cells or mucosal sections. Fifty-five of the strains (59%) had an auto-aggregative ability with thirty of these being strongly aggregative. Strains with a positive aggregation profile were chosen as a basis for the seventy–six strains taken forward for tests on tolerance to gastric conditions. The remaining twenty-two strains were included from the facultative heterofermentative (FHEL) and obligatory heterofermentative (OBEL) lactobacilli to increase the diversity of the strains to and even out the number from the respective fermentative groups. Nearly all strains tolerated high bile concentrations (2.0 % w/v) and all were unaffected by high levels of pancreatin (0.5 % w/v), reflecting their gastrointestinal origin. Half the strains also tolerated incubation for up to four hours at a pH 3.0 or less as a test for resistance to gastric passage. Twenty four strains were taken forward for testing for antibiotic susceptibility, primarily based on aggregation profile and resistance to low pH as the two other markers provided little discrimination among the strains.

The selected strains were tested for resistance against 13 commonly used antibiotics. All of the tested *Lactobacillus* showed an intrinsic resistance to metronidazole and the majority of the strains were resistant to cefoxitin (14 of 24 strains), vancomycin (12 strains), ciprofloxacin (13 strains), and trimethoprim-sulfamethoxazole (16 strains). This is similar to what has previously been reported and could suggest a high natural resistance towards these antibiotics (208, 209). Plasmid-encoded erythromycin, tetracycline and chloramphenicol resistance has been reported in lactobacilli and are a reason for growing concern (210-212). Based on a phenotypic test it is not possible to confirm the presence or absence of transferrable resistance genes as the origin of the antibiotic resistance. However, a high level of resistance that is not shared within the

species should be a reason for worry and warrants further studies of the origin of resistance. One strain of *L. buchneri* showed resistance to tetracycline and was excluded from further studies. The issue of antibiotic resistance within probiotic strains is two sided. The general notion is that strains with antibiotic resistance located on mobile elements should be avoided when selecting probiotic strains, both to avoid the risk of transfer of resistance to gastrointestinal pathogens, but also as antibiotic treatment would be the safeguard against, however rare, translocation of probiotics resulting in adverse effects. However, in cases where the resistance is through mutations or intrinsic resistance mechanisms, these strains could potentially aid the re-colonization of the gut after antibiotic treatment due to their natural resistance to the used antibiotic.

Antagonistic effect towards pathogens is often a parameter when selecting prospective probiotic strains. In this study, this was not analyzed as the primary objective was to select strains that could be used for therapeutic expression against multiple targets. A previous screen on strains used in this study have shown that individual strains inhibited growth of potential gastrointestinal pathogens (213). For optimal therapeutic effect against a specific pathogen, a desirable synergistic effect could possibly be achieved by using a strain with antagonistic activity as vector for delivery of the therapeutic molecules.

To test the strains for their capacity for storage, the ten strains with the highest overall score in the previous tests were subjected to lyophilisation. Six strains, *L. gasseri* E16B7, *L. gasseri* 177, *L. paracasei* 317, *L. paracasei* 1-4-2A, *L. fermentum* 338-1-1 and *L. acidophilus* 821-3, all yielding at least 1010 CFU/g, were taken forward for safety studies in mice as the last parameter of selection.

Oral administration for five days with a mixed dose of the six strains (107 CFU/day of each) caused no adverse effects on the general health and activity of the treated mice. The heart blood, liver, kidney and lung samples obtained by autopsy on day five all showed no translocation of the lactobacilli. For one mouse, the spleen culture was positive and showed growth of both *L. paracasei* and *L. plantarum*. The *L. paracasei* was confirmed to be the strain 1-4-2A and this strain was thus discarded due to its potential for translocation. For the *L. plantarum* isolate, the AP-PCR typing showed no similarity to the fingerprint patterns of any of the six strains and had likely originated from the indigenous flora of the mouse. Generally, translocation of probiotic *Lactobacillus* in humans is rare and in the cases it does occur, the effects are rarely detrimental. Due to a long history of safe use, *Lactobacillus* has earned a status as

GRAS for consumption. However health damaging effects have been observed in some immunocompromised patients and suggests caution when selecting strains for therapeutic use (214). This highlights a duality where some probiotics have been shown to improve the barrier function (215-217) whereas others, in the case of underlying disease, have shown an ability to translocate (214).

