From THE DEPARTMENT OF LABORATORY MEDICINE
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LACTOBACILLUS BASED ORO-MUCOSAL THERAPIES AGAINST ROTAVIRUS

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Cover Image: A color enhanced scanning electron micrograph of rotavirus (blue) bound to lactobacilli expressing anti-rotavirus VHH fragments (brown).

The right questions are half the battle in life

- Kena Upanishad

ABSTRACT

Rotavirus is the most important agent for severe infantile diarrhea, responsible for over 2 million diarrhea episodes and 600,000 deaths annually, mainly in the developing countries. Introduction of successful vaccine programs could help reduce the burden of rotavirus disease. However, at a deplorable rate of 70 children dying every hour there is an urgent need of finding alternative treatments. Passive immunization with orally delivered protective antibodies provides immediate protection against rotavirus. Over the last few decades the role of probiotics especially from *Lactobacillus spp* in managing rotavirus diarrhea has also been increasingly recognized. The objective of this work has been to optimize oral delivery of passive immunity against rotavirus using antibodies directly or through genetically engineered lactobacilli that express antibody fragments.

Oral combination treatment of different lactobacilli with bovine anti-rotavirus antibodies was evaluated in mouse pups challenged with rotavirus (paper I). *Lactobacillus rhamnosus* GG, a well known probiotic was found to synergize with antibodies and helped in early recovery from diarrhea in mice while saving up to 90% of antibodies. These components do not require special storage conditions and could be used to complement existing therapies.

Members of the family *Camilidae* express unconventional IgG antibodies composed of only heavy chains. The variable part of these antibodies (VHH) is composed of a single polypeptide and has remarkable thermo- and acid-stability allowing its use in the gastrointestinal tract. A phage display library of llama VHH fragments against rotavirus was constructed. VHH fragments were selected by stringent panning and were expressed in yeast. Purified VHH proteins were tested in mouse pups challenged with rotavirus. A VHH fragment with high neutralizing activity *in vitro* and ability to reduce diarrhea *in vivo* was identified (paper II).

Genetically engineered lactobacilli that can sustain continuous *in situ* production of antibodies are suitable candidates for delivery of passive immunity against rotavirus. The VHH fragment selected in paper II was expressed in *Lactobacillus paracasei* both in cell surface anchored and in secreted forms. VHH fragments produced by lactobacilli conferred significant reduction of infection in cell culture. Oral delivery of lactobacilli expressing cell surface anchored VHH fragments alleviated diarrhea symptoms and reduced viral load in the intestine. Genetically engineered lactobacilli expressing functional VHH fragments may thus form the basis of a novel form of therapy against rotavirus (paper III). Targeting a single epitope has limitations in terms of cross-reactivity to the circulating serotypes of virus and typically requires high amounts of antibodies for neutralization. Therefore, two VHH fragments (VHH1 and VHH3) that recognize unique epitopes on rotavirus and in combination synergistically reduce infection were expressed in *L. paracasei* as cell surface anchored dimers. The VHH3-VHH1 dimer expressing lactobacilli had stronger affinity for rotavirus than lactobacilli expressing monomers. These lactobacilli reduced diarrhea symptoms when administered to mice challenged with rotavirus. Thus VHH fragments can be used to build modular designs that can be expressed by lactobacilli (paper IV).

LIST OF PUBLICATIONS

I. **Effective prophylaxis against rotavirus diarrhea using a combination of *L. rhamnosus* GG and antibodies.**
Pant N., Marcotte H., Brüssow H., Svensson L., Hammarström L.
BMC Microbiology. 2007, 7(1):86.

II. **Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments.**
van der Vaart J.M., **Pant N.**, Wolvers D., Bezemer S., Hermans P.W., Bellamy K., Sarker S.A., van der Logt C.P.E, Svensson L., Verrips C.T., Hammarström L., van Klinken B.J.W.
Vaccine. 2006, 24: 4130-4137.

III. **Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus induced diarrhea.**
Pant N.*, Hultberg A.*, Zhao Y., Svensson L., Pan-Hammarström Q., Johansen K., Pouwels P.H., Ruggeri F.M., Hermans P., Frenken L., Boren T., Marcotte H., Hammarström L.
The Journal of Infectious Diseases, 2006, 194: 1580-1588.

IV. **Protection against rotavirus-induced diarrhea by dimerized lactobodies.**
Pant N., Marcotte H., Hermans P., Bezemer S., Frenken L., Johansen K., Hammarström L.
Submitted, 2008.

CONTENTS

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	Basepair
CDR	Complementarity determining region
CFU	Colony forming unit
DLP	Double layered particle
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric Nervous System
FFU	Focus forming unit
GI tract	Gastro-intestinal tract
GRAS	Generally regarded as safe
GMO	Genetically modified organism
HBC	Hyper-immune bovine colostrum
Ig	Immunoglobulin
IS	Intussusception
kDa	kilo Dalton
LAB	lactic acid bacteria
LPS	Lipo-polysaccharide
MRS	Mann Rogosa Sharpe lactobacilli media
NSP	non-structural protein
ORT	Oral rehydration therapy
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RRV	Rhesus rotavirus strain RRV
scFv	single-chain variable antibody fragment
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SIgA	Secretory IgA
SGLT	Sodium-Glucose linked transporter
TLP	Triple layered particle
VHH	variable heavy chain from *Camilidae*
VP	viral structural protein

1 INTRODUCTION

1.1 ROTAVIRUS AND INFECTIOUS INFANTILE DIARRHEA

Rotavirus is the single most important etiologic agent of severe diarrhea in infants and young children worldwide causing more than 111 million episodes of diarrhea annually. Regardless of the social and economic status, nearly all children will be infected with rotavirus by 5 years of age. Over 600,000 children die every year from rotavirus infection, primarily from developing countries, and many more have severe diarrhea that requires hospitalization, incurring direct and indirect costs of more than 1 billion dollars [1]. Given the severity and scope of rotavirus infection, there is an urgent need for an effective treatment.

In the developing countries infections with rotavirus happen throughout the year but in the temporal climate of the west, a peak in rotavirus infections is observed during the winter. Rotaviruses are primarily transmitted by the fecal-oral route, but unlike other bacterial and protozoan agents of diarrhea, improvement in sanitary conditions does not seem to protect against transmission of rotavirus. This could be both due to the small infectious dose (estimated to be less than 100 particles) and the recalcitrance of the rotavirus virions to inactivation in the environment. Once ingested, virus not neutralized by stomach acid attaches to the proximal small intestine and primarily infects the mature enterocytes on the tips of the villi. During the incubation period of 18-36 hours, the virus first produces a potent enterotoxin - NSP4 that can induce diarrhea and then go on to destroy the epithelial surface leading to blunted villi, extensive damage and shedding of massive quantities of virus (10^{12} particles per g) in stools. The outcome is profuse watery diarrhea with loss of fluids and electrolytes that can last 2-7 days and might lead to severe or fatal dehydration. Aggressive rehydration with oral or intravenous fluids can correct these imbalances and sustain a child until diarrhea stops.

1.2 ROTAVIRUS STRUCTURE

Rotaviruses are non-enveloped viruses with complex architecture and belong to the Reoviridae family. The viral capsid is composed of three concentric protein layers surrounding a genome of 11 segments of double-stranded (ds) RNA (Fig 1). The segmented dsRNA genome encodes 6 structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6). The outer capsid layer consists of VP4 and VP7, the intermediate layer is composed of VP6 and the viral core is made up of a shell protein VP2 as well as enzymes VP1 and VP3 (a guanylyltransferase and methylase). The viral core contains all of the enzymatic activities needed for synthesis of full length, capped mRNA transcripts of the 11 genome segments.

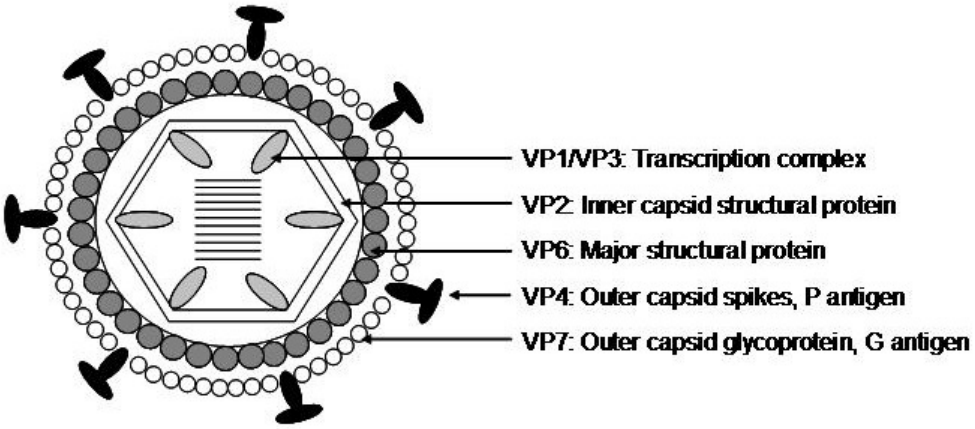

Fig 1. A schematic representation of a triple-layered mature rotavirus virion.

