

PROBIOTICS AND VIRUS INFECTIONS:
The effects of *Lactobacillus rhamnosus* GG
on respiratory and gastrointestinal virus infections

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ACADEMIC DISSERTATION

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“That which does not kill us makes us stronger”

- Friedrich Nietzsche

1844–1900

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (Studies I-V) and some unpublished data.

- I **Lehtoranta, L.**, Villberg, A., Santanen, R. and Ziegler, T. A novel, colorimetric neutralization assay for measuring antibodies to influenza viruses. *J Virol Methods* 2009; 159; 271-276.
- II **Lehtoranta, L.**, Pitkäranta, A., Korpela, R. The effects of probiotic combination *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* Lc705 in cytokine and chemokine response in human macrophages. *Int J Probiotics & Prebiotics* 2012; 7; 17-22.
- III Ventola, H.* and **Lehtoranta, L.***, Madetoja, M., Simonen-Tikka, M-L., Maunula, L., Roivainen, M., Korpela, R., Holma, R. The effects of the viability of *Lactobacillus rhamnosus* GG on rotavirus infection in neonatal rats. *WJG*. In press. **Equal contribution.*
- IV Kumpu, M.* and **Lehtoranta, L.***, Hatakka, K., Roivainen, M., Rönkkö, E., Ziegler, T., Söderlund-Venermo, M., Kautiainen, H., Järvenpää, S., Kekkonen, R., Hatakka, K., Korpela, R., Pitkäranta, A. *Lactobacillus rhamnosus* GG and viral findings in the nasopharynx of children attending day care. Submitted. **Equal contribution.*
- V **Lehtoranta, L.**, Söderlund-Venermo, M., Nokso-Koivisto, J., Toivola, H., Blomgren, K., Hatakka, K., Poussa, T., Korpela, R., Pitkäranta, A. Human bocavirus in the nasopharynx of otitis-prone children. *Int J Pediatr Otorhinolaryngol* 2012; 76; 206-211.

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MAIN ABBREVIATIONS

APC	Antigen presenting cell
ADV	Adenovirus
AOM	Acute otitis media
B.	<i>Bifidobacterium</i>
cfu	Colony forming unit
DC	Dendritic cell
ELISA	Enzyme linked immunoabsorbent assay
GG	<i>Lactobacillus rhamnosus</i> GG
GI	Gastrointestinal tract
HBoV	Human bocavirus
HEV	Human enterovirus
HI	Hemagglutination inhibition test
HRV	Human rhinovirus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP-10	IFN-inducible protein 10
L.	<i>Lactobacillus</i>
Lc.	<i>Lactococcus</i>
Lc705	<i>Lactobacillus rhamnosus</i> Lc705
MCP-1	Monocyte chemoattractant protein-1
NK	Natural killer
NO	Nitric oxide
NPS	Nasopharyngeal swab
NT	Neutralization test
ORS	Oral rehydration solution
PBMC	Peripheral blood mononuclear cell
PIV	Parainfluenza virus
PCR	Polymerase chain reaction
pfu	Plaque forming unit
PJS	<i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RTI	Respiratory tract infection
RV	Rotavirus
TLR	Toll-like receptor
TNF	Tumor necrosis factor

ABSTRACT

Viral respiratory and gastrointestinal infections are a major health problem, in particular among children. A large range of etiologic agents and increasing antiviral and antibiotic resistance, challenge the development of efficient therapies. Accumulating evidence suggests that specific probiotic bacteria are able to decrease the risk and symptoms of these infections. This thesis investigated the effects of specific probiotics, in particular *Lactobacillus rhamnosus* GG, on respiratory and gastrointestinal virus infections in a cell model *in vitro*, in a rat model *in vivo*, and in children. A particular focus was on questions, whether viability of a probiotic is an important factor in probiotic-virus interaction, and whether a combination of probiotics is more effective than single strains.

A novel colorimetric neutralization assay was developed for measuring influenza virus antibodies in human sera. The method was applied to measure antibody response after the administration of a seasonal, inactivated, trivalent influenza vaccine. The results were compared with those obtained with a traditional hemagglutinin inhibition test. The results obtained with both assays correlated well. Moreover, neutralization test proved to be more sensitive and specific than the hemagglutinin inhibition test. Thus, the method is valid for influenza virus research, and it could be applied for studying immune adjuvant effects of probiotics on serum influenza antibody titers in the future.

Immunomodulatory effects of probiotics were screened in human macrophage model *in vitro*. After 24 hours of bacterial stimulation, probiotic combination of *L. rhamnosus* GG and *L. rhamnosus* Lc705 was not able to significantly induce higher macrophage cytokine and chemokine production (TNF- α , IL-1 β , IL-6, IL-10, and IL-12, MCP-1, IP-10) over individual *L. rhamnosus* strains. However, cytokine responses induced by this combination were stronger than responses induced by traditional starter culture bacterium *Lactococcus lactis* ARH74, highlighting that immunomodulatory effects of probiotics are strain specific.

The effects of live and unviable *L. rhamnosus* GG in rotavirus infection were investigated in a neonatal rat model. Consistency of feces, animal weight, colon weight and the rotavirus colonization of plasma and intestinal tissues were considered as indexes of infection severity. Nonviable *L. rhamnosus* GG had beneficial effects in rotavirus infection in terms of reducing rotavirus induced body weight reduction and colon weight increase. However, live *L.*

rhamnosus GG was more effective in reducing significantly viral load in the gastrointestinal tract.

The effects of *L. rhamnosus* GG alone or probiotic combination containing *L. rhamnosus* GG on the occurrence of viral respiratory infections was assessed in a six month intervention trial in children or in otitis-prone children. Children receiving only *L. rhamnosus* GG had fewer days with respiratory tract symptoms during the intervention period. However, *L. rhamnosus* GG did not reduce viral occurrence in the nasopharynx, suggesting that *L. rhamnosus* GG is able to reduce respiratory virus symptoms through enhancing immune response.

In otitis-prone children, *L. rhamnosus* GG in a combination with *L. rhamnosus* Lc705, *Bifidobacterium breve* 99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS significantly reduced human bocavirus load in the nasopharynx three to six months after intervention.

In conclusion, probiotics and their combinations differ in their ability to elicit immunomodulatory effects *in vitro*. Viability of a probiotic is an important factor in virus infection. The probiotic *L. rhamnosus* GG reduced days with respiratory tract symptoms. In children, *L. rhamnosus* GG alone was not effective in reducing viral occurrence in the nasopharynx. However in otitis-prone children, *L. rhamnosus* GG in a combination reduced the numbers of human bocavirus.

INTRODUCTION

Viral respiratory and gastrointestinal infections in children pose a considerable health **and economic burden in terms of hospitalizations, medical costs, doctor's consultations, and** absenteeism from work and school. Currently, the only effective antivirals and vaccines for the prevention and treatment of respiratory virus infections are available against influenza viruses. Large varieties of other etiologic agents and increasing antibiotic and antiviral resistance challenge the development of efficient therapies. Consequently, it is of importance to find alternative and safe ways to reduce the risk of these infections. Moreover, with constantly evolving viruses and the introduction of novel viruses, there is a clear need for new sensitive and specific methods for identifying virus infections in order to provide more accurate virological diagnoses and properly direct antiviral therapy.

Probiotics are defined as live microorganisms that confer a health benefit on the host (FAO/WHO, 2002). The most common types of microbes used as probiotics are lactobacilli and bifidobacteria, which are generally consumed as part of fermented foods, such as in yogurts or as dietary supplements. Considering beneficial effects of probiotics in virus infections, specific probiotics have been suggested to be effective in alleviating the duration and severity of acute rotavirus gastroenteritis (Guarino et al., 2009). In addition, probiotics are able to reduce the risk of respiratory tract infections in children (Hatakka et al., 2001, Cobo Sanz et al., 2006, Hatakka, 2007., Hojsak et al., 2010a, Hojsak et al., 2010b, Taipale et al., 2011), which in most cases are of viral origin. However, the mechanisms behind these beneficial effects are largely unknown. Probiotics are likely to have an impact through gut mucosa by balancing the local microbiota (Madden et al., 2005), by inhibiting the growth of pathogenic microorganisms (Servin, 2004), and by enhancing local and systemic immune responses (Bodera and Chcialowski, 2009). They may also influence the composition and activity of microbiota in the intestinal contents. However, there are virtually no comparative clinical studies of probiotics effectiveness against respiratory tract infections.

In recent years products containing multispecies probiotics have been launched into markets. However, evaluation data whether they elicit synergistic beneficial effects or antagonistic effects over single strains is scarce. In addition, increasing evidence shows that killed/nonviable bacteria, products derived from bacteria, or end products of bacterial growth could provide some health benefits (Kataria et al., 2009, Lahtinen and Endo, 2011). These nonviable bacteria would serve as a great potential for food industry in terms of

providing new product applications, increasing product shelf life, and reducing storage costs. However, more data concerning health effects of nonviable probiotics are necessary.

The aim of this thesis was to characterize the effects of probiotics especially *L. rhamnosus* GG on respiratory and gastrointestinal virus infections in experimental models and in children. A particular focus was on questions, whether viability of a probiotic is an important factor in probiotic-virus interaction, and whether a specific combination of probiotics is more effective than single strains.