The five final strains from the screening described here were taken forward to a second step where they were tested for safety and persistence in the gastrointestinal tract of human volunteers (218).

The production and delivery of therapeutic components by engineered lactobacilli is to a large extent dependent on the expression system applied. Several criteria need to be met for an expression system for optimal delivery of therapeutic proteins in the gastrointestinal tract: flexible targeting of proteins, high level of expression, lack of antibiotic markers and stable expression. In paper II, a flexible expression system that facilitates chromosomal integration was developed for expression of antibody fragments and delivery of passive immunity. The expression system was based on the transcriptional framework of the aggregation-promotion factor (*apf*) gene from *L. crispatus* M247 (219). The *apf* gene encodes a cell surface gene attached non-covalently to the cell wall (220) that was initially thought to be connected with aggregation (221) but later shown to be involved in maintenance of cell shape (222). A series of translational fusions with a scFv antibody fragment against the SAI/II adhesion of *Streptococcus* mutants and the apf gene was constructed to optimize the level of expression, secretion and localization of the scFv. Eight constructs were made with the apf gene together with an additional three constructs including a C-terminal PrtP anchoring domain (40) (For details see paper II).

Three constructs, pAF100, pAF400 and pAF900 resulted in markedly different localization, with the scFv being produced as a secreted protein, a covalently cell wall-anchored protein or both secreted and cell wall-attached protein respectively. These constructs were selected for delivery of passive immunity in subsequent studies. The choice of delivery and display for therapeutic molecules from lactobacilli depends to a vide extent on both the target and the nature of the therapeutic molecule. For delivery of passive immunity, both secreted and cell wall anchored display of antibody fragments have been applied. The majority of the studies have used a cell wall anchored display which has been shown to be successful against rotavirus (VHH) (41, 51) and *Streptococcus mutants* (scFvs and VHH) infection (40, 47, 50). For secreted production,

functional scFvs have been produced in *Lactobacillus* against rotavirus (41, 49) and HIV (223) but the only secreted antibody fragments tested in an animal model so far, a VHH against rotavirus, failed to provide protection (41). Both methods of delivery should have the potential to deliver a passive immunity but at the moment only cell wall-anchored display have been functional *in vivo*.

For therapeutic applications, chromosomally integrated expression constructs are considered a prerequisite. The primary reason is to reduce the risk of horizontal transfer of either the antibiotic marker or the recombinant gene. In addition, it also significantly improves the stability of the expression constructs and obviates the need for either antibiotic of autotroph markers to maintain the plasmid in the *Lactobacillus*. To generate stable food grade strains devoid of antibiotic markers, the expression cassettes were inserted into the chromosome of *L. paracasei* BL23 using the phage A2 integrase (paper II). In a subsequent step, the non-food grade DNA, including the antibiotic marker, was excised using a β-recombinase, leaving only the integrated expression cassette on the chromosome. The strain with the chromosomally integrated cassette producing surface anchored VHH (ARP1) (L. paracasei EM233) was shown to be stable for more than 50 generations. A significant improvement compared with previous studies where a secreted scFv produced from the non-integrated plasmid pAF100 were lost after 15 generations upon removal of the antibiotic pressure (unpublished data). One of the challenges with chromosomal integrated constructs is the significant reduction in expression that can be expected when going from a multi-copy plasmid to a single chromosomally integrated gene (224). In the present study, only a 6 to 10 fold reduction in expression of the anchored and secreted construct was observed when integrated on the chromosome which is significantly less than expected considering that the pAF plasmids are derived from a the high copy number plasmid, pIAV7 (162 copies/cell) (225). However, it is highly likely that expression from a strong promoter, like the apf promoter, will encounter several bottlenecks within translation, secretion and anchoring of the proteins and therefore do not benefit from copy number in a linear fashion. Most important, the level of ARP1 antibody fragment displayed in *L. paracasei* EM233 was sufficient for the modified lactobacilli to reduce infection when tested in an animal model of rotavirus infection. The protection level was similar between the chromosomally-integrated and plasmid expression systems validating the use of the chromosomally-integrated system for subsequent applications.