1.3 REPLICATION OF ROTAVIRUSES

The exact mechanism or the receptors by which rotaviruses gain entry into enterocytes is not known, however two opposing hypotheses have been proposed; through direct entry or fusion [2] and through Ca^{2+} dependent endocytosis [3]. Rotavirus entry is accompanied by the loss of the VP4 and VP7 outer layer, thereby converting triple layered particles (TLPs) to double-layered particles (DLPs). The DLPs contain the RNA dependent RNA polymerase enzyme (VP1 and VP3 complex) which functions as a transcriptase to synthesize the 11 viral plus-strand RNAs. The plus-strand RNAs are extruded from DLPs through channels at the vertices that extend through both the VP2 and VP6 protein layers. The plus-strand RNAs contain 5' caps but lack 3' poly(A) tails and are translated to give rise to six structural proteins (VPs) and six nonstructural proteins (NSPs). The plus-strand RNAs also function as templates for the synthesis of the dsRNA genome segments. RNA replication occurs concurrently with the packaging of the genome segments into newly formed cores and is coordinated such that the 11 segments are produced at equimolar levels. The localization of several viral proteins to viroplasms and the observation that subviral particles seem to bud directly into the endoplasmic reticulum (ER) indicate that the viroplasm are the site for assembly of DLPs. NSP4 and VP7 are synthesized on ribosomes in close relation to the ER and are co-translationally inserted into these membranes. The rest of the structural and non-structural proteins are synthesized on free ribosomes in the cytoplasm.

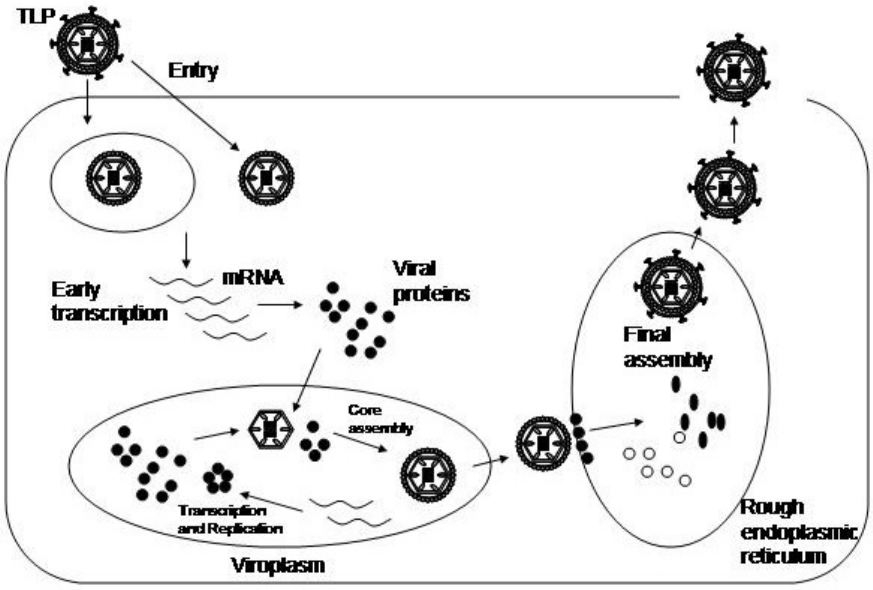

Fig 2. A schematic representation of replication of rotaviruses

1.4 COMMON SEROTYPES

Rotaviruses are divided into 6 distinct groups determined by the VP6 protein (A-F). Group A, B and C rotaviruses are those that are found in both humans and animals with Group A rotaviruses mostly infecting human infants. Upon infection, the outer capsid proteins VP7 and VP4 independently elicit the production of neutralizing antibodies and thereby determine virus G and P serotype respectively. There are currently 15 G serotypes and 26 P genotypes known [4] [5] of which 10 G types and 11 P types have been found in human infections. Because rotavirus proteins are encoded by different dsRNA segments that can reassort readily in doubly infected cells, theoretically this could lead to 110 different G and P combinations. Fortunately, this level of reassortment has not been seen and five G-P combinations (G1P[8], G2[P4], G3[P8], G4[P8] and G9[P8]) are commonly detected and account for more than 80 % of all human rotavirus infections globally [5]. However unusual serotypes of rotaviruses do emerge and are endemic in some areas mainly in the developing countries. In some areas of India, Brazil and Africa, G9[P6], G5, and G8 are more frequent than elsewhere [6] [7]. The extensive diversity of rotavirus strain types in developing countries may be related to more frequent genomic re-assortment as a consequence of a larger number of mixed infections and possibly, reassortment between human and animal rotaviruses [6]. The diversity among strains along with geographical and temporal variations and emergence of new strains potentially poses a challenge in the development of vaccines and other forms of immune therapy.

1.5 REASONS FOR DIARRHEA

The pathophysiological mechanisms underlying the fluid loss seen during rotavirus infection has been attributed to different causes. Some of the current hypotheses that have been proposed are-

1. Carbohydrate malabsorption
   Rotavirus infection decreases the expression of brush border disaccharidases such as lactase. The undigested sugars may induce an osmotic diarrhea.
2. Diminished absorptive capacity of the intestinal epithelium
   Atrophy of the mature enterocytes due to rotavirus mediated damage may result in the replacement of absorptive epithelium by 'crypt like' secretory epithelium creating an imbalance and resulting in net secretion [8].
3. Rotavirus enterotoxin
   The non-structural protein 4 (NSP4) of rotavirus as been implicated to be a viral enterotoxin. Intraperitoneal injection of the full length NSP4 or a 7 kDa cleavage product (which is also secreted by infected cells) induces diarrhea in newborn mice. Electrophysiological studies suggested that NSP4 potentiates chloride ion secretion by triggering a calcium-dependent signaling pathway. However, it remains to be established whether NSP4 from human rotavirus strains also functions as an enterotoxin [9].
4. Involvement of the enteric nervous system (ENS)
   The clinical picture of rotavirus disease in not limited to diarrhea but involves vomiting and nausea which indicates participation of the nervous system. According to this hypothesis, rotavirus or its products may directly activate the ENS leading to secretion of serotonin by the enterochromaffin cells in the crypts which causes vomiting and diarrhea [10].

It is likely that the fluid and electrolyte secretion caused by rotavirus is not explained by one single mechanism but is a multifaceted event. Each of these pathogenic mechanisms offers different avenues for the prevention or treatment of rotavirus diarrhea.

1.6 IMMUNITY AGAINST ROTAVIRUS

Despite three decades of research, the mechanism of immunity to rotavirus remains unclear. Repeated infections seem to build resistance; however, immunity after natural rotavirus infection is incomplete and may depend on the time between two exposures, the properties of the rotaviruses involved in those exposures and the immune status of the human host at the time of each exposure. The best protection has been observed in the developed countries, especially when re-exposure is to rotaviruses serotypically similar to those that caused the initial infection [11]. In developing countries where several G types circulate simultaneously, many children experience two or more episodes of rotavirus infections within the first year of life [12]. Rotavirus antibodies have been examined as a possible correlate of immunity following natural infection in many human studies and numerous associations have been reported. Local immunity in

the gut with the presence of neutralizing secretory IgA seems to be crucial for protection and accordingly, levels of fecal (copro) rotavirus IgA have been associated with protection [13] [14] [15]. Although, IgA deficient individuals do resolve rotavirus infection probably by compensating with higher serum titers of specific IgG antibodies [16], there might be other arms of the immune system that may also play a role in resolving infection. These effectors include natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), cytokines and other chemical mediators. CTLs have been shown to provide both active and passive immunity against rotavirus disease in animal models by helping in clearance of infected cells and thereby resolving infection [17].

VP6 is the most immunodominant protein in the viral capsid, however antibodies against VP4 or VP7 are likely to be more crucial in imparting protection at mucosal surfaces. It has previously been demonstrated that antibodies directed to VP4 prevent viral attachment to target cells thereby abrogating infection [18]. Antibodies against VP7 have been shown to prevent virion decapsidation and hence inhibit functional double layered particle (DLP) formation [19]. SIgA antibodies against VP6 are the most interesting as they have been shown to neutralize the virus intracellularly during transcytosis from the basolateral to the apical surface [20]. Antibodies against VP6 can also target more central aspects of rotavirus replicative cycle like inhibition of genome replication [21].

1.7 CURRENT TREATMENTS

The first rotavirus vaccine, RotaShield™, a tetravalent rhesus–human reassortant vaccine was licensed in the United States in 1998. However, within a year of licensure the vaccine was voluntarily withdrawn on grounds of temporal association with inducing intussusception (IS) [22]. The second generation of vaccines has undergone development and two live, oral, attenuated rotavirus vaccines were licensed in 2006: a live pentavalent human-bovine reassortant (RotaTeq™, comprising G1, G2, G3, G4 and P-type P1A[8]) and an attenuated human monovalent rotavirus vaccine (Rotarix®, comprising a single strain P1A[8],G1). Both vaccines have demonstrated good safety and efficacy profiles in large clinical trials in industrialized western countries and in Latin America [23] [24]. Careful surveillance has not revealed any increased risk of intussusception in the vaccinated groups with either vaccine. The new rotavirus vaccines are now being introduced for routine use in a number of industrialized and developing countries.

In the absence of specific antiviral therapy, Oral Rehydration Therapy (ORT) has served as a useful treatment that is rapidly distributed, does not require specific storage conditions and is cheap. However, even after achieving a substantial reduction in the mortality from dehydration, ORT has little or no effect on the course of diarrhea or nutritional morbidity. Oral rehydration solutions typically comprise of a low osmolarity mixture of glucose and sodium chloride that work by co-transporting glucose with sodium ion across the epithelia, leading to a net absorption of water. Interestingly, rotavirus infection downregulates sodium glucose linked transporter activity (SGLT), the transporter through which ORT helps in restoring fluid and electrolyte loss [25]. Additionally, rotavirus infection and diarrhea often induces emetic response, thereby making oral rehydration difficult. Other non-specific therapies tested, but not currently used, include anti-motility agents (e.g. loperamide), anti-secretory drugs (e.g. 5-

hydroxytryptamine3 (5-HT3)-receptor antagonist), anti-viral drugs, supplemental zinc therapy and probiotics [26].