REVIEW OF THE LITERATURE

1 COMMON VIRAL INFECTIOUS DISEASES IN CHILDREN

1.1 RESPIRATORY VIRUS INFECTIONS

Viral respiratory tract infections (RTI) are a major cause of morbidity and mortality worldwide particularly in children (Denny et al., 1986, Heikkinen and Järvinen, 2003). On average children suffer annually from 5-10 RTIs during the first years of life (Lumio, 2010). According to global annual estimates two million children die from acute RTIs, which account for 10-20 % of all childhood deaths (Williams et al., 2002). The social and economic impact of respiratory viral disease is substantial due to hospitalizations, medical costs, missed work, and school and day care absences. For instance, viral RTIs lead to over 400,000 annual hospitalizations in children under 18 years of age in the United States alone (Nichols et al., 2008). Children attending day care are especially at risk for acquiring RTIs (Denny et al., 1986, Louhiala et al., 1995) as close physical contact among children in day care favors the transmission of infectious diseases.

RTIs are typically classified into upper and lower RTIs. Upper RTIs affect the nose, sinuses, throat, and the ear, whereas lower RTIs affect airways and lungs. Common infections of upper RTIs are acute upper RTI (common cold), otitis media, otalgia, tonsillitis, sinusitis, and laryngitis involving symptoms such as cough, sore throat, runny nose, nasal congestion, headache, low grade fever, and sneezing (Eccles, 2007). The majority of lower RTIs are bronchitis and pneumonia, which are clinically characterized by a variety of symptoms like cough, fever, wheezing, chills, and chest pain (Marrie, 2000). The most common viral RTIs in children are the common cold and acute otitis media (AOM).

1.1.1 Etiology and clinical manifestations

In humans over 200 types of viruses may cause RTIs. In upper RTIs, human rhinovirus (HRV), respiratory syncytial virus (RSV), and parainfluenza virus (PIV) are considered the major pathogens, followed by human enterovirus (HEV), influenza virus, and adenovirus (ADV). Influenza virus, PIV, and RSV cause more symptoms in the lower respiratory tract (**Table 1**) (Heikkinen and Chonmaitree, 2003, Nokso-Koivisto et al., 2006, Ruuskanen et al., 2011). Although viruses tend to have some variation in their typical clinical manifestation, it is not possible to identify the causative virus on the basis of symptoms.

Most respiratory viruses follow seasonal occurrence: In the Northern hemisphere, the frequency of viral RTIs increases rapidly in the autumn, reaching peak incidence during winter, and decreases again in the spring (Butz et al., 1990, Rautakorpi et al., 2006).

Table 1. The most common viral causative agents of RTIs in children.

Virus	Upper RTIs				Lower RTIs		
	Common cold	Otitis media	Tonsillitis	Laryngitis	Bronchitis	Bronchiolitis	Pneumonia
Rhinovirus	+	+					
Enterovirus	+		+				
Influenza virus	+	+	+	+	+	+	+
Respiratory syncytial virus		+	+	+	+	+	+
Parainfluenza virus	+			+	+	+	+
Metapneumovirus						+	
Adenovirus			+				+
Bocavirus		+					+
Coronavirus	+						

Rhino- and enteroviruses

Human rhinoviruses (HRV) and enteroviruses (HEV) are non-enveloped, positive-stranded RNA viruses. HRVs are also the largest group of respiratory viruses, including over 100 serotypes. In children the predominant illness caused by HRV is the common cold. Frequently HRV infection results in a mild illness characterized by a runny and stuffy nose, a sore throat, coughing, and hoarseness (Lina et al., 1996, Arruda et al., 1997, Monto et al.,

2001, Gwaltney Jr., 2002). More severe symptoms resembling influenza may also occur (Boivin et al., 2002). HRV is very common in children with AOM as well (Pitkäranta et al., 1998, Nokso-Koivisto et al., 2004), and is associated with acute lower RTIs infections such as wheezing, bronchiolitis, and pneumonia (Chidekel et al., 1997, Kim and Hodinka, 1998, Hayden, 2004).

HEVs are commonly associated with clinical manifestations ranging from mild respiratory symptoms to serious conditions, including aseptic meningitis, encephalitis, neonatal sepsis, and acute flaccid paralysis. In addition, HEVs are a common cause of upper RTI and AOM (Ruohola et al., 2000, Nokso-Koivisto et al., 2004).

Influenza viruses

Influenza viruses are RNA viruses with segmented genome. Influenza viruses are divided into three distinct types namely A, B, and C based on their structural and antigenic properties. Influenza A viruses are divided further into subtypes based on the variation on viral glycoproteins hemagglutinin and neuraminidase. Most human RTIs are caused by types A and B, which are responsible for annual influenza epidemics due to antigenic drift. Influenza A and B viruses cause infections ranging from asymptomatic infections and common colds to serious illnesses with systemic complications such as pneumonia (Mäkelä et al., 1998, Zambon, 1999). Influenza virus infection also disposes children to secondary bacterial infections such as sinusitis, otitis media, and pneumonia (Heikkinen and Chonmaitree, 2003). Typically, the attack rates during the annual outbreaks of influenza are highest in children, affecting on average of 20–30% of the pediatric population (Fraaij and Heikkinen, 2011).

Respiratory syncytial virus

Both RSV and PIV belong to the family of *Paramyxoviridae*, which are enveloped RNA viruses with single-stranded (ss) genome. There are two major groups of RSV (A and B), and both strains may circulate and cause concurrent infections (Peret et al., 1998). RSV is the leading cause of bronchiolitis and acute wheezing in young children accounting also for approximately 50% of all pneumonia cases (Langley and Anderson, 2011). In immune-compromised patients, RSV infection may cause respiratory failure leading to mortality rates exceeding 70% (Glezen et al., 1986, Hertz et al., 1989). In the United States, 60% of infants

are infected during their first RSV season, and nearly all children have been infected with the virus by 2–3 years of age (Glezen et al., 1986).

Parainfluenza virus

Four types of PIV are infecting humans. PIV 1-3 occur worldwide and among persons from all age groups, whereas PIV 4A and 4B are much less frequent. PIV can cause a broad spectrum of respiratory diseases, ranging from mild upper RTIs to pneumonia, but are most often associated with laryngitis (Knott et al. 1994). PIV infections are most severe in infants and become less severe with age. PIV 1-3 are the main causes of croup in infants and young children under five years of age, and these viruses also cause viral pneumonia and bronchiolitis (Knott et al., 1994, Marx et al., 1997, Laurichesse et al., 1999, Rihkanen et al., 2008).

Metapneumoviruses

Human metapneumovirus (hMPV) is an enveloped ssRNA virus, which was originally isolated from respiratory tract samples of children with respiratory disease in 2001 (Van den Hoogen et al., 2001). hMPV causes upper and lower RTIs in all ages, but mostly in children under five years of age (Jartti et al., 2012). Currently, hMPV is recognized second to RSV as a cause of bronchiolitis in early childhood (Mullins et al., 2004, Williams et al., 2004, Chano et al., 2005, Feuillet et al., 2012).

Adenoviruses

Human ADVs are non-enveloped DNA viruses with double-stranded (ds) genome. ADVs are divided into seven species (A-G) containing 56 serotypes (Robinson et al., 2011). Certain clinical presentations of ADV RTIs are linked to particular serotype. For instance respiratory illnesses are attributed mainly to species ADV B and C, whereas ADV-F types 40, 41, and ADV-G type 52 are responsible for gastroenteritis. In children, the usual clinical presentation of RTI caused by ADV includes upper respiratory symptoms (rhinorrhea, cough) and lower respiratory tract symptoms (Rowe et al., 1953). In addition, ADV infection is frequently associated with pharyngoconjunctival fever and epidemic conjunctivitis (Edwards et al., 1985).

Bocaviruses

Human bocavirus (HBoV) is a non-enveloped, ssDNA virus. HBoV1 was discovered in 2005 using molecular screening from nasopharyngeal aspirates of children with RTIs (Allander et al. 2005). Later on, HBoV 2-4 **have been identified in children's fecal samples** (Arthur et al. 2009, Kapoor et al. 2009, Kapoor et al. 2010). HBoV1 has been detected globally mostly in respiratory samples with 1.5–19% prevalence but also in stool, serum, tonsillar, saliva, and urine samples (Jartti et al., 2012). HBoV1 is associated with upper and lower RTIs, and most notably with pneumonia, otitis media, and acute wheezing in children (Allander et al., 2007, Kantola et al., 2008, Söderlund-Venermo et al., 2009, Kantola et al., 2010, Meriluoto et al., 2012). HBoV2-4 have been found mainly in fecal samples of children, and HBoV2 seems to be the most prevalent of causing gastroenteritis (Jartti et al., 2012).

Coronaviruses

Human coronaviruses are enveloped viruses with a large positive-stranded RNA genome. Altogether five types of coronaviruses have been identified causing respiratory illnesses in humans. The first two, 229E and OC43 were identified in the 1960s (Tyrrell and Bynoe, 1965, McIntosh et al., 1967). In 2003-2005, three new coronaviruses SARS, HKU1 and NL-63 were identified from patients with respiratory symptoms (Drosten et al., 2003, Van der Hoek et al., 2004, Woo et al., 2005). Coronaviruses 229E, OC43, HKU1 and NL-63 have been in continuous circulation since their first isolation, and cause annually a large number of upper and lower RTIs in children (Brodzinski and Ruddy, 2009, Principi et al., 2010).