Oral challenge with the toxins of *Bacillus anthracis* was chosen to explore the possibility of delivering passive immunity by engineered *Lactobacillus* against toxins in the gastrointestinal tract. Targeting the protective antigen (PA) of *B. anthracis* with antibodies, as a mean to prevent the assembly and uptake of the toxin complexes, neutralizes effectively both the lethal factor and the edema factor. Deriving single chain antibodies from neutralizing monoclonal antibodies is a way to obtain antibody fragments suitable for expression in bacterial expression systems.

Here, a dual strategy was applied using both a previously characterized scFv, 1H scFv, with neutralizing activity against PA of *B. anthracis* (226) as well as a scFv constructed from a neutralizing anti-PA mAb. The fusion of the $V_H$ and the $V_L$ from the anti-PA mAb with a decapentapeptide $(Gly_4Ser)_3$ linker generated a scFv that could be expressed both secreted and anchored in *Lactobacillus* (data not shown). The mAb derived scFv could be displayed but bound with very low affinity towards PA compared to the mAb from which it was derived. This illustrates a commonly encountered problem when constructing a scFv from mAb, that despite maintaining the binding specificity, the scFv often has a reduced affinity compared to the parent mAb. This is primarily due to difficulties to obtain the correct conformational folding and stability of the scFv once the variable domains are removed from the structural support provided by the constant domains. Improvement of the affinity can be obtained by either mutational evolution or grafting the antigen specificity onto a variable domain framework providing better stability and folding (227). The other anti-PA scFv, developed through molecular evolution of a scFv with lower affinity (226), showed good high affinity when expressed in *L. paracasei* BL23 and was chosen as the basis for the rest of the work.

The scFv was cloned into the three previously constructed expression vectors for expression as a secreted protein, a cell wall anchored protein or a secreted and attached protein (referred to as attached construct). All three constructs directed the scFv to the expected cellular location and showed binding to PA. For secreted production, the highest level of scFv was found in the supernatant of the construct producing the scFv in the attached form which had three times higher levels than the secreted construct. This construct utilizes a non-covalent anchor and a significant proportion of the scFv is found in the supernatant, possibly due to either shedding from the cell wall or saturation of the binding sites. For the cell wall binding, both the anchored and attached construct displayed the scFv on the bacterial surface.

*In vitro* neutralization was tested with purified scFvs from the supernatant of the secreting and the attached construct. Both were able to protect macrophages from the cytotoxic effects of anthrax LT. The secreted scFv provided the best protection with only a 3.5 fold molar excess needed for complete for neutralization of the LT. The attached scFv needed a slightly higher dose, a 5-10 fold molar excess, to confer protection against the LT. This corresponds with the ELISA data, where a 3-fold higher production of the scFv in the supernatant of the secreted and attached construct shows a binding that is comparable to the secreted construct. This suggests that the secreted 1H scFv might have a slightly higher binding affinity than the 1H scFv produced from the attached construct.

The *in vivo* protection of three constructs was tested in a mouse model of prophylactic protection against an oral challenge with the *B. anthracis* edema toxin. Anthrax edema toxin causes a significant fluid accumulation in the small and large intestine leading to a 10-15% increase in total intestinal weight. The three expression constructs provide a choice for the mechanism of neutralization 1; anchored and attached scFv constructs immobilizing PA on the cell wall of the lactobacilli and clearing of bound PA from the intestinal tract by gastric emptying 2; secreted scFv expression as seen both using the secreted construct and the attached construct where a significant proportion of the scFv are non-cell wall attached, leading to diffusion of the neutralizing scFvs in the gastrointestinal lumen with subsequent binding and inactivation of PA. Mice treated with either *L. paracasei* BL23 expressing the 1H scFv in either anchored or secreted form failed to show any significant difference in fluid accumulation when compared to mice receiving only ET. For mice treated with *L. paracasei* BL23 expressing the 1H ScFv in the attached form, the intestinal weight was in the same range as the negative control and significantly lower compared to mice receiving ET only ($P<0.05$). These results indicate that attached scFv can prevent the uptake of the ET in the intestine by blocking the binding of the ET complex to the receptor. The reason that the attached construct provided protection when the secreted and anchored showed no significant effect, remains to be elucidated. In this case, it is particularly interesting as three constructs are producing the same anti-PA scFv and are transcribed from the same promoter. One explanation might be the dual function of the attached construct where the scFv is both cell wall displayed and secreted into the supernatant. The secreted part of the 1H scFv produced by the attached construct would have an unbound cell wall attaching domain, allowing it to re-attach to the cell wall of lactobacilli after binding to PA. This could theoretically provide a therapeutic advantage as the resident lactobacilli