1.8 PASSIVE IMMUNITY AGAINST ROTAVIRUS

Passive antibody therapy was the first consistently effective antimicrobial strategy and in the absence of specific treatments in the pre-antibiotic era, antibody therapies were developed against a wide variety of infectious diseases. The passive transfer of maternal secretory IgA (SIgA) through breast milk has important implications in the neonatal period for protection against a variety of infections, including rotavirus. The increased risk of contracting rotavirus infection in infants more than 6 months of age is at least partially related to weaning and the decline in maternal antibodies. Consequently, transfer of passive immunity against rotavirus through orally delivered immunoglobulins is a viable prophylactic strategy. Over the last two decades, oral administration of specific antibodies prepared against a variety of enteric pathogens, has been tested with various degrees of success, both in animal models and in humans [27], [28], [29]. Besides the use of human gamma globulin, antibodies against rotaviruses have been derived from heterologous sources such as bovine colostrum and chicken egg yolk [30], [31].

1.8.1 Bovine Antibodies

In nursing cows, antibodies are normally transported from serum to the colostrum to protect the neonate from infections. Immunization of pregnant cows with antigens of choice results in high concentrations of specific antibodies in the colostrum. A cow produces about 1.5 kg of antibodies in a few days after calving, thus making it an attractive source for high scale production of immunoglobulins. Such hyper-immune bovine colostrums (HBC) have been successfully used prophylactically against enterotoxigenic *Escherichia coli* infection and therapeutically against rotavirus [29] [30]. These antibodies have excellent stability profile and do not require special storage conditions once lyophilized. Even though treatment with bovine colostrum antibodies is highly effective, their wide-scale use is prohibitively expensive and therefore there is a need to find alternative sources of antibodies with inexpensive production costs.

1.8.2 VHH Antibody fragments

An alternative to using conventional immunoglobulins is the use of monovalent fragments derived from heavy chain antibodies found in *Camelidae* [32]. These antibody fragments (VHHs) are devoid of the light chains and constitute the smallest naturally occurring antigen-binding molecule known to date (Fig 3). VHHs exhibit several advantages over the analogous single chain fragments (scFvs) that are derived from conventional antibodies by linking the heavy and light chain with a peptide linker. VHH's are smaller, markedly more acid- and heat-resistant, and, as they are formed by a single polypeptide, easier to express in a recombinant form with an intact spatial structure [33], [34]. Additionally, VHHs have been shown to have longer projecting CDR loops allowing them to target cryptic immuno-evasive sites [35]. Expression of

VHH in yeast is highly efficient and results in the secretion of functional antibody fragments (VHH) in the growth medium [33]. Several VHHs are now being studied for use in various areas, including infectious diseases [34]. Given their excellent stability over a wide range of physical conditions, VHHs are well suited for use in the gastro-intestinal tract.

The simplicity and small size of VHH fragments makes it not only possible to express them at high levels in bacteria like lactobacilli but also to build modular designs that incorporate VHH fragments with complementing functions. Dimeric VHH fragments have already been generated against TNF, a trimeric molecule, thereby increasing the avidity 500 fold compared to a monovalent VHH [36].

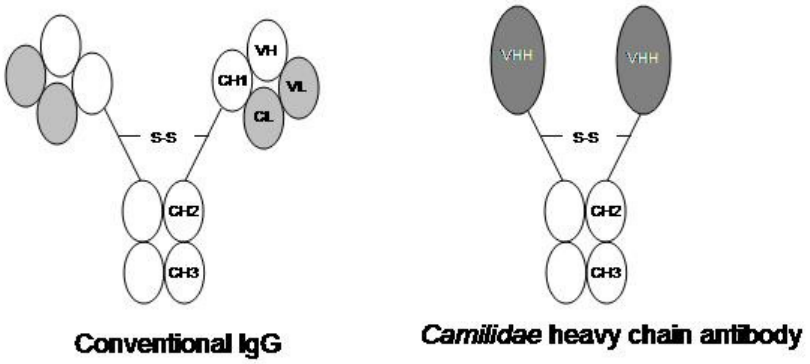

Fig 3. The variable region of a *Camilidae* heavy chain antibody is a single polypeptide (VHH).

1.9 PROBIOTICS

There is a currently growing appreciation for the potential for commensal and mutualistic organisms to influence host health. The concept that certain microorganisms, when supplied in sufficient quantities, confer direct benefits to the host is scientifically proven and probiotics are getting recognition.

Most probiotics in use today belong to the genus *Lactobacillus* and/or *Bifidobacterium*. Lactobacilli are characterized by their production of lactic acid and are predominant participants in many industrial and artisanal plants involving meat and dairy fermentations. In addition, lactobacilli are indigenous inhabitants of the human gastro-intestinal (GI) tract and are thought to be the dominant colonists of the small intestine. Correspondingly, the GI tract is the site where the probiotics are believed to perform most consumer-health modulating activities, although probiotic applications at other locations of the body (e.g. respiratory, subcutaneous, and vaginal) have also shown promise. The mechanisms by which probiotics beneficially affect human health are typically divided into one of a number of general categories, including strengthening of the intestinal barrier, modulation of the immune response [37], and antagonism of pathogens either by the production of antimicrobial compounds [38] or through competition for mucosal binding sites. In case of rotavirus infection,

compensation of the brush border lactase deficiency by bacterial lactase may also alleviate symptoms of diarrhea. Primary clinical interest in the application of probiotics has so far been in the prevention or treatment of infectious diseases including bacterial and viral-associated diarrhea [39], [40], [41]. Several randomized control trials have explored the use of probiotics, mainly from the genus *Lactobacillus*, for the management or treatment of rotavirus diarrhea [42]. The consensus that has so far emerged regarding the use of probiotics in children suffering from diarrhea is [43]

1. Administration of probiotics to children with acute diarrhea in developed countries is safe and reduces diarrhea duration by 24 hrs.
2. The effect is seen particularly in young children and is more pronounced if probiotics are administered early in the course of illness.
3. The single probiotic most consistently effective is *Lactobacillus rhamnosus* GG
4. The efficacy of probiotics is evident in viral diarrhea (and especially in infections by rotavirus).

1.9.1 Lactobacilli as antibody delivery system

Most pathogens that cause disease do so by first establishing local sites of initial infection mostly at the mucosa of the lungs or the intestines. Full blown infections and systemic spread of a pathogen usually results when this initial build-up of inocula at local sites goes unchecked. Therefore a strategy that precludes the establishment of localized site of pathogen multiplication will be ideal for mitigating a variety of mucosal infections.

Lactobacilli that are normal inhabitants of the human oro-gastrointestinal tract are ideal candidates to achieve this purpose. Their long history of safe use in the food industry has led to their status as Generally Regarded As Safe (GRAS) microorganisms. This GRAS status has led to reports in which live lactobacilli were suggested as carriers for different biomolecules and vaccinations [44]. Most lactobacilli are acid resistant and certain strains are able to survive through the stomach. Since they lack LPS, there is no risk of endotoxic shock. For use as a vaccine carrier, a suitable strain of lactobacilli should be non-pathogenic and genetically amenable for modifications. In addition, it should be a good colonizer of the mucosal site in question i.e. it should be able to adhere to the mucosa and persist for certain amount of time. Genetically engineered lactobacilli, expressing antibody fragments against different mucosal pathogens can help build up the arsenal against invading pathogens. Lactobacilli expressing antibody fragments have been used to successfully deliver passive immunity against *Streptococcus mutans* [45], rotavirus [46] and *Porphyromonas gingivalis* [47]. Since under natural conditions, infections tend to be initiated by relatively small inocula of a pathogen, whether inhaled or ingested, even a small population of resident lactobacilli expressing specific antibody fragments can achieve the objective of abrogating infection.

2 AIMS

2.1 GENERAL AIM

The aim of these investigations has been the development of lactobacilli expressing antibody fragments against rotavirus as a system for oral delivery of passive immunity against rotavirus-induced diarrhea.

2.2 SPECIFIC AIMS

1. Evaluation of therapeutic potential of different probiotic bacteria and their combination with polyclonal anti-rotavirus antibodies for the treatment of rotavirus diarrhea.
2. Evaluation of yeast produced anti-rotavirus monovalent llama VHH antibody fragments for treatment of rotavirus diarrhea.
3. Generation of lactobacilli expressing anti-rotavirus llama VHH antibody fragments against rotavirus.
4. Generation of lactobacilli expressing multimerized llama VHH fragments against rotavirus.

3 MATERIALS AND METHODS

Details concerning the different methods can be found in the papers I –IV.

3.1 STRAINS OF LACTOBACILLI AND GROWTH CONDITIONS

Four different lactic acid bacteria were obtained from Nestec, Nestlé, Lausanne, *L. casei* strain NCC 2461 (ST11), *L. rhamnosus* strain GG (ATCC 53103), *L. johnsonii* strain NCC 533 (La-1), *L. rhamnosus* strain NCC 596 and *Streptococcus thermophilus* strain NCC 2496. The *L. reuteri* strain ATCC 55730 (SD2112) was obtained from Biogaia, Sweden (paper I).

Lactobacillus paracasei (previously named *L. casei* 393 pLZ15^{-}) [48] was obtained from Peter Pouwels (TNO Institute, the Netherlands). This bacterium has been used for expression of antibody fragments in papers III and IV. Lactobacilli were reconstituted and cultured in MRS broth (Difco, Sparks, MD, USA) in standing aerobiosis conditions at 37º C.