1.1.2 Pathogenesis

The understanding of the pathogenesis of respiratory viruses has important implications for the development of therapies against common colds. The pathogenetic mechanisms of respiratory viruses differ between the viruses (**Figure 1**). Transmission of the virus may occur by direct inoculation of contagious secretions from the hands, or by large or small particle aerosols into the eyes and nose, and requires close or direct contact with large droplets or fomites (Boone and Gerba, 2007). Respiratory viruses target mainly the epithelial and bronchial cells of the upper and lower respiratory tract. The incubation period takes approximately 2-15 days depending on the virus. For instance, in HRV infection the first symptoms occur soon after virus entry into the nasopharynx and peak on 2-3 days of infection (Brownlee and Turner, 2008). HRV infection typically starts with a sore throat,

soon followed by watery nasal discharge, and later by nasal congestion and cough, which may persist for over three weeks (Eccles, 2005). RSV and PIV replicate in the nasopharyngeal epithelium, and spread to the lower respiratory tract 1-3 days later possibly via direct spread along the respiratory epithelium, or through the aspiration of nasopharyngeal secretions, or through macrophages (Domachowske and Rosenberg, 1999). Influenza viruses predominantly attach to the tracheal and bronchial epithelial cells causing typically a high fever (40°C), headache, nausea, and chills within 1-4 days (Peltola et al., 2003). In addition, efficient virus replication, maintenance of viral protein synthesis, shut-down of host protein synthesis, and production of viral particles lead usually to cytolytic death of cells at 20–40 hours of infection (Julkunen et al., 2001). In the host, viral replication and cellular damage evokes inflammatory and immune responses, leading to vasodilatation, increased vascular permeability and cellular infiltration through the release of inflammatory mediators. Increased concentrations of proinflammatory and chemotactic cytokines in nasal lavage result in a cascade of inflammatory reactions necessary for viral eradication (Van Kempen et al., 1999, Julkunen et al., 2001). The characteristic inflammation of RSV bronchiolitis is necrosis and sloughing of the epithelium of the small airways with edema and increased secretion of mucus, which obstructs flow in the small airways. The resulting clinical findings are the hallmarks of bronchiolitis: hyperinflation, atelectasis, and wheezing (Hall, 2001). Viral infections also dispose to secondary bacterial infections such as *Streptococcus pneumoniae*, leading to bacterial complications in the upper respiratory tract (such as AOM and sinusitis) (Osur, 2002, Heikkinen and Chonmaitree, 2003), or in the lower respiratory tract (such as bronchitis and pneumonia) (Peltola et al., 2004, Peltola et al., 2006). Viral infections upregulate receptors utilized by bacteria, which facilitate bacterial adherence and colonization (Hament et al., 1999, Peltola and McCullers, 2004). Moreover, virus induced physical damage in the respiratory epithelium may impair the local defense mechanisms, which leads to increased translocation of bacteria through the epithelial barrier of the respiratory cells.

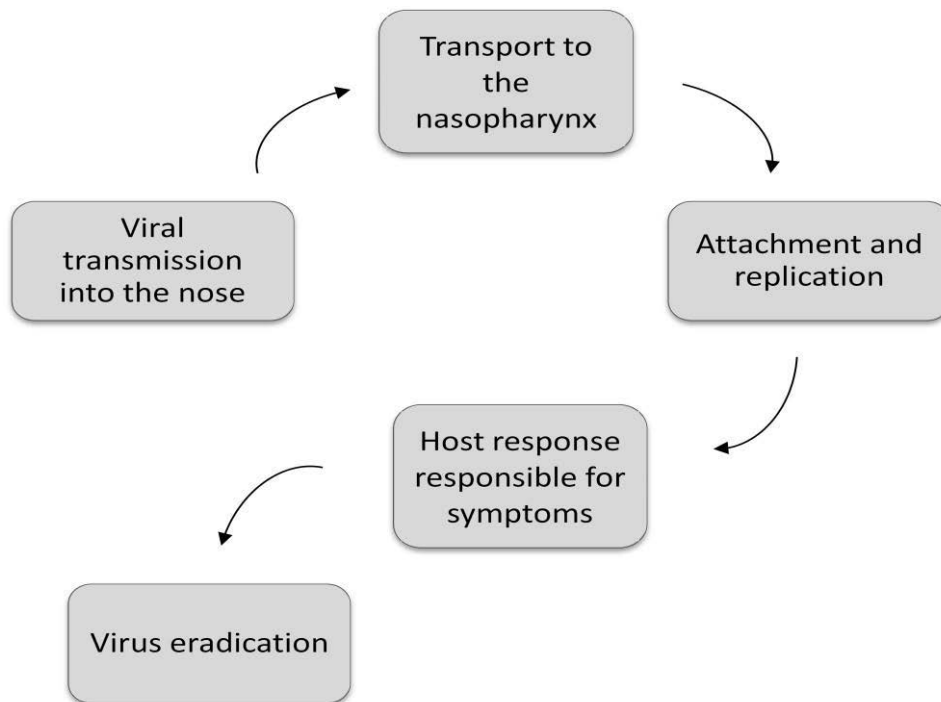


Figure 1. Schematic illustration of pathogenesis of respiratory viruses in the host. Adapted from Fields' virology 2007 (Fields et al., 2007).

1.2 GASTROINTESTINAL VIRUS INFECTIONS

1.2.1 Etiology and clinical manifestations

Acute viral gastroenteritis is a worldwide cause of infant morbidity and mortality in developed and developing countries. Viruses that cause gastroenteritis in humans include rotaviruses (RV), caliciviruses (noroviruses), sapoviruses, astroviruses, enteric ADVs (serotypes 40 and 41), and Aichi virus (Ciarlet and Estes, 2001). Among these, RV is the etiologic agent of greatest medical and epidemiologic importance in young children and infants accounting annually for nearly 450 000 child deaths worldwide (Tate et al., 2012). In Finland after the introduction of RV vaccine in 2009, norovirus infections have become as common as RVs as the causative agents of acute gastroenteritis in young children (Puustinen et al., 2011, Räsänen et al., 2011a).

RVs are dsRNA viruses belonging to the *Reoviridae* family. RV form seven antigenically different main groups named A-G. Group A-C infects humans, of which group A is the most common. Other groups (E-G) have been identified only in animals. There are also different serotypes within RV A group, and the classification is based on two structural proteins on the

surface of the virion. The glycoprotein VP7 defines G-types and the protease-sensitive protein VP4 defines P-types. In temperate latitudes RV infection occurs most frequently in winter. The disease is characterized by vomiting and watery diarrhea for 3-8 days. Fever and abdominal pain occur frequently. Vomiting generally lasts for 24 hours from the onset, whereas watery diarrhea persists longer, and is the predominant sign of RV infection. Immunity after infection is incomplete, but repeat infections tend to be less severe than the original infection (Rodriguez et al., 1977, Velazquez et al., 1996).

1.2.2 Pathogenesis of rotavirus infection

Life cycle of rotavirus

As a distinction for respiratory virus life cycle, RVs replicate mainly in the gut, and infect enterocytes of the villi of the small intestine leading to structural and functional changes of the epithelium. RV virion consists of triple protein coats, which provide resistance to the acidic pH of the stomach and the digestive enzymes in the gut. Several cell-surface molecules have been implicated in the early interactions of RV with its host cell, including sialic acid, various integrins, heat shock protein 70, and gangliosides. RV enters into the cells by receptor mediated endocytosis and forms an endosome vesicle. Viral proteins in the third layer (VP7 and VP4) disrupt the membrane of the endosome, creating a difference in the calcium (Ca) concentration, and leading to the disruption of outer layer proteins. This event triggers the uncoating of the virus to a double-layered particle, and the activation of the viral transcriptase. A special viroplasm is formed around the cell nucleus, where viral RNA is replicated, translated proteins accumulate, and the double-layered RV particles are assembled. These particles migrate to the endoplasmic reticulum where they obtain third outer layer (formed by VP7 and VP4). Finally, the progeny of viruses are released from the cell by lysis (Greenberg and Estes, 2009).

Pathogenesis

RV is transmitted by the fecal-oral route via contact with contaminated hands, surfaces and objects (Butz 1993) **with small infectious doses (\geq one plaque forming unit, pfu)** (Graham et al., 1987). During the incubation period of approximately two days, RV enters the epithelial cells and replicates causing diarrhea. RV continues to destroy the epithelium leading to extensive damage, and shedding of extensive masses of virus in the stools (Glass et al., 2006). The diarrhea is caused by multiple activities of the virus (Greenberg and Estes, 2009, Liu et

al., 2009): a) RV replication inside the enterocytes causes altered metabolism of enterocyte membrane proteins inducing malabsorptive or osmotic diarrhea, b) RV increases the concentration of intracellular calcium (Ca) disrupting the cytoskeleton and the tight junctions, and raising paracellular permeability, c) RV produces NSP4, a toxin that induces a Ca- dependent signal transduction pathway leading to increasing Ca²⁺ concentration. In addition, NSP4 produced by the infection disrupts tight junctions allowing paracellular flow of water and electrolytes, d) RV can stimulate the enteric nervous system, inducing secretory diarrhea and increasing intestinal motility, e) enterocyte cell death also contributes to malabsorptive or osmotic diarrhea. Primary RV infection in the host leads to a serotype-specific humoral immune response in the intestine and serum providing initial monotypic protection. During the first two years of life, children are repeatedly infected with various types of RV, resulting in a more complex immune response, which seems to provide partial heterotypic protection (Velazquez et al., 1996). RV-specific secretory IgA antibodies in the serum seem to provide the best protection (Franco et al., 2006).

1.3 LABORATORY DIAGNOSIS OF VIRUS INFECTIONS

The laboratory diagnosis of virus infections is based on the identification of viruses or virus particles from clinical samples, or demonstration of a rise in virus specific antibody titers between acute and convalescent serum samples. Diagnosis of RV infection normally follows diagnosis of acute gastroenteritis as the cause of severe diarrhea. Most children admitted to hospital with gastroenteritis are tested for group A RV (Patel et al., 2007). Reliable diagnosis of respiratory virus infections, however, is challenging because of the high number of potential viral agents causing similar signs of symptoms of respiratory illness. It is potentially of importance to rapidly diagnose respiratory viruses in order to properly direct antiviral therapy, as some antivirals (e.g. against influenza) are only effective if administered in the early stages of infection. Rapid viral diagnosis significantly also decreases length of hospital stays and unnecessary laboratory testing (Henrickson, 2005). In addition, understanding the actual cause of disease also decreases unnecessary use of antibiotics in viral RTIs (Gonzales et al., 2001). Prior the development of molecular diagnostics such as polymerase chain reaction (PCR), viruses were primarily identified by virus isolation in tissue culture, or antibody and antigen detection using immunological methods, including enzyme linked immunoabsorbent assay (ELISA) or direct and indirect immunofluorescence (IF) assays. The comparison of novel and traditional virological methods are summarized in

Table 2.