in the gastrointestinal microbiota could function as a binding reservoir for the attached 1H scFv, mopping up and immobilising PA. We have shown in this paper that the re-attachment of the 1H scFv to L. paracasei is indeed possible, but further studies would be needed to determine if this is a parameter involved in the neutralization seen with the attached construct. Homologous binding domains are also found in several other Gram-positive bacteria so re-attachment could hypothetically involve other members of the gastrointestinal flora (219, 228). The protection by engineered *L. paracasei* expressing high affinity scFv against PAin the gastrointestinal tract provides an indication that infections with toxin producing gastrointestinal pathogens could be targeted successfully.

*C. difficile* is one of the most prominent toxin producing gastrointestinal pathogen and represents an obvious target for delivery of passive immunity against bacterial toxins. *C. difficile* produces two exotoxins, toxin A and toxin B, which are the causative elements of C. difficile associated diarrhea. Only one non-neutralizing scFv targeting toxin B has previously been produced (229). To generate neutralizing antibody fragments against both toxin A and toxin B, two llamas were immunized with inactivated toxin A and B. For both llamas, a good humoral response against both toxins were seen when testing the post immune sera at day 28. Two VHH specific phage libraries were constructed from the pooled peripheral blood lymphocytes and subsequently panned for toxin binding VHH. The isolated VHH showing best binding to either of the toxins were tested for their capacity to neutralize toxin in a cell based *in vitro* assay. Through several rounds of selection, four VHH neutralizing toxin B were isolated, VHH-B2, VHH-E2, VHH-G3 and VHH-D8. For the toxin A, fifteen unique anti-toxin VHH were isolated and tested in the *in vitro* neutralization assay. For all 15 VHH, a leaky protective effect were seen where the toxin was slowly released over time leading to killing of the cells. This transient protection could indicate that the binding affinity of the selected VHHs were either too low to compete with the toxin receptor or that not all of the relevant epitopes for preventing toxin processing were blocked. Interestingly, recent work on the receptor binding domain of toxin A has begun illuminating the structural base for *C. difficile* toxin neutralization. This work, conducted with anti-toxin A VHH fragments suggests that multiple epitopes on the receptor binding domain of toxin A are involved in toxin binding and neutralization (230). Two distinct epitopes were identified, with one of the epitope potentially repeated up to seven times in the highly repetitive structure of the toxin A receptor binding domain. Slight

sequence variations in the repeated epitope suggest that multiple antibodies could be needed for complete neutralization, an observation that helps to explain previous observations that using a mix of several antibodies binding to both non-overlapping and overlapping epitopes of toxin A can improve neutralization (231, 232). In the present study, no improvement in the toxin neutralization could be seen when using combinations of up to 11 of the selected anti-toxin A VHH, pointing again to the VHH either having too low binding activity or not blocking the relevant epitopes. It still leaves the question why no neutralizing VHH could be isolated despite that a high sera toxin neutralization titer was found in the llamas. One possible explanation could be the mixed composition of the llama sera with heavy chain only antibodies constituting 10-25 percent of the total IgG and conventional IgG making up the rest and providing the neutralization observed (233). Successful isolation of toxin A neutralizing VHH are possible though, as shown by a previous study, but could require a different immunization strategy, using for example only the receptor binding domain of toxin A for immunization (232).

However, several neutralizing anti-toxin B VHH were isolated and showed strong neutralizing activity against the dominant virulence factor, toxin B. The protective concentration of these for neutralizing 20 ng of toxin B *in vitro* ranged from 80-320 ng/ml to 5.12 µg/ml, which corresponds to 55-220 fold molecular excess of VHH to toxin B for complete neutralization by the best neutralizing VHH, VHH-G3. This protective range is comparable, or better, than previously isolated therapeutic anti-toxin B HuMAb tested for neutralization in a similar assay (116), suggesting that the anti-toxin B VHH isolated in the present study could be suitable for therapeutic use. The anti-toxin B VHH were cloned into the previously constructed expression plasmids for both secreted and cell wall anchored expression. The four VHH showed good expression with three of the four VHH (VHH-E2, VHH-G3 and VHH-D8), secreting between 1-2 µg/ml of VHH which is around 10-20 fold higher than what we generally can achieve with well-expressed scFv (234), illustrating the advantage of VHH for therapeutic expression in *Lactobacillus*.