3.2 ANTI-ROTAVIRUS HYPERIMMUNE BOVINE COLOSTRUMS ANTIBODIES

The Hyperimmune Bovine Colostrum (HBC) used was produced by vaccination of pregnant cows in a Swiss dairy farm with human strains of rotavirus, i.e. Wa, RV3, RV5 and ST3, representing serotypes 1 to 4. The preparation and the antiviral activity of the HBC concentrate is described in detail elsewhere [30]. The freeze-dried anti-rotavirus HBC concentrate was stored at room temperature and used in paper I.

3.3 GENERATION OF LLAMA VHH LIBRARY AGAINST ROTAVIRUS STRAIN RRV

The llama VHH library against RRV was generated in paper II. A llama (*Llama glama*) was immunized subcutaneously and intramuscularly at day 0, 42, 63, 97 and 153 with $5x10^{12}$ pfu of rhesus-monkey rotavirus serotype G3, strain RRV. Animal experiments were performed under the supervision of the animal experiment committee at ID Lelystad as described before [33]. The immune response was followed by titration of serum samples in ELISA with RRV rotavirus coated at a titer of $4x10^{6}$ pfu/ml in 0.9% NaCl following the protocol described before [33], [49]. Total cellular RNA was isolated from an enriched lymphocyte population and first strand cDNA synthesis was performed using random hexamer primers. VHH genes were amplified by PCR using specific primers. The amplified products were cloned in a phagemid vector (pUR5071). Rescue with helper phage VCS-M13 and PEG precipitation was performed as described before [50]. Selection of VHH expressing phages was performed by a bio-panning method using reducing number of rotavirus particles while increasing stringency.

3.4 VHH FRAGMENT PRODUCTION IN *SACCHAROMYCES CEREVISAE*

The genes encoding VHH fragments were isolated from pUR5071 and cloned in a yeast episomal vector (pUR4547) under the *SUC2* signal sequence and the *GAL7* promoter. The *S. cerevisae* strain VWK18gal1 was transformed and induced for antibody expression and secretion. Antibody fragments were secreted into the supernatant and were purified and concentrated by filtration over microcon filters with 10 kDa cut-off (Amicon, US).

3.5 CLONING OF VHH FRAGMENTS IN *LACTOBACILLUS PARACASEI*

Two different promoters have been used for the expression of VHH fragments in *L. paracasei*. For paper III, the gene encoding VHH1 was fused to an E-tag and cloned in the pLP501 vector and the expression was driven by the lactate dehydrogenase promoter. To generate cell surface anchored antibody fragments, the VHH1 gene was fused to the anchor sequence from proteinase P of *L. casei*. To generate the secreted VHH1 antibody fragment, a stop codon (TAA) was inserted by PCR amplification after the E-tag and the product was introduced into pLP501.
For paper IV, the expression of the VHH fragments was driven by the high activity APF promoter of *Lactobacillus* in the expression vector pIAV7 [51] (Marcotte *et al*, unpublished). Dimers of VHH fragments (VHH1-VHH1, VHH3-VHH3 or VHH3-VHH1) were generated by fusing the two fragments, end to end, by PCR and fused to an E-tag encoding gene. For bacterial surface expression, the same anchor sequence as in Paper III was introduced after the E-tag encoding gene.
Transformation of *L. paracasei* (previously named *L. casei* ATCC 393 pLZ15$^-$) was performed as described previously [45]. The transformed lactobacilli were cultured in MRS broth containing 5 μg/ml erythromycin.

3.6 PURIFICATION OF ANTIBODY FRAGMENTS PRODUCED BY LACTOBACILLI

Purification of secreted VHH1 and irrelevant VHH antibody fragments was performed using the RPAS Purification module (Amersham Biosciences). The purity of antibody fragments was verified on SDS-PAGE and the concentration of total protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories) (paper III).

3.7 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

RRV ELISA was used in papers I, II, III and IV for verification of binding of antibodies or antibody fragments to RRV. Ninety-six-well ELISA plates were coated with rabbit anti-human rotavirus antiserum (1:1000) followed by incubation with rhesus rotavirus stock (RRV) (1:100). Antibodies, transformed lactobacilli culture supernatant or cell homogenates were added to the plates. Plates were then incubated with mouse anti-E-tag antibodies (Amersham Pharmacia Biotech) (1:1000) followed by alkaline phosphatase (AP)-conjugated goat anti-mouse antibodies (DAKO A/S) (1:1000).

3.8 SCANNING ELECTRON MICROSCOPY (SEM)

SEM was used in paper III. Cultures of lactobacilli expressing VHH1 fragments anchored to the surface and non-transformed *L. paracasei* were mixed with RRV, fixed in 2% glutaraldehyde and analyzed by SEM (JEOL JSM-820; Jeol) at 15 kV.

3.9 FLOW CYTOMETRY

Flow cytometry was used to estimate the expression level of different VHH proteins by lactobacilli (in papers III and IV) and to evaluate binding and compare avidity to RRV (in paper IV). Lactobacilli were grown to an OD_{600} of 1 and were stained with a 1:200 dilution of a mouse anti E-tag monoclonal antibody (Amersham Biosciences) for 30 minutes on ice. Anti-mouse Cy2 conjugate (1:200) was used as secondary antibody (Jackson Immunoresearch Laboratories). The samples were analyzed using a FACS Calibur machine (Becton Dickinson).

To ascertain binding to RRV, lactobacilli grown to an OD_{600} of 0.8 were incubated with a 10 fold excess of RRV. The lactobacilli were then incubated with a 1:200 dilution of rabbit anti-rotavirus serum, followed by a 1:200 dilution of anti-rabbit PE conjugate antibody (Jackson Immunoresearch Laboratories). Incubations were performed on ice for 30 minutes. The lactobacilli were fixed using 2 % paraformaldehyde and analyzed using a FACS Calibur machine (Becton Dickinson).

3.10 VIRUS PRODUCTION AND PURIFICATION

Rhesus rotavirus (RRV) was cultivated in MA104 cells as previously described [52]. The virus titre was determined by immunoperoxidase staining of MA104 cells challenged with RRV.

3.11 NEUTRALIZATION ASSAY

A neutralization assay was used in papers I and III. Briefly, antibodies or antibody fragments expressing *L. paracasei* was incubated with RRV and directly used for infection of MA104 cells. The infection of cells was scored by immunoperoxidase staining and counting of infected cells. A reduction in the number of RRV-infected cells by more than 60 % relative to the control suggested significant neutralization.

3.12 *IN VITRO* ROTAVIRUS INHIBITION ASSAY: WESTERN BLOT

In vitro rotavirus inhibition assay was used in paper IV to determine the synergistic interaction of purified VHH1 and VHH3 fragments in reducing the rate of infection in MA104 cells. MA104 cells were seeded in 24 well plates a day before infection at 1×10^5 cells/ml in DMEM with 5 % FCS. 10^5 FFU of trypsinized RRV was added to dilutions of purified VHH1, VHH3 or a combination of both, prepared in OptiMEM to a final volume of 50 µl. The virus was incubated with the antibodies at RT for 15 minutes and used for infection of MA104 cells after adjusting the volume to 250 µl with DMEM for 1 hr at 37° C. After removing the virus, the cells were washed with DMEM, supplemented with DMEM + 10 % FCS and cultured for 14 hrs at 37° C with 5 % CO_2. Cells were lysed and the extracts boiled with SDS loading dye for protein gels. 12 % SDS-PAGE gels were cast and the proteins were separated by electrophoresis (Bio-Rad Laboratories). The proteins were electro-blotted on to

nitrocellulose membrane using wet transfer (Bio-Rad Laboratories). The VP6 protein of rotavirus was detected using rabbit anti-VP6 antisera (1:1000), followed by anti-rabbit HRP conjugated antibodies (DAKO A/S) (1:1000). The reaction was developed using the ECL chemiluminescence kit (GE Healthcare).

3.13 *IN VITRO* ROTAVIRUS INHIBITION ASSAY: FLUORESCENCE MICROSCOPY

In vitro rotavirus inhibition assay by fluorescence microscopy was used in paper IV to verify the mechanism by which surface VHH expressing lactobacilli may reduce infection in cells. MA104 cells were seeded on chamber slides (Becton Dickinson) a day before infection at 1×10^5 cells/ml in DMEM with 5 % FCS. On the day of infection, lactobacilli were grown to an OD_{600} of 0.8 and 50 µl of the culture was incubated with a 100 fold excess of trypsin activated RRV in a final volume of 100 µl for 20 minutes on ice. After adjusting the volume to 500 µl with OptiMEM, the mixture was used for infection of the cells for 1 hr at 37° C and 5 % CO_2. The cells were washed and supplemented with DMEM with 10 % FCS and incubated for 14 hrs at 37° C and 5 % CO_2. The cells were fixed with chilled methanol for 10 minutes at RT and washed with PBS. Double immunofluorescence staining was performed to detect lactobacilli (using anti-E-tag antibodies) and rotavirus VP6 protein (using rabbit anti-VP6 antisera) which is present in the rotavirus virions and also accumulates in the infected cells.

3.14 RRV VP7 REAL TIME PCR

Real time PCR was used for quantifying rotavirus load in small intestines of infected and treated mice in papers I, III and IV. Total cellular RNA was isolated from small intestinal tissue using RNeasy Kit (Qiagen), treated with RNase-free DNase® (Qiagen) and analyzed by real-time PCR using the EZ RT-PCR® core reagent kit (PE Applied Biosystems). Rotavirus *vp7* mRNA or viral genomic RNA was amplified at 58°C (ABI 7000 cycler, Applied Biosystems) in the presence of 600 nM primers, 300 nM probe, and 5 mM Mn, to generate a 121-bp-long amplicon. The sense primer (VP7f: 5'-CCAAGGGAAAATGTAGCAGTAATTC-3'; nucleotides (nt) 791-815), the antisense primer (VP7r: 5'-TGCCACCATTCTTTCCAATTAA-3'; nt 891-912), and the probe (5'-6FAM-TAACGGCTGATCCAACCACAGCACC–TAMRA-3'; nt 843-867) were designed based on the *vp7* gene sequence of RRV (GenBank AF295303). A standard curve was generated using a RRV *vp7* gene containing plasmid and the lowest level of detection of the PCR was 10 viral RNA copies. The RNA samples from each animal were normalized against the GAPDH gene [53]. The presence of less than 10 copies of *vp7* RNA was defined as clearance of infection.