1.3.1 Traditional detection and isolation methods

The principal classical techniques for detecting and isolating respiratory and gastrointestinal viruses are indirect cell culture methods and direct electron microscopy (EM) (Mahony, 2008, Beck and Henrickson, 2010). Most respiratory viruses can be detected by virus specific cell lines allowing both quantification and isolation of the virus. The presence of a replicating virus in the cell culture can be detected with cytopathic effect or haemadsorption. Virus culture is beneficial for culturing a wide variety of viruses (including novel and unknown viruses), and obtaining infectious virus particles for biological characterization with relatively low cost. In addition, biological responses such as resistance to antivirals are obtained only with the cell cultures or inhibition assays. However, disadvantages with the virus culture are taking time to results, expertise required for result interpretation, careful sample preservation, and low sensitivity. For the improvement of these characteristics, IF or molecular tests have been utilized for confirmation and identification. In addition, modified culture methods (e.g. shell vial culture) which allow faster detection, are commonly used for isolation of respiratory viruses.

In EM, virus particles are detected and identified on the basis of virus morphology. EM is mainly used for the diagnosis of viral gastroenteritis such as RV. The main problem with EM is the expense involved in purchasing and maintaining the facility. In addition, the sensitivity of EM is often poor, as at least 10^5 to 10^6 virus particles per ml in the sample is required for visualization. As reliable antigen detection and molecular methods for identification of all species and serotypes of gastrointestinal viruses from environmental samples, serum, and cerebrospinal fluids have been developed, EM is becoming less and less widely used (Wilhelmi et al., 2003).

1.3.2 Immunological methods

The majority of immunological methods are based on viral antigen detection using monoclonal antibodies, which rely on IF or ELISA technologies. IF is widely used for respiratory virus detection from nasopharyngeal specimens, and ELISA for detection of RV antigen in feces. IF utilizes specific fluorescent labeled monoclonal antibodies which bind to viral antigens. ELISA techniques are based on enzymatic reaction, where coated primary antibody binds to viral antigen from the sample, and the positive reaction is identified using enzyme conjugated secondary antibody. The main advantage of IF and ELISA is that they are rapid to perform with the result being available within a few hours. However, IF is difficult to

read and interpret with sometimes poor specificity and sensitivity. A number of commercial products are available for the diagnosis of RV and respiratory viruses, including screening kits for the detection of up to seven respiratory viruses (Beards et al., 1984, Landry and Ferguson, 2000).

1.3.3 Serology

Serology allows the identification of virus specific antibodies from serum samples, such as recognition of IgM in primary/acute infection, and the detection of rising titers of antibody between acute and convalescent stages of infection (IgG immunity). Hemagglutination inhibition test (HI), ELISA, radioimmunoassay, complement fixation test, and virus neutralization tests (NT) are widely used. HI is based on the ability of a certain virus (e.g. influenza) to agglutinate the erythrocytes of mammalian or avian species causing hemagglutination of the cells. Hemagglutination is prevented if serum contains antibodies against the viral protein responsible for hemagglutination. By serially diluting the sample, the amount of antigen can be quantified in an unknown sample by its titer. In addition HI allows subtyping of influenza A virus. However, the specificity of the HI test varies with different viruses. In NT method, neutralization of a virus is defined as the loss of infectivity through binding reaction of a specific antibody to a virus. When virus and serum sample are mixed under appropriate condition and inoculated into cell culture, the presence of unneutralized virus may be detected by cytopathic effect, enzyme immunoassay, or plaque formation. The sensitivity and specificity of the assays depend greatly on the antigen used. For respiratory and gastrointestinal virus infections, which produce clinical disease prior appearance of antibodies, serological diagnosis is retrospective. For the diagnosis of influenza infections, however, serology may be important and cost-effective during an influenza epidemic.

1.3.4 Molecular diagnostics

Diagnosis of viral infections has been revolutionized by the development of molecular techniques, principally with the applications of PCR. PCR techniques allow rapid identification of viral genomes from clinical specimens within 2-24 hours from sample collection. Another significant advantage is sensitivity, as PCR methods can detect several orders of magnitude less of a target virus than tissue culture. In general, viral diagnosis with PCR involves three main steps: First, viral nucleic acid (DNA or RNA) is extracted from the clinical sample. Second, fragments of the genome are amplified using virus-specific primers.

Prior amplification, detection of RNA viruses generally requires also an additional step where RNA is reverse-transcribed (RT) into cDNA. Third, the amplified viral genome is visualized with either traditional stained agarose gel or in a real-time PCR assay. Real-time PCR assays have additional advances over conventional PCR, as the inclusion of an additional fluorescent probe detection system allows increased sensitivity, the ability to confirm the amplification product, and to quantitate the target concentration. More recently, multiplex detection strategies have been developed, which allow sensitive detection of over ten viral pathogens simultaneously (Templeton et al., 2004, Li et al., 2007, Wang et al., 2009). Although PCR assays are sensitive, PCR is highly prone to contamination, which may result in false positive results. This may lead to inaccurate diagnosis, and to more serious complications. Instead of infective virus, PCR assays detect genomic material from the specimen. As some respiratory viruses are detectable in asymptomatic patients, the results may also indicate a past infection (Jartti et al., 2004, Winther et al., 2006, Wright et al., 2007).

In summary, many virological methods are available for virus diagnostics depending on the study material used. Molecular diagnostics has significantly improved the laboratory's ability to diagnose viral RTIs. In order to provide more accurate virological diagnoses, existing methods with increased sensitivity need to be constantly improved. In addition, new diagnostic tests will be required to determine the impact of novel virological agents, and assist clinicians in the management of patients.

Table 2. Traditional and molecular virological diagnostic methods.

Diagnostic methods	Technique	Discovery	Target	Advantages	Disadvantages
Virus isolation	Cell culture	1940s ^a	Cytopathic effect, Hemabsorption	Detection of wide variety/novel viruses Biological characterization Low cost	Time consuming Requires expertise Low sensitivity
	Electron microscope	1930s ^b	Virus morphology	Specific	Mainly for gastrointestinal viruses Low sensitivity High maintenance costs
Immunological methods	IF	1950s ^c	Virus antigen detection	Rapid Screening of multiple viruses	Difficult result-interpretation Poor sensitivity and specificity
	ELISA	1960s ^d	Virus antigen detection	Rapid Sensitive	Specificity may vary
Serology	HI NT ELISA	1950s ^e 1970s ^f	Virus specific antibodies	Identification of primary infection Viral subtyping Sensitive and specific	Specificity may vary Requires expertise
Molecular diagnostics	PCR-techniques	1980s ^g	Virus genome	Rapid Sensitive and specific Screening of multiple viruses	High contamination risk

^a Enders et al., 1949^b Hazelton and Gelderblom, 2003^c Marshall Jr., 1951^d Yalow and Berson, 1960^e Donald and Isaacs, 1954^f Schmidt et al., 1976^g Shampo and Kyle, 2002, Zaia and Rossi, 1989

1.4 TREATMENT AND PREVENTION

Children are at increased risk for acquiring respiratory and gastrointestinal infection in day care centers and during a hospital stay (Denny et al., 1986, Raymond et al., 2000, Lu et al., 2004). Viral infections are preventable by interrupting viral transmission by maintaining good hygiene, and cleaning all surfaces with suitable disinfectants. For instance, respiratory viruses can survive few hours in contaminated surfaces (Vasickova et al., 2010), and RV for several days depending on the environment (Ansari et al., 1991). Environmental spread of viruses can be minimized with the use of certain disinfectants (Anderson and Weber, 2004, Kramer et al., 2006). Clinical trials also demonstrate that improved hand hygiene, alcohol-based sanitizers, and disinfectants are effective in reducing viral respiratory and gastrointestinal illnesses in children and adults (Uhari and Möttönen, 1999, Mott et al., 2007, Gray et al., 2008, Savolainen-Kopra et al., 2012) There are also many options for the treatment of respiratory and gastrointestinal virus infections, which are described in more detail below.

1.4.1 Vaccines

Immunization against respiratory and gastrointestinal virus infections is the most efficient way to reduce severe disease incidence. For respiratory viruses, commercially available vaccines globally are against influenza. In Finland, the influenza vaccine is also included (since 2007) in the routine vaccination programme for all children aged 6-35 months, which effectively prevents symptomatic influenza infections (Heinonen et al., 2011). Influenza vaccination reduces otitis media in the pediatric population as well (Heikkinen et al., 1991, Ozgur et al., 2006). In addition, pneumococcal vaccine in Finland has also contributed to the reduction of AOM cases in children (Eskola et al., 2001, Kilpi et al., 2003). Live vaccines against ADV types 4 and 7 have been approved in the United States for ADV-associated acute RTI, but only for military population 17-50 years of age. Vaccine development against the most common RTI viruses RSV, HRV, and PIV has been ongoing without any real success (Sato and Wright, 2008, Hurwitz, 2011, Papi and Contoli, 2011).