Mapping of the four best neutralizing anti-toxin B VHH on the purified domains of toxin B revealed that all four bound to the cell wall binding domain thereby indicating that their neutralizing effect most likely arises by blocking the toxin binding to the receptor and preventing cellular toxin uptake. This is in line with previous observations that many *C. difficile* neutralizing antibodies appear to be directed towards the receptor

binding domain of the toxins despite immunizations having been conducted with the full length toxins (116, 118, 235-237). From a passive immunity perspective, antibodies binding to the receptor binding domain are desirable as they would prevent uptake of the toxin and the neutralized toxin would thus remain in the colon to be eliminated together with the feces.

Both the secreted and the cell wall anchored VHH expressed from *Lactobacillus* could provide protection in the *in vitro* neutralization assay, validating that the engineered strains were warrant testing in a hamster model of CDI.

A prophylactic oral treatment model was chosen for the current study as it would be the most likely application for *Lactobacillus* mediated toxin neutralization for treatment against *C. difficile* infection. Syrian golden hamsters were challenged with spores of a toxin $A^-B^+$ strain of *C. difficile* 24hours after disruption of the indigenous flora with clindamycin.
To establish the efficacy of toxin neutralization, the model was initially tested with a mixture of the three best *in vitro* neutralizing VHH (VHH-G3, VHH-D8 and VHH-B2), produced and purified from yeast. Surprisingly no protective effect was seen despite that high levels of anti-toxin B VHH were given continuously during the treatment (375 ug twice daily). The hamsters succumbed to the infection at the same rate as the control groups receiving either an irrelevant VHH preparation or *C. difficile* spores only.

To test if the lack of protection seen with the yeast purified VHH fragments could be overcome by *in situ* production of the toxin neutralizing VHH fragments, the hamster protection model was repeated with engineered *Lactobacillus* expressing toxin neutralizing VHH. With the yeast purified VHH fragments failing to provide protection at concentrations that exceeds what could likely be achieved by *L paracasei* BL23 secreting VHH fragments, it was decided to focus on toxin neutralization through a cell wall anchored display. Two strains of *Lactobacillus* displaying VHH fragments binding to non-overlapping epitopes (VHH-B2 and VHH-G3) were used in combination. Neutralization was tested in a prophylactic hamster protection model receiving a combined dose containing $5x10^9$ CFU of each of the two *Lactobacillus* strains, KKA413 and KKA416, twice daily for the duration of the experiment.

For the groups receiving spores only or treated with a non-expressing strain of *L. paracasei* BL23, the course of infection for all animals was brief with the hamsters succumbing to the infection within 24 hours, with vegetative C. difficile being detected

in their stool samples. For the hamsters treated orally with the two engineered strains of *L. paracasei* BL23 displaying VHH-B2 and VHH-G3, a delay in development of infection and partial protection against killing were on the contrary observed ($p<0.05$). Fifty percent of the hamsters in this group also survived until termination of the experiment at day 5 and showed either no damage or limited inflammation of the colonic mucosa despite having been colonized with *C. difficile* for up to 4 days. The complete absence or very limited mucosal damage in the hamsters despite having been colonized with *C. difficile* is significant, considering the rapid progression of the disease and the extensive damage to the colonic mucosa seen in *C. difficile* infections in hamsters. These results again suggest that the binding of the toxin to the cell wall displayed VHH fragments has a potential to efficiently neutralize the cytotoxic effects of toxin B.