3.15 HISTOPATHOLOGICAL ANALYSIS

This was performed in papers I and III. Sections of small intestine from mice were excised and perfused with formalin. The sections were kept immersed in formalin for a day after which they were transferred to 70 % ethanol. The samples were embedded in paraffin and sections were stained with hematoxylin and eosin using standard protocols. The sections were analyzed blindly for signs of rotavirus infection associated pathology [54].

3.16 INFANT MOUSE MODEL OF ROTAVIRUS (RRV) DIARRHEA

The infant mouse model of rotavirus-induced diarrhea was used in papers I, II, III and IV. All animal experiments were approved by the local ethical committee of the Karolinska Institutet at Karolinska University Hospital, Huddinge. Pregnant BALB/c mice were purchased from Møllegard Breeding Center, Denmark. Four-day-old pups were used for the study. The experiments were performed in two setups, prophylactic or therapeutic. Briefly, antibodies, VHH fragments or lactobacilli were administered to pups once daily in a 10 µl volume, starting on day –1 (for prophylactic treatment) and continuing until day 3. Infections were made orally on day 0 using 2×10^7 ffu RRV (20 diarrhea doses (DD_{50})), a dose which causes diarrhea in more than 90 % of inoculated animals. For therapeutic intervention, the first dose of VHH fragments or lactobacilli were administered to pups 2 hrs after infection and then continued once daily until day 3. Occurrence of diarrhea was recorded daily until day 4. Pups were euthanized on day 4 and sections of small intestine were stabilized in RNAlater® (QIAGEN) for RNA isolation and fixed in neutral buffered formalin for histopathological analysis.

3.17 STATISTICS

Diarrhea in the pups was assessed on the basis of consistency of feces. Watery diarrhea was given a score of 2 and loose stool was given a score of 1, no stool or normal stool was given a score of 0. Presence or absence of diarrhea was compared among treatment groups in a day-wise manner by Fischer's exact test and was presented as percentage diarrhea in graphs. Severity was defined as the sum of diarrhea scores for each pup during the course of the experiment (severity = Σ diarrhea score (day 1 + day 2 + day 3 + day 4)) and duration was defined as the total sum of days with diarrhea. Both severity and duration were analyzed by Kruskal-Wallis and Dunn tests. Differences in the intestinal virus load as assessed by real-time PCR were tested using the Mann-Whitney test.

4 RESULTS

4.1 PAPER I

Oral delivery of specific immunoglobulins provides passive immunity and is a fast acting treatment for rotavirus diarrhea. Probiotic bacteria have also gained considerable attention lately as treatment for rotavirus diarrhea. In this paper we report an evaluation of the therapeutic potential of different probiotics and their combination with anti-rotavirus antibodies in a mouse model of rotavirus diarrhea. Of the six lactic acid bacteria tested, *Lactobacillus rhamnosus* strain GG had the strongest influence in reducing prevalence, duration and severity of diarrhea and was therefore chosen for combination treatment with immunoglobulins. The combination treatment reduced the diarrhea outcome measures significantly, prevented histopathological changes and reduced the virus load in the intestines. The advantages associated with immunoglobulins and probiotics based therapy is that the treatment provides a rapid therapeutic effect and is cost efficient. These components do not require special storage conditions and could potentially complement the rehydration therapy that is currently used.

4.2 PAPER II

Fragments derived from llama heavy chain antibodies (VHH) are the smallest derivative of natural antibodies capable to binding to their targets with high affinity. In this paper we have generated a phage display library of llama VHH fragments against rotavirus strain RRV. The VHH fragments were subsequently produced in *Saccharomyces cerevisae* and the purified VHH fragments were tested for neutralization of RRV. We have selected a llama antibody fragment, VHH1 (or 2B10) that neutralizes RRV very efficiently. VHH1 is acid stable and may therefore survive gastro-intestinal environment. Oral administration of VHH1 in mouse pups challenged with RRV reduced the morbidity of diarrhea and aided in early recovery from disease. VHH fragments may represent a novel approach to the management and treatment of rotavirus diarrhea.

4.3 PAPER III

In this paper we have developed a system for passive immunotherapy against rotavirus where recombinant lactobacilli constitutively express the neutralizing llama VHH1 antibody fragments against rotavirus. Heavy chain variable domains (VHH1) from llama were expressed in *Lactobacillus paracasei,* both in secreted and cell surface anchored forms. Electron microscopy was used to investigate the binding efficacy of the VHH expressing lactobacilli. To investigate the *in vivo* function of the VHH1 expressing lactobacilli, a mouse pup model for rotavirus infection was used. Efficient binding of the VHH1 antibody fragments to rotavirus was shown by ELISA and scanning electron microscopy. VHH1 fragments expressed by lactobacilli conferred significant reduction of infection in cell cultures. When administered orally, lactobacilli producing surface expressed VHH1 markedly shortened disease duration, severity and viral load in a mouse model of rotavirus-induced diarrhea both when given fresh or in a freeze-dried form.

4.4 PAPER IV

In this paper we have tested the effect of multimeric antibody fragments as prophylaxis against rotavirus infection. Two unique VHH fragments, VHH1 and VHH3, with non-overlapping epitopes as suggested by ELISA were tested in combination against rotavirus infection. The combination of purified VHH1 and VHH3 proteins acts synergistically and drastically reduces infection rate by rotavirus *in vitro* and *in vivo*. The fragments were cloned in a plasmid expression vector of *Lactobacillus paracasei* and expressed as monomers or dimers (homo/hetero-dimers) anchored on the lactobacillus surface. The expression of the fragments and avidity of binding to rotavirus was tested by flow cytometry. Dimers of VHH fragments were efficiently expressed by lactobacilli with comparable level of expression for both monomers and dimers. Lactobacilli expressing heterodimers of VHH3-VHH1 showed the highest avidity among all the constructs to bind rotavirus and were also superior at reducing the rate of rotavirus infection *in vivo*. Multimerization of VHH fragments helps in increasing avidity and binding capacity and is more efficacious than monomer expressing lactobacilli.

5 DISCUSSION AND FUTURE PERSPECTIVES

5.1 GROUNDS FOR ALTERNATIVE THERAPIES FOR ROTAVIRUS: ONE SIZE DOESN'T FIT ALL

Even though the incidence of rotavirus diarrhea among diarrheal diseases is similar both in developed and developing countries, there are significant differences that should not be ignored [55]-

1. The age at which significant infections first occur: much younger children in developing countries (2-3 months vs. > 6 months in developed countries)
2. Frequency of diarrhea: 6-7 episodes/year vs. 1 /year
3. Severity of disease: higher in developing countries with 30-40 % cases requiring rehydration therapy compared to 10 % in developed countries
4. Seasonality of virus circulation: all year round compared to winter months in developed countries
5. Serotype prevalence and mixed infections: high and sometimes incongruous diversity in developing countries
6. Access to medical care and cost of disease: limited in developing countries
7. Mortality: majority of deaths occur in developing countries

The two newly licensed rotavirus vaccines have excellent efficacy records, however, the acid test would be their capacity to prevent rotavirus mortality in the least developed countries of the world, particularly in Africa and Asia [6]. Previous studies have shown that oral rotavirus vaccines are less efficient in developing than in developed countries [6]. Factors which could affect the efficacy or safety of these vaccines include malnutrition, pre-immune (maternal) antibodies, parasitic infections, other enteric infections, or immune suppression. No safety or efficacy data are available for the administration of RotaTeq and Rotarix to infants who are potentially immunocompromised, or with a history of gastrointestinal disorders [6], [56]. Although precautions should be taken when the vaccine is administered to immunodeficient patient, the immunodeficiency or HIV infection status is often unknown in developing countries. The most deterring fact about the current vaccination program is the exclusion of children more than 3 months of age due to the risk of developing intussusception [23] [24]. Although this precautionary measure is required, it would create an inexhaustible pool of unprotected children, since access to medical care in the developing countries is limited.

ORT has sustained the burden of diarrheal diseases and, to a large extent, has been successful. In relation to rotavirus diarrhea, ORT and active vaccination are two polarized treatment modalities. ORT is completely non-specific and exclusively treats the symptoms of diarrhea. Active vaccination, in being highly specific, lacks the immediacy of action that may often be required. The compelling question that has to be answered here is whether treating the symptoms (through ORT) is enough in cases where active vaccination is not a choice (especially in view of the repeated diarrheal episodes in developing countries). A need for fast acting, specific preventive/therapeutic measure can clearly be identified. To improve the management of rotavirus diarrhea, characterization of substances that could shorten the period of

diarrhea, nutritionally benefit the patient, and strengthen the mucosal barrier would be an important breakthrough. Passive immunization is currently the only available intervention that provides immediate protection and may thus represent the prophylaxis of choice for selected groups of children such as hospitalized children, immunocompromised patients, and children in which vaccination is contraindicated (those more than 3 months).