Since 1984, RV vaccine has significantly reduced the number of severe acute RV gastroenteritis in children (Vesikari et al., 1984, De Vos et al., 2004, Vesikari et al., 2010). Currently, two live RV vaccines containing an attenuated human monostain (Rotarix®) or a combination of five bovine human reassortant strains (RotaTeq®) are approved in most countries, and introduced in national immunization programmes of several American,

European, Eastern Mediterranean countries, and in Finland (WHO, 2009). In addition, based on data from clinical trials evaluating vaccine efficacy in high child mortality countries, WHO recommends inclusion of RV vaccination of infants into all national immunization programmes.

1.4.2 Antiviral drugs

All events in the respiratory virus life cycle can be interfered with antiviral agents (**Table 3**). Few studies show promising results in blocking viral attachment. For instance in RSV infection, binding of monoclonal antibody (palivizumab) to RSV fusion protein inhibits fusion of the viral particle with the cell membrane (Scott and Lamb, 1999). Palivizumab is currently approved antiviral for the prevention of RSV-associated hospitalization for high-risk infants. Moreover, a novel peptide molecule with antiviral activity inhibits the binding of influenza virus hemagglutinin to its cellular receptor *in vitro* (Jones et al., 2006). In addition, soluble intercellular adhesion molecule-1 (ICAM-1) (tremacamra) molecule inhibits HRV for binding to the membrane-bound ICAM-1 on host cells by binding to HRV particles (Turner et al., 1999). Pleconaril has been shown to block ICAM-1 receptor, and inhibit HRV attachment and HEV uncoating preventing viral replication (McKinlay et al., 1992). However, due to the modest efficacy, and drug interactions of these soluble ICAM-molecules, they have not been approved for the treatment of common colds (Nichols et al., 2008). Another drug, a capsid binding compound BTA-798, was successful in reducing the incidence and severity of HRV infection (Thibaut et al., 2012). Adamantanes (amantadine and rimantadine) prevent influenza A virus uncoating by inhibiting the M2 membrane protein ion channel activity, and block viral replication at an early stage of infection. However, due to the development of high levels of resistance to adamantanes among circulating influenza A viruses, they are no longer recommended for the prevention of influenza virus infections (Fiore et al., 2011). Viral replication and translation can also be inhibited with specific antivirals. For instance, cidofovir inhibits ADV DNA synthesis, and shows good clinical efficacy in the eradication of ADV and alleviation of symptoms (Waye and Sing, 2010). However, the toxicity profile of the substance has limited its clinical use. Some options for blocking or degrading viral mRNA molecules are also available, such as small interfering RNAs (siRNA) against RSV, PIV, and influenza viruses (Ge et al., 2003, Bitko et al., 2005). Recently, a randomized clinical trial in humans with intranasal siRNA targeting RSV nucleoprotein gene showed protective activity (DeVincenzo et al., 2010). Moreover, ribavirin blocks the attachment of viral mRNA of RSV, PIV, and ADV to ribosomes (Snell, 2001, Gavin and Katz, 2002). Currently, ribavirin as an aerosol is approved for the treatment of RSV bronchiolitis in children in the United States.

For many respiratory viruses processing of translated polyproteins into smaller biologically active proteins with proteases plays a pivotal role in viral gene expression and replication. Several different inhibitors of such proteases (e.g rupintrivir) have been described for coronaviruses, HRV, and HEV (Rohde, 2007). For the inhibition of viral release from the host cells, there are available specific neuraminidase inhibitors against influenza virus infection. Zanamivir and oseltamivir are approved drugs against influenza A and B virus infections. Both drugs reduce significantly the duration and severity of symptoms with infrequent adverse effects (Rohde, 2007).

Table 3. Options for antiviral drug targets in the treatment of respiratory virus infections.

Virus	Antiviral targets				
	Attachment	Entry/ Uncoating	Replication/ Translation	Posttranslational processing	Release of viral particles
Rhinovirus	ICAM-1 receptor blocking (pleconaril)	-	-	Protease inhibitor (rupintrivir)	-
Enterovirus	-	Capsid uncoating inhibitor (pleconaril)	-	-	-
Influenza virus	Hemagglutinin inhibitor	M2 activity inhibitor (adamantanes)	-	-	Neuraminidase inhibitors (zanamavir, oseltamivir)
Respiratory syncytialvirus	F protein inhibitor (palivizumab)	-	siRNA Viral mRNA attachment blocker (ribavirin)	-	-
Parainfluenza virus	-	-	siRNA	-	-
Adenovirus	-	-	DNA synthesis inhibitor (cidofovir) Viral mRNA attachment blocker (ribavirin)	-	-
Bocavirus	-	-	-	-	-

1.4.3 Interferons

In response to the presence of a virus infection, host cells produce interferons (IFN), which act by inhibiting protein synthesis and stimulating host defense mechanisms including cellular and humoral immune responses. Several studies have evaluated the efficacy and safety of intranasal recombinant IFN 2b in the prevention or treatment of HRV infections (Nichols et al., 2008). In these studies, only prophylactic use showed modest efficacy, and was ineffective against symptomatic HRV infection. In addition, prolonged use induced histological changes in the nose.

1.4.4 Oral rehydration therapy

In general, viral gastroenteritis is treated with counteracting the dehydration by correcting the fluid loss and electrolyte imbalance (Cheng et al., 2005, Gadewar and Fasano, 2005). Mortality from acute diarrhea has decreased substantially due to worldwide campaigns of treatment with oral rehydration therapy. However, rehydration therapy does not shorten the diarrhea. Rehydration therapy encompasses two phases: a rapid oral rehydration phase, in which water and electrolytes are administered as oral rehydration solution (ORS) to replace existing losses, and a maintenance phase, which includes both replacement of ongoing fluid and electrolyte losses, and adequate dietary intake with normal foods appropriate to the age of the subject (King et al., 2003). According to the guidelines of European Society for Paediatric Gastroenterology, Hepatology, and Nutrition, specific probiotics (mainly lactobacilli) with proven efficacy in viral diarrhea in children together with rehydration therapy may reduce duration and severity of diarrheal symptoms of acute gastroenteritis (Guarino et al., 2008).

2 CRITICAL HOST FACTORS IN VIRUS INFECTIONS

The mucosal surfaces are the primary portals of entry for respiratory and gastrointestinal viruses. Innate and adaptive immune responses are the most important defense mechanisms of the host in order to combat against virus infections. Responses may act directly on the virus, or indirectly on virus replication by altering or killing the infected cell. Innate responses are unspecific and function early, and include physical, chemical, and microbiological barriers, as well as many elements of the immune system (monocytes, macrophages, natural killer (NK) cells, and virus-induced cytokines). Adaptive responses are highly specific, but the induction requires several days or weeks. In virus infections adaptive responses mainly depend on cytotoxic T cells and antibodies. Hallmark of adaptive immune response is the development of immunological memory. Although the host defense mechanisms involved in a particular viral infection vary depending on the virus, dose and portal of entry, critical host factors and immunological events in virus infections are summarized in **Figure 2**.

2.1 INNATE IMMUNE SYSTEM

The innate immune system is essential for the initial detection of invading viruses and subsequent activation of adaptive immunity. As the initial infection is established in epithelial cells lining the respiratory or gastrointestinal (GI) tract, epithelial cells as well as tissue resident macrophages and dendritic cells (DCs) detect the presence of invading viruses through pattern-recognition receptors (PRRs). There are three classes of receptors, which sense viral components: retinoic acid-inducible gene I like receptors (RLRs), toll-like receptors (TLRs), and nucleotide oligomerization domain-like receptors (NLRs) (Takeuchi and Akira, 2009, Rathinam and Fitzgerald, 2011). RLRs are cytoplasmic proteins sensing viral dsRNA. TLRs detect viral components outside of cells and in cytoplasmic vacuoles after phagocytosis or endocytosis. In humans TLR2, TLR3, TLR4, TLR7, and TLR9 are involved in the recognition of viral components. On the plasma membrane TLR2 and TLR4 recognize viral envelope proteins. TLR3, TLR7, and TLR9 are localized on cytoplasmic vesicles such as endosomes and the endoplasmic reticulum. TLR3 recognizes dsRNA, and TLR7 and TLR9 recognize products of viral replication such as ssRNA and DNA with CpG motifs. NLRs are cytoplasmic proteins, which play a role in the production of mature interleukin (IL)-1 β in response to the dsRNA stimulation. The recognition of viral components by these receptors initiates a cascade of signals that results in the production of type I IFNs (including IFN- α

and IFN- β), and proinflammatory cytokines. IFNs are key antiviral cytokines which play an essential role in both innate and adaptive immune responses to viruses (Iwasaki and Medzhitov, 2004). Inflammatory signals trigger also the production of chemokines by epithelial cells, macrophages, and DCs that attract more innate immune cells to the infection site. The most important innate leukocytes involved in viral infection include the NK cells, which upon activation destroy virus infected cells by releasing small cytoplasmic granules of proteins called perforin and granzyme inducing cell apoptosis (Alter and Altfeld, 2006). Macrophages and DCs phagocytose infected cells, and process viral antigens functioning as antigen-presenting cells (APCs), and initiating the adaptive immune response.