That the hamsters receiving the *Lactobacillus* displaying the toxin neutralizing VHH conferred a protective effect in the hamster model when the yeast purified VHH fragments failed to have an effect, raises interesting questions. The dose of VHH administered to hamsters was 100-fold lower when using engineered lactobacilli compared to the purified VHH. Several non-mutually exclusive possibilities may explain why cell wall anchored expression of the VHH could be advantageous compared to the yeast purified VHH. The anchoring of the VHH on the surface of the *Lactobacillus* would hugely increase the footprint of the VHH when bound to the toxin and make a larger part of the receptor binding domain inaccessible for binding to the receptor. Likewise, the bound toxin would be less free to diffuse in the gastrointestinal tract when immobilized on the cell wall of the *Lactobacillus* and the close proximity of the VHH molecules on the cell wall could provide an avidity effect. The use of a mixture of two *Lactobacillus* expressing VHH antibody fragments binding two different epitopes could also contribute to increase avidity, promote agglutination and clearance of the toxins. Possible VHH proteolysis might also be overcome by the direct *in situ* production of the VHH fragment in the colon where the VHH would be replenished as long as the lactobacilli were growing vegetatively.

This study also seems to confirm a trend when using *Lactobacillus* for delivery of passive immunity where several studies have shown protective effect using anchored constructs *in vivo* (40, 41, 47, 50, 51). It is possible that anchored delivery of antibody binding domains is more effective for reasons outlined above but it could also be an artifact of the models used. When administering the engineered lactobacilli orally, the

supernatant containing the secreted antibody fragments are removed whereas the cell wall displayed are maintained on the surface of the lactobacilli. This gives an advantage when administering lactobacilli with cell wall displayed antibody fragments as they offer immediate protection whereas the protective effect of the secreted constructs require de novo production of the antibody fragments. In the describe studies, expression promoters that have been characterized for *in vitro* production have been used and though we expect them to be active *in vivo* their expression levels remains to be determined. Work has been undertaken to identifying promoters activated in the gastrointestinal tract and future expression constructs could likely be based on these (22).

# 5 CONCLUSIONS

The aim of this thesis has been to explore the possibilities for using engineered lactobacilli for delivery of passive immunity against pathogenic bacteria in the gastrointestinal tract. The conclusions drawn from the individual papers in the thesis are:

1. **From a collection of lactobacilli, five human intestinal *Lactobacillus* strains were found to have properties required for a potential probiotic, suggesting that they would be suitable as vectors for delivery of passive immunity in the gastrointestinal tract. (Paper I)**

2. **An integrative stable food grade expression system could be constructed based on the expression frame work of the apf gene of *Lactobacillus* crispatus. Furthermore, when expressing an anti-rotavirus VHH, the integrative expression system was equally protective as a plasmid based system in providing protection against rotavirus in a mouse pup model of infection.(Paper II)**

3. **Expression of a high affinity anti-anthrax PA scFv by engineered L. paracasei BL23 can provide in situ protection against anthrax lethal toxin in the gastrointestinal tract. (Paper III)**

4. **L. paracasei BL23 engineered for expression of a cell wall anchored anti-toxin B VHH could significantly delay and partly protect against a lethal challenge with spores of a toxin $A^-B^+$ strain of Clostridium difficile. (Paper IV)**

# 6 FURTHER PERSPECTIVES

The presented work has described the progress towards utilizing *Lactobacillus* for delivering passive immunity against gastrointestinal pathogens with a focus on the toxin producing pathogen *C. difficile*. It is the first example describing engineering lactobacilli for providing direct *in situ* toxin neutralization to prevent the cytotoxic effect of a toxin producing pathogen. Further work still needs to be carried out to bring this strategy to a stage where it can potentially be used as a supplement to the existing treatments against *C. difficile* infections. In the longer perspective there are also several other strategies that can be applied using engineered *Lactobacillus* for targeting *C. difficile* or other gastrointestinal pathogens.

For the current strategy to be relevant in a clinical setting, neutralization against toxin A will likely also be needed to achieve complete protection against CDI (116, 180). In the current study we were unsuccessful in isolating a neutralizing anti-toxin A VHH despite a high toxin neutralizing serum titer and extensive panning of the phage library. However, other studies have shown that it is possible to isolate toxin A neutralizing VHH (232) but it could potentially require another immunization strategy, using either the receptor binding domain of toxin A or non-inactivated toxin A.

As stated previously, chromosomal integration of the expression cassettes, removal of non-food grade DNA and inclusion of a containment system is today more or less a prerequisite for therapeutic application of engineered strains of *Lactobacillus*. This technology is currently available and can be easily adapted for the expression of VHH, as shown in paper II.