5.2 PASSIVE IMMUNOTHERAPY IN THE TREATMENT OF ROTAVIRUS DIARRHEA: A JUSTIFICATION

In the early 1890's Behring and Kitasato discovered that transferring sera from animals immune to diphtheria or tetanus to naïve animals imparted protection against the deleterious effects of bacterial toxins upon challenge. These very early findings laid the grounds for the modern day passive immunotherapy. Although the use of 'serum therapy' could not be continued, mainly due to adverse reactions to transferred heterologous sera (serum sickness) and a subsequent shift in the scientific interest to antimicrobials and antibiotics, these experiments established that transfer of antibodies was an excellent way of imparting immediate protection against many infections. Antibodies are very interesting biomolecules in the immune defense of various species and have specialized during the course of evolution to meet different biological requirements. Nature seems to have ensured the passive transfer of maternal antibodies into the neonates of different species by various mechanisms (pre-transferred IgY in egg yolk in birds, breast milk in humans/cows). By using the maternal immune system as a probe for possible environmental pathogens, this is an excellent scheme used by nature to protect the neonate from potential infections. Thus, in the neonatal period, passive immunotherapy can be envisaged to mimic the natural response to infections.

Mucosal surface represents the major interface between host and environment. It constitutes the point of entry of most infectious agents, and is in contact with potentially injurious antigens in ingested or inhaled substances. The mucosa must defend itself against insult without jeopardizing vital functions. The mucosal immune system contains more than 80 % of all Ig-producing cells in the body, and the major product of these cells is IgA. By serving as an external barrier capable of inhibiting attachment of microbes to luminal surface of the mucosal epithelial lining, IgA antibodies form the first line of immune defense. Mucosal SIgA is an important determinant in protection against rotavirus. IgA is basically non-inflammatory with the major role of the Fc portion of the antibody to transport IgA across mucosal epithelial cells and not, as in the case of other classes of antibody, to activate secondary phenomenon of the kind that contribute to inflammation [57]. Thus it can be said that at the mucosal surfaces, SIgA mainly works by 'antigen binding' mediated through the variable part of the antibody alone. This might especially be true in the case of rotavirus where several investigations using antibodies from multiple sources indicate that protection could be extended across the species barrier [30] [31], again suggesting that the main principle involved is binding and blocking of adherence of the virus, and not interaction with cells in the immune system of the recipient. This observation opens a whole new area for the treatment of rotavirus diarrhea, because where on one hand it makes possible to make use of antibodies from alternative sources, it also makes one think of the best possible way to achieve virus binding and blocking of adherence.

Polyclonal antibodies for commercial purposes have been produced in different mammalian hosts such as mice, rats, rabbits, sheep, goats and horses. Traditionally, serum is collected post-immunization and the immunoglobulin fraction is purified. Large-scale production/purification of antibodies cannot be achieved this way because of difficulties in obtaining large quantities of blood and the invasiveness of the procedure. Passive transfer of large amounts of IgG antibodies to the colostrums in cows presents with the possibility of hyperimmunizing pregnant cows with a given antigen and subsequently collecting colostrums, a collection procedure which is completely non-invasive. Antibodies can be purified from the colostrums preparation and once lyophilized typically have long shelf life [58]. Bovine colostrum-derived antibodies have been raised against a variety of mucosal pathogens [28] [29]. Bovine colostrum antibodies against rotavirus have been shown to be efficacious at reducing the duration of diarrhea and aiding in early recovery from rotavirus in infected children [30]. However, treatment for rotavirus diarrhea purely based on bovine colostrum antibodies, however effective, is not practical and may be offset by the costs involved for mass prophylaxis. It is thus imperative to find alternative methods to make immunoglobulin based therapy economically viable.

5.3 THE COMMENSAL FLORA AND PROBIOTICS: THE UNSUNG HEROES

Hundreds of microbial species live in association with the gastro-intestinal tract of higher animals. In terms of sheer numbers, bacterial populations have been estimated to exceed the number of somatic cells associated with the human body by 10-fold. This staggering figure prompts one to view the microbiota as an indispensable and important organ system, performing and assisting collectively in vital functions that sustain the 'normal' physiology of the gut [59]. Germ-free animals are known to have poor immune function, an effect directly attributable to the lack of the commensal flora. One can say with reasonable certainty that the evolution of humankind has been paralleled by the co-evolution of the human commensal flora. While some of these commensals are probably on the way to becoming pathogens [60], there are others that might be losing pathogenicity [61]. However in the vast realm of possibilities, the unifying theme of evolution has been to ensure survival, whether by pathogenesis or commensalism. It is therefore reasonable to assume that some microbes deem it important to maintain human health in order to ensure their survival. The concept of probiotics (literally meaning 'for life' or supporting life) may therefore stem from this notion. This hypothesis is most interesting for mucosal infections, like rotavirus, where the pathogen and the probiotic/commensal bacteria can have an intimate encounter. It is as yet difficult to say exactly how probiotics work. The health promoting effects of the probiotic bacteria could be due to a specific inhibition of the pathogen in question or a reflection of a more complex interaction between host, pathogen and the probiotic. Stimulation of the mucosal immune system resulting in increased circulation of local mucosal antibodies like SIgA and SIgM has been observed and is probably of importance in rotavirus infection [62] [63].

Another way in which supplementation with lactobacilli might reduce rotavirus diarrhea has been suggested by Isolauri *et al* [64]. It has been suggested that rotavirus infection gives rise to a biphasic diarrhoeal illness, first causing osmotic

diarrhoea and later an overgrowth of urease producing bacteria. Rotavirus infection causes patchy lesions in the small intestine mucosa leading to malabsorption of carbohydrates, and to osmotic diarrhoea, which turns the colonic contents acidic. The acidic stools convert ammonia to ammonium ions, which are poorly absorbed from the colon. Unabsorbed ammonium ions provide nitrogen to many enteric bacteria, including urease producing bacteria. Overgrowth of urease producing bacteria might predispose to further mucosal damage, initiated by rotavirus infection. Following this hypothesis, an intervention that reduces ammonia content or bacterial overgrowth in the intestine could decrease the severity of rotavirus infection. Oral administration of lactobacilli prevents overgrowth of urease producing bacteria in the colon thereby preventing worsening of diarrhea symptoms.

Clinical data on the use of probiotics in treating rotavirus diarrhea in developing countries is sparse. In comparison, the use of probiotic bacteria against rotavirus in developed countries is well documented but not very effective, allowing a reduction of only 24 hrs in diarrhea duration in children. However, considering the high morbidity and mortality of the infection in developing countries, such reduction seems desirable and it would also afford considerable savings in terms of loss of working days and direct health costs. It should also be considered that probiotics may reduce the risk of spreading rotavirus infection by shortening diarrhea duration and volume of watery stool output.

5.4 COMBINATION OF PASSIVE IMMUNOTHERAPY AND PROBIOTIC BACTERIOTHERAPY FOR TREATMENT OF ROTAVIRUS DIARRHEA (PAPER I)

The immediacy of action of antibodies in treating rotavirus disease is desirable but is offset by high costs. Attempts must be made to make passive immunotherapy a more affordable and realistic alternative. Using a combination of probiotic bacteria with specific antibodies is one way of achieving this. In paper I, four lactobacilli with wide ranging anti-rotavirus properties were administered in combination with polyclonal bovine antibodies to mouse pups challenged with rotavirus. Combination treatment of *L. rhamnosus* GG and antibodies was the most potent among all other combinations tested and saved up to 90 % of antibodies per treatment, if antibodies were to be used exclusively. A treatment combining antibodies with probiotic bacteriotherapy has the dual advantage of 1.) cutting the costs drastically while retaining the immediacy of action from antibodies 2.) of approaching the treatment of disease in two different ways, thereby being complementary/holistic. The immunostimulatory effect of *L. rhamnosus* GG in increasing the levels of circulating IgA has been documented before [62] [63]. In the context of rotavirus infection, a good proportion of this IgA response can be expected to be directed against rotavirus. However, complementation with a small dose of exogenous antibodies blunts the initial infection and provides the protective umbrella under which this response may develop. Other probiotic bacteria may have a different mode of action against rotavirus which may or may not complement with antibodies. One of the most desirable features of including probiotics and antibodies as treatment alternatives is that these components do not require special

storage conditions and could potentially be added as a supplement to the rehydration therapy that is currently used.

5.5 LLAMA VHH FRAGMENTS AGAINST ROTAVIRUS (PAPER II)

Camelids produce functional antibodies devoid of light chains of which the single N-terminal domain is fully capable of antigen binding. These single domain, heavy chain antibody fragments (VHHs), have several advantages for biotechnological applications including facile expression in microbial systems and high stability and solubility. Antibodies play an important role in limiting virus infectivity *in vivo* by a multitude of mechanisms. However, to escape immunosurveillance, many pathogenic viruses have evolved narrow cavities (canyons) in their surface, which are poorly accessible to conventional antibodies and are thus largely immunosilent. This 'blind spot' of the antibody response is caused by the limited diversity of complementarity-determining region (CDR) loop lengths, which constrains the displayed antigen-binding surfaces to mostly flat or concave topologies [65]. VHH domains are well suited to target these cryptic sites as their projecting CDR loops may be able to access deep recesses on the virus capsid. Indeed, camelid single VHH domains have demonstrated improved penetration against cryptic (immuno-evasive) target antigens such as trypanosome surface glycoproteins [35] and make potent enzyme inhibitors by specifically targeting the active site [66].

A llama VHH library was generated against rotavirus strain RRV by parenteral immunization. After stringent panning rounds which included selection under acidic condition (to mimic the gastro-intestinal environment), VHH1 was selected. The VHH1 fragment has great efficiency at neutralizing rotavirus *in vitro* and protects mice against severe diarrhea *in vivo*, albeit at high doses (>10 µg/dose). Although, the epitope on rotavirus which VHH1 recognizes is not known, there is a high possibility that it is one of the outer capsid proteins, VP7 or VP4, since both immunization and selection were done with triple layered particles (TLPs). These proteins are present in the virus capsid in multiple copies (260 trimers of VP7 = 780 and 60 dimers/trimers of VP4 = 120/180). Taking the monovalency and the small size of VHH into account (1/10 of a complete antibody), it is not surprising that it took a high dose of VHH1 to ensure activity *in vivo*.