2.2 ADAPTIVE IMMUNE SYSTEM

Adaptive immune system acts against both viral particles and infected cells. The most important leukocytes of the adaptive immune system consist of T and B lymphocytes. T-cells are intimately involved in cell-mediated immune responses, whereas B cells play a major role in the humoral immune response. Adaptive immunity to a virus infection initiates as naive T lymphocytes recognize viral antigens from the APCs through specific receptors located on the surface of T-cells. APCs present viral antigens bound to the major histocompatibility complex (MHC) proteins. CD4⁺ T lymphocytes recognize antigen on the MHC class II receptors, expressed only on APCs. CD8⁺ T lymphocytes recognize antigen on the MHC class I receptors, expressed on all nucleated cells. Upon specific antigen recognition T-cells undergo clonal amplification and progressively acquire differentiated functions (Kidd, 2003, Izcue and Powrie, 2008). CD8⁺ T cells mature into cytotoxic T lymphocytes (CTL), which specifically kill pathogen-infected cells. CD4⁺ helper T cells (Th) mature into two major subsets of effectors based on their cytokine expression profiles. Th1 cells coordinate the host response to intracellular pathogens, and have a central role in phagocyte activation, which promotes viral killing (Soghoian and Streeck, 2010). Th2 cells promote humoral immunity leading to activation of B cells (plasma cells) and the release of antibodies (immunoglobulins, Ig) into blood and tissue fluids. Other subsets include T regulatory cells, which negatively control the T-cell responses by producing specific cytokines and Th17 cells, which regulate the cellular immune response to influenza infection. As B cells and T cells are activated and begin to replicate, some of their offspring will become long-lived memory cells. Immunological memory can be in the form of either passive short-term memory or active long-term memory.

2.2.1 Cytotoxic mechanisms

Cytotoxic mechanisms are effective against virus infected cells. Once CTL has recognized viral antigen in a complex with the MHC I receptor, it migrates to the infection site. When an activated CTL contacts infected cells, it releases cytotoxins such as perforin, which form pores on the plasma membrane of a target cell, allowing ions, water and toxins to enter. The entry of protease granulysin induces the target cell to undergo apoptosis (Radoja et al., 2006). CTL cytotoxic activity is particularly important in preventing the replication of viruses. CTLs also release antiviral substances such as IFN- γ (Kagi et al., 1994), which enhance, and promote proliferation, activation, and prolonged survival of T cells.

2.2.2 Antibody responses

The most important mechanisms against viral particles are antibodies. After recognition of viral antigen on Th2 cells, B cells differentiate into plasma cells and begin to produce specific antibodies. Antibodies are produced against many epitopes on multiple virus proteins. A subset of these antibodies can block virus infection by neutralization, which inhibits virion binding to the receptors and uptake into cells, prevents uncoating of the viral genomes in endosomes, or cause aggregation of virus particles. Alternatively, antibodies can be elicited by virion fragments, or by viral proteins that are released from dying, infected cells (Hangartner et al., 2006). Antibodies that bind to surface-accessible determinants have been shown to help to control certain virus infections by activating the complement system, augmenting phagocytosis and/or promoting antibody-dependent cellular cytotoxicity. The main antibody isotypes in the virus specific humoral immune response are IgA, IgM, and IgG. For most viruses, specific IgA antibodies play a key role in clearing the virus from mucosal sites during primary and secondary infection (Russell 1999). IgG and IgM antibodies are more predominant in viremic infections. Serum IgAs are also produced after influenza virus infection (Voeten et al., 1998, Rothbarth et al., 1999).

Figure 2. A schematic presentation of innate and adaptive immune responses during viral infection.

3 PROBIOTICS IN THE PREVENTION OF RESPIRATORY AND GASTROINTESTINAL VIRUS INFECTIONS

3.1 OVERVIEW OF PROBIOTICS

According to WHO probiotics are live microorganisms, which administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). Probiotics must be able to survive in the GI tract and to proliferate in the gut, and be resistant to gastric juices and bile. In addition, they should exert benefits to the host through growth and/or activity in the human body. In order to confer health benefits, they should be non-pathogenic and non-toxic, and provide protection against pathogenic micro-organisms by means of multiple mechanisms (FAO/WHO, 2001). In addition, probiotics should be lacking transferable antibiotic resistance. Different bacterial strains of the same genus and species, verified also by genomic information, may exert completely different effects on the host. The most promising health effects of probiotics in human intervention studies include amelioration of acute **diarrhea in children, reduction of the risk of RTIs, relief of children's milk allergy/atopic dermatitis, and relief of irritable bowel syndrome** (Wolwers et al., 2010, Aureli et al., 2011). Probiotics may exert their beneficial health effects by normalization of microbiota, modulation of immune response, and metabolic functions. They may also enhance the resilience of microbiota against detrimental outside factors. However, the molecular mechanisms behind the effects are largely unknown (Marco et al., 2006). The most commonly investigated and commercially available probiotic species are mainly lactic acid bacteria of *Lactobacillus* ssp. and *Bifidobacterium* ssp. In addition, several other species of the genus such as *Propionibacterium*, *Streptococcus*, *Bacillus*, *Enterococcus*, and yeasts are used. *Lactobacillus rhamnosus* GG (ATCC 53103) is one of the most extensively studied probiotic strains in humans and experimental studies (**Figure 3**). Since its isolation from an adult human in 1985, it has gained a safe history of use in food products since 1990. The strain provides excellent survival in and transient colonization of the GI tract, which is attributed to its adhesion capacity to the intestinal mucus and epithelial cells (Alander et al., 1999, Saxelin et al., 2010).

In the industry, probiotics are mainly incorporated into fermented foods (milk, cheese, yoghurt), but also in non-dairy products such as chocolate, cereals, and juices (Anal and Singh, 2007). Probiotic supplements are also available in different formulations such as capsules, sachets or tablets, and with and without prebiotics such as fructo- and galacto-

oligosaccharides. Ingested probiotic strains do not become established members of the normal intestinal microbiota, but generally persist only for the period of consumption and for some months thereafter (Gueimonde et al., 2006, Corthésy et al., 2007). Probiotics must also retain their viability during storage, manufacturing process of the functional food, and transit through the stomach and small intestine. The concentration of probiotics in research trials and in commercial products varies significantly, and no international standards are available regarding the levels of required bacteria (Parvez et al., 2006).

Probiotic bacteria are often a part of/members of the normal gastrointestinal microbiota, and therefore probiotic therapy is generally considered as safe (Boyle et al., 2006). However, probiotic therapy has raised potential safety concerns including systemic infections, toxic or metabolic effects on the GI tract, and the transfer of antibiotic resistance in the gastrointestinal microbiota (Sanders et al., 2010). In Finland, increased consumption of probiotic products containing *L. rhamnosus* GG has not resulted in significant increase in *Lactobacillus* bacteremia (Salminen et al., 2002). In addition, *L. rhamnosus* GG consumption is regarded as safe in immunocompromised HIV-infected patients (Salminen et al., 2004). In addition, clinical studies show that *L. rhamnosus* GG is safe treatment for neonates and infants (Isolauri et al., 1991, Van Niel et al., 2002, Grandy et al., 2010, Szajewska et al., 2011, Luoto et al., 2010). However, in rare cases, some studies have reported *Lactobacillus* septicaemia in children (Land et al., 2005), or in immunocompromised subjects (Kalima et al., 1996), and detrimental effects in subjects with hepatitis (Besselink et al., 2008). Moreover, the European Food Safety Authority has concluded that there are no specific safety concerns regarding *Lactobacillus*, *Bifidobacterium*, or *Propionibacterium* strains as they have a long history of safe use in food (EFSA, 2011). However, it should be taken into consideration that the safety of probiotics has not been as systematically investigated as in drugs and the safety evaluation is partly based on long term experience.

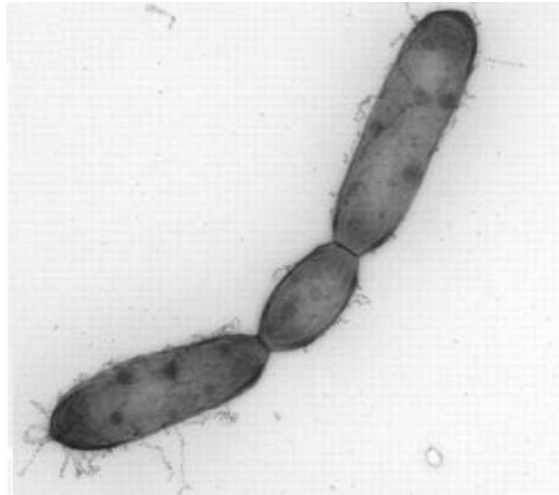


Figure 3. The illustration of *L. rhamnosus* GG with electronic microscope. Adapted from Kankainen et al. 2009 (Kankainen et al., 2009).

3.2 MECHANISMS OF ACTIONS OF PROBIOTICS IN VIRUS INFECTIONS

Clinical and animal studies have demonstrated that specific probiotics are effective in viral infections, but the underlying mechanisms are not completely understood. Additionally, the strain to strain variation may be relatively large concerning strain properties and efficacy. Possible antiviral mechanisms of probiotics include 1) hindering the adsorption and, 2) cell internalization of the virus, 3) production of metabolites and substances with a direct antiviral effect, and 4) crosstalk (immunomodulation) with the cells in establishing the antiviral protection. The possible mechanisms by which probiotics may influence in virus infections are presented in **Figure 4**.