For optimal delivery of the VHH in the gastrointestinal tract, a strain with the ability to colonize for a prolonged period of time could potentially give a better and more durable protective effect. In the current study, we have used *L. paracasei* BL23 as a vector for delivery of the anti-toxin VHH. This is a laboratory strain that has been widely applied for expression of heterologous proteins due to its ease of manipulation. It has been connected with both probiotic effect (238, 239), adhesion to the mucus membrane (240) and shown to colonize the gastrointestinal tract in mice (241). However, regarding the ability to persist in the human gastrointestinal tract, there are other strains with more well documented colonizing characteristics that could be used for production of the

VHH, like *L. rhamnosus* GG (242). Bifidobacteria constitutes another group of gastrointestinal bacteria that could be utilized for optimizing the therapeutic delivery, being more abundant than *Lactobacillus* in the gastrointestinal tract and with higher numbers in the colon where *C. difficile* colonizes (243, 244). The tools for engineering of Bifidobacteria are steadily improving and engineering for therapeutic purposes are becoming a possibility.

In the current study, the anti-toxin VHH were raised against toxin A and B from the *C. difficile* VPI10436 strain. To ensure the best protective effect, either VHH with a broad toxin neutralizing activity against several toxinotypes should be selected or alternatively, multiple toxin neutralizing VHH should be used in combination. From a clinical point of view, VHH neutralizing the toxins from the hypervirulent PCR ribotype 027 strain would be especially relevant for delivering passive immunity.

The two most likely applications for engineered *Lactobacillus* as a mean to delivering passive immunity against CDI would be either prophylactic treatment or treatment against recurrent infections. Prophylactic treatment could be used for patients belonging to defined risk groups for contracting CDI prior to scheduled hospitalization. This treatment would ideally start before or at the time of hospitalization and continue throughout the stay. Treatment against recurrent infections could likewise be based on a predefined risk profile for contracting re-current CDI and given as follow up treatment once antibiotic treatment is terminated.

In our work we have directly targeted the two dominant virulence factors, toxin A and toxin B, to obtain the most significant effect. Several other approaches to engineering of *Lactobacillus* could also be used to target *C. difficile* to prevent the onset of CDAD either used on its own or in combination with strains of *Lactobacillus* producing toxin neutralizing VHH.

*Blocking of adhesion*

Expression of antibody fragments against *C. difficile* cell surface proteins (245) could be used to prevent the adhesion of *C. difficile* to the mucosa. Expressed as cell wall anchored VHH in *Lactobacillus,* they could both prevent adhesion of *C. difficile* to the mucosa and, possibly even form large aggregates that would promote clearance of the bacteria from the gastrointestinal tract.

*Utilizing phage endolysins*

Endolysins are phage encoded enzymes that towards the end of the lytic cycle are produced together with a holin to release the assembled phage particles by lysis of the bacteria. The holing forms large pore structures in the inner cell wall giving the endolysin access to reach and degrade the peptidoglycan layer, leading to lysis of the cell. The endolysin is a two domain protein with a C-terminal binding domain responsible for the binding to the peptidoglycan and an N-terminal catalytic domain responsible for the cleavage of the peptidoglycan bonds. They are to a wide extend, species or subspecies specific with the specificity being defined by the binding domain. Supplied externally, they can target Gram-positive bacteria directly without the need for the holin due to the lack of an outer membrane in Gram-positive bacteria. An endolysin cloned from a *C. difficile* bacteriophage produced and secreted from *Lactobacillus* could thus be used to directly target and lyse *C. difficile* in the gastrointestinal tract.

*Inhibiting the growth of C. difficile by production of bacteriocins*

Bacteriocins are produced by bacteria to prevent the growth of closely related species. Engineering of *Lactobacillus* for expression of bacteriocins could help to prevent the outgrowth and colonization of *C. difficile* in the gastrointestinal tract in individuals where the indigenous flora is damaged by antibiotic use.

*Toxin receptor blockage*

As well as neutralizing the toxins with *Lactobacillus* produced VHH, the toxin binding receptor could be targeted by expressing the binding domains or peptides containing the binding epitopes of the toxins. It has previously been shown that the C-terminal receptor binding domain from toxin A can effectively compete for binding to the toxin receptor and prevent the cytotoxicity of toxin A (235). This strategy would be difficult to combine with the expression of toxin neutralizing VHH and would need to stand alone or be combined with another approach.

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