Recently it has come to our observation that VHH1 depicts an unusually broad cross-reactivity to different serotypes of rotavirus (Einerhand *et al.*, unpublished). The broad cross-reactivity of the VHH1 fragment may suggest that it might be directed against a conserved epitope that is shared by different serotypes of rotaviruses. Attempts to map the epitope by standard laboratory techniques like western blotting, have failed suggesting that the epitope may be conformation dependent. There is currently an ongoing phase I clinical trial in Bangladesh where the VHH1 fragment is being evaluated.

Rotavirus administered to the llama via parenteral immunization was certainly immunogenic and high activity VHH fragments were selected from the resulting phage-display library. However, it is tempting to speculate whether a different (better?) immune response could have been elicited had the immunization been done through mucosal priming. Llamas have been shown to get infected in infancy by coronavirus and rotavirus and develop diarrheal disease [67]. Oral/enteric immunization with RRV

rotavirus could have mimicked a natural response (given that the RRV strain is permissive in llama intestinal cells) and a mucosal immune response characterized by VHH fragments would have followed.

5.6 GENETICALLY ENGINEERED PROBIOTICS: IS FRANKENSTEIN AT LARGE?

Genetic engineering opens to us the possibility of engendering novel properties in an otherwise conventional genome. Commensal flora, by virtue of its close interaction with the epithelia at mucosal surfaces, represents ideal vehicles for delivering biomolecules with various functional activities. A large body of literature exists on the use of lactic acid bacteria as live vaccine delivery vehicles [44]. In a similar attempt to modulate host immune response, *Lactococcus lactis* genetically engineered to express human IL-10 were found to be efficacious at treating inflammatory bowel disease in a phase I clinical trial [68]. Lactic acid bacteria expressing neutralizing antibody fragments against mucosal pathogens have been used as anti-infective agents to block mucosal adhesion of pathogens such as *Streptococcus mutans* [45] and rotavirus [46]. An upcoming strategy to abrogate mucosal infections is the expression of microbiocides against mucosal pathogens such as the Human Immunodeficiency Virus by lactobacilli [69] [70]. In a novel approach to incorporate anti-microbial properties in commensal bacteria, researchers have expanded the system to include expression of foreign enzymes thus allowing new metabolic pathways to take shape. These modified commensal bacteria express sugar structures that mimic host cell surface receptors for toxins from enterotoxigenic *Escherichia coli* [71].

The science of genetically modifying (rather, fortifying) lactic acid bacteria (LAB) with customized activities is progressing well; however, there are regulatory concerns, the most pertinent being- how can we see these modified bacteria transit from bench to bedside. Should genetically modified (GM) LAB be considered a GM food product or a pharmaceutical product? Social acceptance of genetically modified (GM) foods or ingredients is not uniform in developed countries which, over the past few years, have established mechanisms for adjudicating on the safety of novel foods before they are marketed. In 1998, the EU introduced a *de facto* moratorium on the import and production of GM foods and in March 2003, the European Commission upheld the moratorium and is standing firm on the decision that any food containing 0.9% of a GM product should be labeled [72]. This includes the use of a LAB modified with an exogenous gene. The main issue for GMO's, is the evaluation of the risk to human health of uncontrolled product expression following transfer of the transgene into a commensal bacterium. Undoubtedly, the use of genetically engineered lactobacilli for medical purposes must guarantee their stability, safety and containment within the host. Furthermore, the heterologous gene(s) must be integrated into the chromosome of the carrier lactobacilli without any antibiotic selection marker to ensure maximum safety. Additionally, the risk of accumulation in the environment and lateral dissemination of the foreign gene to other bacteria must be minimized by use of biological containment systems. Unless a disseminated transgene offers a massive selective advantage to a wild population, it will not be selected and propagated, and therefore, may not pose a threat, however, precautions do need to be taken.

5.7 LACTOBACILLI EXPRESSING VHH FRAGMENTS (PAPER III AND PAPER IV)

Conventional antibody fragments (scFvs) against a variety of mucosal pathogens, like *Streptococcus mutans* [45], *Porphyromonas gingivalis* [47] and rotavirus [73] have been expressed in lactobacilli. However, it is extremely difficult to guarantee the functional expression of scFvs in lactobacilli and the rate of success is daunting, the most common problem being aggregation and improper folding of the heavy (VH) and light (VL) chains. By virtue of their simple structure, VHHs are expressed in lactobacilli at much higher levels and in a functional conformation compared to scFvs. In paper III, the VHH1 fragment against rotavirus strain RRV was expressed in *L. paracasei* under control of the lactate dehydrogenase promoter in a plasmid vector. The VHH1 protein was expressed either as a secreted product or as a cell surface anchored product. As evident from the *in vitro* assays, VHH1 purified from the supernatant of VHH1-secreted lactobacilli or those expressed on the surface of VHH1-anchored lactobacilli were effective at binding to and neutralizing RRV. Nevertheless, only lactobacilli expressing VHH1-anchored successfully reduced viral load, normalized pathology and mitigated diarrhea in the challenged mice. Monovalent VHH1 fragments are potent at reducing rotavirus disease rate, but only when administered in high doses (more than 10 µg daily). The rate of secretion of monovalent VHH1 fragments by lactobacilli was estimated to be 1 µg/ml (after 8 hrs of culture) which probably was too low. An important feature of natural mucosal antibodies (SIgA or SIgM) is their multivalent nature. By increasing the avidity of interaction, multivalency ascertains that upon finding the right epitope on a pathogen, the chance of dissociation of the antibody-antigen complex is reduced drastically, leading to aggregation. Aggregation reduces the number of virions able to initiate an independent infectious event, thereby reducing the overall infectivity. This is probably how lactobacilli expressing anchored VHH1 reduced rotavirus disease in mice. The numerous antibody fragments expressed on the bacterial surface result in the formation of "biological beads" which allow high-avidity binding due to multivalency, thus promoting strong agglutination and subsequent clearance of the virus. In addition, freeze-dried VHH1–anchored lactobacilli were also shown to be protective against rotavirus diarrhea, suggesting that the anti-viral activity is retained after freeze-drying, thereby making stockpiling of VHH expressing lactobacilli possible.

An emerging concept in the field of antibody therapies is the use of oligoclonal antibody preparations which can mimic a polyvalent antibody mixture while retaining the specificity of monoclonal antibodies. For effective protection against common rotavirus strains, an oligoclonal antibody preparation targeting multiple conserved epitopes simultaneously is probably an ideal solution. Despite having a segmented genome, the level of reassortment seen with rotaviruses in nature is lower than what theory would predict. Clearly, not all reassortants are viable and therefore not selected in nature. It may therefore be possible to target a majority of rotaviruses with a combination of monoclonals with broad and complementing reactivities. In paper IV, we employed two VHH fragments, VHH1 and VHH3 which by themselves impart partial protection against RRV infection *in vitro* and *in vivo* but in combination, the fragments reduce infection synergistically. We also expressed the VHH1 and VHH3 fragments as homo- and heterodimers on the surface of lactobacilli. The level of

expression of dimers was comparable to monomers, however parameters like mRNA stability and polypeptide folding may be crucial determinants.

Unlike the high protection seen when using a combination of purified VHH1 and VHH3 fragments for prophylaxis, a mixture of VHH1 and VHH3 monomer expressing lactobacilli was only marginally protective against diarrhea. This is interesting because even though VHH1 and VHH3 fragments may interact synergistically in solution, expressing them as anchored proteins on lactobacilli may restrict their activity. The mode of action of free VHH fragments in solution can be different from the same VHH fragments expressed as anchored products on the surface of lactobacilli.

VHH3-VHH1 dimer expressing lactobacilli showed the highest avidity and was 3.5 fold better than VHH1 monomer expressing lactobacilli at binding rotavirus. Prophylactic treatment with these lactobacilli reduced diarrhea symptoms in challenged mice and reduced the viral load in the intestines. Therapeutic administration of the combination of the yeast-produced VHH1 and VHH3 proteins was not as effective as prophylactic administration in alleviating diarrhea, probably because VHH3 may target a 'transient' epitope, one that is exposed shortly before infection. Paradoxically, VHH3-VHH1 dimer expressing lactobacilli effectively protected against diarrhea development when administered therapeutically. Fusing VHH3 to VHH1 may have aided in improving the targeting of the 'transient' epitope of VHH3 through initial binding of rotavirus by VHH1.

VHH domains are versatile and can be used to build modular designs that incorporate complementary biological functions in one molecule. The expression of multimerized VHH domains in lactobacilli was successfully achieved without any significant loss in expression. It is thus possible to extend the use of VHH and VHH fusion proteins for various other applications using lactobacilli as delivery vehicles. New, biologically safe contained expression systems where the antibody gene, devoid of antibiotic selection markers, is integrated into the chromosome of lactic acid bacteria are currently being developed in our laboratory. Tailor-made "lactobodies" may thus represent a new and versatile system for passive immunization at mucosal surfaces, and may be of major medical importance – especially in developing countries.

5.8 FUTURE PERSPECTIVES

Lactobacilli have a unique place in the treatment of rotavirus infection and diarrhea. Where on one hand several species of lactobacilli have been shown to limit diarrhea by probiotic mechanisms, in other studies lactobacilli have been successfully used to deliver various biomolecules at different mucosal sites. From this juncture, two divergent but equally interesting roads may lead to a better understanding and development of novel therapeutics against rotavirus.