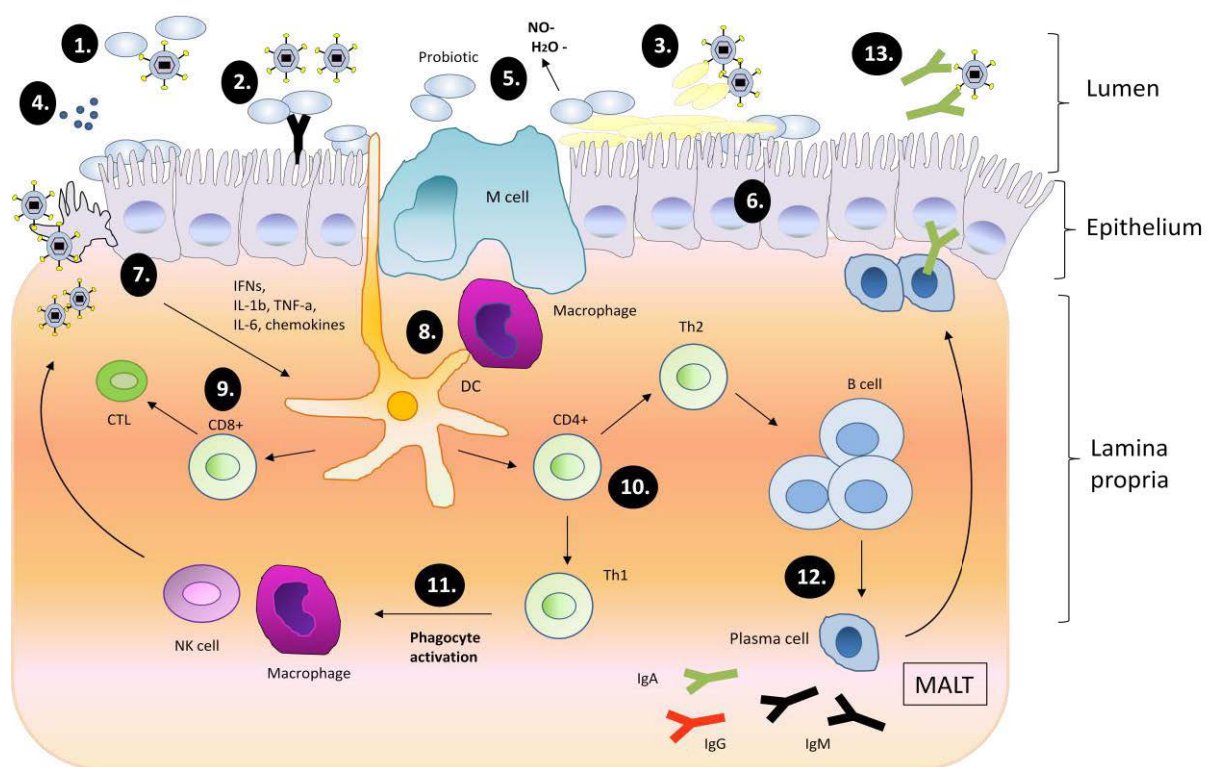


Figure 4. Probiotics may prevent viral infections through several mechanisms.

- 1 Probiotic bacteria may bind directly to the virus and inhibit virus attachment to the host cell receptor.
- 2 Adhesion of probiotics on the epithelial surface may block viral attachment by steric hindrance, cover receptor sites in a non-specific manner, or by competing for specific carbohydrate receptors.
- 3 Probiotics may induce mucosal regeneration: intestinal mucins may bind to viruses, and inhibit their adherence to epithelial cells and inhibit virus replication.
- 4 Probiotics also show direct antimicrobial activity against pathogens by producing antimicrobial substances.
- 5 Induction of low grade NO production and dehydrogenase production may have antiviral activities.
- 6 Probiotics promote normalization of mucosal barrier and increase integrity of mucosal cells.
- 7 Modulation of immune response through epithelial cells.
- 8 Modulation and activation of immune responses through macrophages and DCs.
- 9 Upon activation CD8+ T lymphocytes differentiate into CTLs, which destroy virus infected cells.
- 10 CD4+ T lymphocytes differentiate into Th1 and Th2 cells.
- 11 Th1 activates phagocytes promoting virus killing.
- 12 Th2 induce proliferation of B-cells, which travel to secondary lymphatic organs in mucosa associated lymphoid tissue (MALT) and differentiate into Ig-producing plasma cells, which may migrate back to the infection site.
- 13 Secretory antibodies neutralize the virus.

3.2.1 Antagonisms to pathogens

Respiratory and GI tract are covered by mucosal epithelial surfaces which are constantly exposed to numerous micro-organisms, and serve as primary ports of entry for most infectious viruses. Virus attachment to a host cell is the first essential step in the disease process, and therefore interruption of this attachment could be beneficial to the host. Probiotic bacteria may bind directly to the virus, and inhibit virus attachment to the host cell receptor. For instance, there is evidence that *in vitro* specific strains of lactobacilli and bifidobacteria are able to bind and inactivate RV (Salminen et al., 2010) and vesicular stomatitis virus (flu-like virus) (Botić et al., 2007). In addition, adhesion of probiotics on the epithelial surface (Juntunen et al., 2001, Ouwehand et al., 2001, Ouwehand et al., 2002) may block viral attachment by steric hindrance, cover receptor sites in a non-specific manner, or inhibit binding of a virus to specific carbohydrate receptors. Luminal secretions (mucus, glycolipids, protective peptides) and antimicrobial peptides (defensins) may also protect epithelial cells from virus infections. Intestinal mucins may bind to viruses through specific mucin-bacterial/viral interaction, inhibit their adherence to the epithelial cells (Deplancke and Gaskins, 2001), and inhibit virus replication (Yolken et al., 1994). Probiotics may induce mucosal regeneration by increasing mitose rate in the small intestine, increasing the numbers of cells in the villi (Banasaz et al., 2002, Pipenbaher et al., 2009), and promoting intestinal epithelial homeostasis via soluble proteins (Yan et al., 2007). Probiotics also show direct antimicrobial activity against pathogens by producing antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, short chain fatty acids, biosurfactants, and bacteriocins (Servin, 2004). In experimental studies in epithelial cells and macrophages metabolic products of specific lactobacilli and bifidobacteria prevented vesicular stomatitis virus infection in a strain specific manner (Botić et al., 2007), and metabolites of yoghurts showed antiviral activity inhibiting influenza virus and enterovirus replication (Choi et al., 2009). Supernatants of *L. plantarum* Probio-38 and *L. salivarius* Probio-37 inhibited cytopathic effect of Transmissible Gastroenteritis Coronavirus (Kumar et al., 2010). Nitric oxide (NO) has also been recognized as a compound with antiviral properties (Kleinert et al., 2004, Xu et al., 2006). Induction of low-level synthesis of NO may be involved in the protective actions of probiotics against viruses in the GI tract or respiratory cells as shown in rat and *in vitro* models (Korhonen et al., 2001, Sobko et al., 2006, Ivec et al., 2007, Pipenbaher et al., 2009, Maragkoudakis et al., 2010). It is widely known that the intestinal permeability increases in gastrointestinal virus infections, as viruses attach to cell receptors below the tight junctions on the basolateral membrane, thus modifying tight junctions and disturbing the barrier (Guttman and Finlay, 2009). One possible mechanism of probiotics is

the promotion of gut defense barrier by normalizing increased permeability and disturbed gut microecology (Isolauri et al., 1993, Otte and Podolsky, 2004).

3.2.2 Immunomodulation

An effectively functioning immune system is important for the maintenance of physiological integrity and health. The immune system provides defence against infections caused by pathogenic microorganisms. It also modulates our health and well-being in many ways sometimes by up- or downregulating the defence system. An optimally functioning immune system is fundamental for protection against infectious diseases. Induction of antiviral cytokines such as IFNs, as well as proinflammatory cytokines and chemokines upon antigen recognition in epithelial cells or underlying effector cells (macrophages, DCs, neutrophils) play a key role in virus infections by initiating cell mediated viral elimination and adaptive immune responses. One possible probiotic mechanism against virus infections could be the stimulation of the gut immune system (Schiffrin and Blum, 2002). In the gut epithelial cells and/or APCs, probiotics are recognised by TLRs (Miettinen et al., 1998, Vinderola et al., 2005, Foligne et al., 2007, Miettinen et al., 2008). Probiotics may therefore modulate cytokine expression patterns through epithelial cells (O'Hara et al., 2006), and through underlying professional APCs, such as macrophages and DCs (Miettinen et al., 2000, Veckman et al., 2003, Veckman et al., 2004, Latvala et al., 2009, Latvala et al., 2011, Weiss et al., 2011). In murine DCs *L. acidophilus* NCFM and *L. acidophilus* X37 were able to trigger the expression of viral defense genes (IFN- β , IL-12, IL-10) (Weiss et al., 2010). Also stimulation of human macrophages or DCs with specific probiotics including *L. rhamnosus* GG, have induced proinflammatory cytokine (tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and IFN- γ) and chemokine (CCL2, CCL3, CCL5, CCL7, CCL20 and CXCL8) production (Miettinen et al., 2000, Veckman et al., 2003, Latvala et al., 2009). Indeed, many experimental studies *in vitro* show that certain strains of probiotics are capable of providing protection against virus infections by stimulating antiviral, cytokine, and chemokine responses in respiratory and gastrointestinal epithelial cells, or immune cells (**Table 4**). In addition, oral administration of lactobacilli in mice may affect respiratory virus infections (such as influenza) by reducing virus titer in the lungs, and increasing survival rate of the animals via stimulating innate immune responses (**Table 5**). There is also evidence that intranasal administration of probiotics is able to protect against respiratory virus infection by stimulating innate immune responses directly in the respiratory epithelium (Hori et al., 2001, Harata et al., 2010, Izumo et al., 2010, Harata et al., 2011, Gabryszewski et al., 2011, Youn et al., 2012).

Oral ingestion of viral antigens also induces in the gut local IgA synthesis. After antigenic stimulation in the Peyer's patches, T and B lymphocytes activate and travel via blood circulation to secondary lymphatic organs in the distal mucosal effector sites of the GI and respiratory tracts (so-called mucosa-associated lymphoid tissue, MALT), where B cells differentiate into Ig-producing plasma cells. By this mechanism orally-ingested probiotic bacteria may initiate an immune response in the gut, which then leads to enhanced responses at other mucosal surfaces. Data from animal studies indicate that several specific lactobacilli and bifidobacteria provide protection against respiratory and gastrointestinal virus infections by inducing the synthesis of virus-specific Igs in intestinal and respiratory secretions, in Peyer's patch cells and in serum (Table 6).