One way is to identify the elusive 'probiotic properties' by looking in the gene pool of closely related bacteria of which only one depicts the protective phenotype. This approach relies on the assumption that nature has already introduced a 'protective phenotype' into a given bacteria/probiotic and that now it is left for us to find out what this phenotype and its associated genotype is. This may sound simpler than it probably will be in reality, even though, one might argue that similar approaches have been used to identify 'virulent factors' in pathogenic strains of bacterial species. Probiotic

mechanisms, most probably, will not be attributable to a single gene event, as virulence often is. This is because selection of virulence had a direct evolutionary advantage for the pathogen whereas 'probiosis' may involve a more complex interaction between the host and the microbe(s) in question. We can, however, be optimistic that probiotic pathways may be identified in the future which subsequently may help at predicting the therapeutic potential of different lactobacilli against various indications. As an example, repression of brush border disaccharidases during rotavirus infection is known to contribute to osmotic diarrhea. The alleviation in diarrhea symptoms after consuming lactic acid bacteria could be due to compensation by bacterial lactase. Thus a strain naturally over-expressing lactase may depict good probiotic characteristics against rotavirus diarrhea.

The other road is that of deliberately introducing known features by genetically engineering a carrier lactobacilli, that may impart a protective phenotype. There is an overwhelming selection of biomolecules or pathways that can be employed here and some preliminary work, proving that the concept works in reality, has already been done. In relation to lactobacilli engineered to express anti-rotavirus antibody fragments, there are obvious technical improvements that can be made. Achieving high level of secretion of antibody fragments by transgenic lactobacilli is one aspect. Lactobacilli expressing anchored VHH fragments have been shown to work by aggregating virions. Although effective, this approach can only work with VHH fragments that identify a surface displayed epitope on the virus. Secreted VHH fragments might show better tissue distribution because of their small size; however the activity of secreted antibody fragment will most probably depend on the target proteins. Neutralization by antibodies that block viral attachment (most likely the mechanism by which antibodies directed against VP4 mediates neutralization) will require high levels of secreted antibodies. Theoretically, a maximum a 120 VHH fragments against VP4 would be necessary to block viral attachment of 1 particle which might be difficult to achieve with lactobacilli secreting VHH. On the other hand, antibodies that affect decapsidation or induce capsid conformational changes affecting viral transcription will most likely be effective at concentrations well below saturation. However, such an activity has been shown to be dependant on antibody bivalency and multimerization of VHH might thus be necessary to achieve such an effect. The small size of secreted VHH fragments may assist in rapid tissue penetration, a feature that is employed widely in screening for tumors. Soluble VHH fragments (secreted by lactobacilli) can therefore be expected to cross the epithelial barrier at the villi tips and to abrogate ongoing virus infection intracellularly. This can be further improved by expressing antibody fragments fused to peptide sequences that facilitate membrane uptake, like protein transduction domains. However, it is not known how many intracellular lactobodies would be required to provide effective intracellular neutralization of rotavirus.

The development of a method for mass induction of passive immunity via the drinking water or food products has enormous implications in an age of emerging pandemic diseases. The modified lactobacilli would be produced in fermentors and would not require elaborate downstream processing, thereby decreasing the cost of production. The vaccine could be distributed in the form of fermented milk products (e.g. yoghurt), dried food (e.g. milk powder) or drinks (bottles of water or soft drinks). Once administered, the bacteria would persist in the intestinal tract allowing production of protective antibodies fragments *in situ*. This procedure circumvents the need for large-scale manufacturing and purification and reduces the need for repeated

administration. It also eliminates the problem of refrigeration (cold chain) of conventional vaccines. The system has also the advantage of combining both the specificity of the antibody with the general antimicrobial activity of the lactobacilli.

The *Lactobacillus* system is currently the only system that would be available for large-scale, rapid induction of protective immunity. Passive administration of lactobodies may represent the therapy of choice for both epidemic and endemic gastrointestinal infections, particularly in young children, those suffering from malnutrition and immunodeficiency and the ageing population. Due to their low cost of production and long shelf life when lyophilized, engineered lactobacilli may thus have a major health impact in both developed and developing countries.

In order to reduce the pathology of the rotavirus infection, other molecules could also be concomitantly expressed with the antibody fragments such as anti-inflammatory or anti-secretory molecules. Conceptually, lactobodies may not only be used against gastrointestinal viruses such as rotavirus or norovirus but also against sexually transmitted viruses such as papilloma virus, herpes simplex virus, and HIV.

6 CONCLUSIONS

The aim of this thesis has been to devise economical, efficacious and fast acting treatment alternatives for rotavirus diarrhea. The conclusions drawn from the individual research papers in this thesis are-

1. **Certain strains of lactobacilli can be used in combination with antibodies to treat rotavirus diarrhea. Complementation of passive immunotherapy with probiotic bacteria may represent a viable treatment alternative for rotavirus diarrhea (Paper I).**

2. **Llama-derived VHH fragments produced by *Saccharomyces cerevisae* reduce the morbidity of rotavirus diarrhea in a mouse model (Paper II).**

3. **Llama VHH fragments can be functionally expressed as anchored or secreted proteins by lactobacilli. Anti-rotavirus VHH expressing lactobacilli were capable of reducing disease duration, severity and prevalence in a mouse model of rotavirus diarrhea (Paper III).**

4. **Llama VHH fragments can be used as modular building units and can be expressed as dimers in lactobacilli without compromising expression efficiency. The dimers retained the functionality of both the domains resulting in a higher avidity for rotavirus and were also superior to VHH monomer expressing lactobacilli at reducing diarrhea symptoms in infected mice (Paper IV).**

7 POPULAR SCIENCE SUMMARY

Rotavirus most often infects infants and young children, and in children aged 3 months to 2 years, it is one of the most common causes of diarrhea, causing more than 600,000 deaths annually. Almost all children have had a rotavirus infection by the time they are 5 years old. Rotavirus infection and resulting diarrhea can rapidly lead to dehydration which, if untreated, can be fatal. There are currently two vaccines licensed against rotavirus, the problems, however, are 1.) The efficacy of the vaccines in developing countries has not been validated and 2.) They are recommended only to children less than 3 months of age leaving a large portion of the population unprotected. The aim of this thesis is to find treatment alternatives against rotavirus.

Antibodies are a part of our natural defense system against invading germs. When we are challenged by an infectious agent, our immune system starts to produce antibodies that specifically recognize and bind to the invading bugs just like a magnet can fish out tiny bits and pieces of iron from a rubble of different material. Unfortunately, mostly antibodies are formed after exposure to a bug, and therefore probably after the disease period is over. This makes our natural antibody response to a given bug a little out of sync in stopping the first infection (though once made, they can protect against re-exposure to the same bug). However if preformed antibodies (from external sources) are given to a person at risk of getting an infection, these antibodies can work just as well and provide protection against infection. This concept is recognized as 'passive immunity' and has been shown to work for prevention/treatment of rotavirus diarrhea.

Antibodies can be made against different infectious agents and from a variety of sources. Traditional sources of antibodies have been animals such as cows, horses or chickens. More recently, through genetic engineering, DNA encoding antibodies can be introduced in bacteria like lactobacilli which can then produce antibodies. Lactobacilli are found in large numbers in our intestines where they help us digest food and also guard us against infectious bugs that can be ingested through contaminated food. Certain types of lactobacilli (probiotics) protect against rotavirus infection by yet unknown mechanisms.

In paper I in this thesis, we tested whether feeding a mixture of probiotic bacteria and antibodies to mouse pups could protect them against diarrhea upon infection with rotavirus. The rationale behind using a mixture of probiotic (*Lactobacillus rhamnosus* GG) and antibodies and not just antibodies by themselves is that the latter treatment is much more expensive and therefore economically unrealistic. We could save 90 % of antibodies, per treatment if we used them in combination with *L. rhamnosus* GG. Since both antibodies and probiotic bacteria can be dried in the form of powder, it is possible to stockpile these and package them for a future need.

In paper II we attempted at finding an alternative source of antibodies against rotavirus. In most animals, including humans, the part of an antibody that binds to a bug (Fv) can be visualized as a pair of chopsticks requiring both the sticks to be working together to pick an object. In llamas, however we find a special kind of antibody, where this part is as simple as a skewer. These antibodies are called the heavy chain antibodies and the binding part is called 'VHH'. VHHs are better at binding a given infectious bug than regular antibodies and are also more durable. Therefore we

made a collection of VHH fragments against rotavirus and subsequently found one VHH fragment (VHH1) which was especially good at reducing diarrhea in mice.

As mentioned before, lactobacilli are generally found in our intestinal tract, which is also the site for rotavirus infection. In paper III, we introduced the DNA coding for VHH1 in lactobacilli (*L. paracasei*). These modified bacteria produced VHH1 so that numerous copies of VHH1 were stuck to the surface of bacteria. The bacteria carrying surface displayed VHH1 were therefore able to bind rotavirus and, when fed to mice, protected them from diarrhea.

When it comes to using antibodies against bugs, it is generally better to use a mixture that can bind to different parts of the bug to ensure that the bug doesn't escape. Therefore, in paper IV we used a combination of two llama antibodies, VHH1 and VHH3 that bind to different parts of rotavirus. The DNA encoding VHH1 and VHH3 was first arranged into a single DNA in tandem (like two Lego units put together) and then introduced in lactobacilli. The modified bacteria made both VHH1 and VHH3 as a single product (like two Lego units put together or in technical terms a 'dimer'). Lactobacilli expressing a dimer of VHH1 and VHH3 were better at reducing diarrhea in mice than lactobacilli expressing just VHH1.

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