Table 4. Effects of probiotic bacteria on innate immune responses against gastrointestinal and respiratory virus infections *in vitro*.

Probiotic strains	Virus	Model/Study design	Main findings	Reference
<i>L. acidophilus</i> NCFM <i>L. rhamnosus</i> GG	Porcine RV, HRV Wa	<i>In vitro</i> : Probiotic incubation of procine epithelial cells (IPEC-J2) before and after infection	Virus infection ↔ Effects with L.GG: After RV infection mucin secretion and IL-6 ↓ Effects with LA treatment: Prior RV infection, RV replication and IL-6 ↑	Liu et al. 2010
<i>L. plantarum</i> 299v	Bovine RV	<i>In vitro</i> : Probiotic incubation of primary bovine intestinal epithelial cells before infection	RV infection ↓ IFN-α ↑, TRL3 ↑	Thompson et al. 2010
<i>L. paracasei/rhamnosus</i> Q85 <i>L. paracasei</i> A14 <i>L. paracasei</i> F19 <i>B. longum</i> Q46	Vesicular stomatitis virus	<i>In vitro</i> : Preincubation of pig alveolar macrophages with bacteria before infection	Strain spesific effects: Cell survival against virus infection ↑ NO production ↑ IL- 6 , INF-gamma ↑	Ivec et al. 2007
<i>L. pentosus</i> S-PT84	Sendai virus	<i>Ex vivo</i> : Oral ingestion of probiotic in diet 14d before experimnt in mice splenocytes and plasmacytoid DCs	IFN-α ↑	Izumo et al. 2011

↑ = increase; ↓ = decrease; ↔ = no effect

Several studies also suggest that specific probiotics may enhance the immunogenicity of viral vaccines in healthy human subjects. Orally administered *L. casei* strain GG improved the immunogenicity of RV vaccine in children by increasing RV-specific IgM secreting cells, and enhancing significantly IgM and IgA seroconversion (Isolauri et al., 1995). Vaccination against *diphtheria* and *tetanus* toxoids, poliomyelitis virus, *Haemophilus influenzae*, and *Bordetella pertussis* showed that infants harboring detectable levels of *B. longum infantis* in the intestine had two months after vaccination higher anti-poliovirus IgA titers (Mullie et al., 2004). Moreover, in healthy adults *L. rhamnosus* GG or *L. acidophilus* CRL431 increased poliovirus vaccine induced neutralizing antibody titers, and increased in serum the formation of poliovirus-specific IgA and IgG (De Vrese et al., 2005). *L. rhamnosus* GG was also effective in inducing protective immune response against H3N2 strain in influenza virus vaccine (Davidson et al., 2011). Moreover, *L. fermentum* CECT5716 ingestion in adults resulted in lower influenza-like illness, increased proportion of NK cells in blood, significantly higher TNF- α , and increased anti-influenza specific IgA, and IgM after influenza vaccination (Olivares et al., 2007). Consumption of *B. animalis* subsp. *lactis* Bb12 or *L. paracasei* ssp. *paracasei* *L. casei* 431431 also showed significantly greater increase in influenza virus vaccine-specific IgG antibodies in plasma and secretory IgA in saliva (Rizzardini et al., 2012). In the elderly the consumption of fermented yoghurt with *L. casei* DN-114 001, increased significantly influenza -specific antibody titers after influenza vaccination, especially against influenza B virus (Boge et al., 2009). These studies suggest that orally-ingested lactobacilli and bifidobacteria have an adjuvant-like effect on the humoral responses.

In summary, based on animal and experimental studies in vitro, specific probiotics may mediate their health effects against viral infections with their ability to exclude viruses, strengthen the tight junctions between enterocytes, produce antimicrobial and potentially antiviral substances, and stimulate host-cell immune defenses. In addition, specific probiotics enhance the immunogenicity of vaccines by stimulating humoral responses. However, the effects of probiotics seem to be highly strain specific.

Table 5. Effects of probiotic bacteria on innate immune responses against respiratory virus infections in animal experiments.

Probiotic strains	Virus	Model/Study design	Main findings	Reference
<i>L. casei</i> Shirota	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=14/group, intranasal administration 3x daily for 3d before infection	IL-12, IFN- γ , TNF- α in MLN cells \uparrow Virus titres in nasal wash \downarrow Mice survival rate \uparrow	Hori et al. 2001
<i>L. casei</i> Shirota	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=5/group, oral administration 5x/week for 3 weeks before infection	Mice survival rate \uparrow Pulmonary NK cell activity \uparrow IL-12 production by MLN cells \uparrow Viral titres in nasal washings \downarrow	Yasui et al. 2004
<i>L. plantarum</i> L-137	IFV A/FM1/47 (H1N1)	C57BL/6 mice, n=6/group, intragastric administration daily 7d before and 6d after infection	Viral titers in the lung \downarrow IFN- β in sera \uparrow	Maeda et al. 2009
<i>L. pentosus</i> S-PT84	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=11-12/group, intranasal administration of S-PT84 1x daily for 3d	Mice survival rate \uparrow (especially orally) virus titter in BALF \downarrow IL-12, IFN- γ in MLN cells \uparrow IL-12, IFN- α in BALF \uparrow NK cell activity \uparrow	Izumo et al. 2010
<i>L. gasseri</i> TMC0356 <i>L. rhamnosus</i> GG	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=13/group, oral administration daily for 19d	Effects with both bacteria: Clinical symptom scores \downarrow Pulmonary virus titres \downarrow Effects with <i>L.gasseri</i> : Peyer's patches: mRNA IL-12, IL-15, IL-21 \uparrow Lungs: mRNA IFN- γ , TNF, IL-12, perforin-1 \uparrow	Kawase et al. 2010 Kawase et al. 2012
<i>L. plantarum</i> 05AM2 <i>L. plantarum</i> 06TCa8 <i>L. paracasei</i> ssp. <i>paracasei</i> 06TCa19 <i>L. paracasei</i> ssp. <i>paracasei</i> 06TCa22 <i>L. paracasei</i> ssp. <i>tolerans</i> 06TCa39 <i>L. plantarum</i> 06TCa40 <i>L. paracasei</i> ssp. <i>paracasei</i> 06TCa43 <i>L. plantarum</i> 06CC2 <i>L. delbrueckii</i> ssp. <i>lactis</i> 06TC3 <i>L. plantarum</i> 06CC9	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=5-10/group, oral administration 2xdaily for 10d starting 2d before infection	Effects with <i>L.plantarum</i> 06CC2: Protected body weight loss of infected mice Virus yields in the lungs \downarrow Mice survival \uparrow No.of macrophages and neutrophils in BALF \downarrow TNF- α in BALF \downarrow INF- α , IL-12, IFN- γ , NK cell activity \uparrow mRNA IL-12 receptor, IFN- γ in Peyer's patches \uparrow	Takeda et al. 2011

Table 5. Continued

Probiotic strains	Virus	Model/Study design	Main findings	Reference
<i>L. gasseri</i> TMC0356 <i>L. rhamnosus</i> GG	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=13-19/group, intranasally administered 3x daily for 3d	<i>L. gasseri</i> TMC0356: Morbidity ↓ Survival rate of mice ↑ mRNA IL-1β, TNF, IL-10, MCP-1 ↑ <i>L. rhamnosus</i> GG: Accumulated symptoms ↓ Survival rate of mice ↑ mRNA IL-1β, TNF, IL-10, and MCP-1 ↑	Harata et al. 2010 Harata et al. 2011
<i>L. fermentum</i> -1 <i>L. brevis</i> -2	IFV A/NWS/33 (H1N1)	BALB/c mice, n=10/group, intranasal or oral administration for 21d before infection	Mice survival ↑ Virus titer ↓ Lung IgA and IL-12 ↑ Lung TNF-α and IL-6 ↓ Lung IFN-γ ↔	Youn et al. 2012
<i>B. longum</i> BB536	IFV A/PR/8/34 (H1N1)	BALB/c mice, oral administration daily for 2 weeks before infection	Symptom score ↓ Loss of body weight ↓ Lung virus titer ↓ Lung IL-10, IL-12 ↔ Lung IL-6, IFN-γ (↓)	Iwabuchi et al. 2011
<i>L. casei</i> Shirota <i>L. fermentum</i>	Murine cytomegalovirus	ICR-mice, N/A	Effects with <i>L. casei</i> : Mice survival ↑ Virus titers ↓ NK cell activity ↑	Ohashi et al. 1989
<i>L. plantarum</i> NCIMB 8826 <i>L. reuteri</i> F275	Pneumonia virus of mice J3666	BALB/c and C57BL/6 mice, n=5-10/group, intranasal inoculation 2 weekly doses 2 weeks before infection	Protection against virus infection ↑ Granulocyte recruitment ↓ CXCL10, CXCL1, CCL2, TNF ↓ Virus recovery ↓	Gabryszewski et al. 2011

IFV = influenza virus; ↑ = increase; ↓ = decrease; ↔ = no effect; MLN = mediastinal lymph node

Table 6. Reported effects of probiotic bacteria on antibody responses against gastrointestinal and respiratory virus infections in animal experiments.

Probiotic strains	Virus	Model	Main findings	Reference
<i>B. bifidum</i> ATCC 15696	Murine RV EDIM-5099	BALB/c mice, n=15-19/group, orally administered in saline 5d before and 23d after infection	RV antigen shedding ↓ Onset of acute diarrhea ↓ Serum RV IgG antibody responses ↔	Duffy et al. 1994
<i>B. breve</i> YIT4064	SA-11 RV	Oral administration of bacteria in mice	Protection against RV diarrhea ↑ anti-RV IgA in milk and feces ↑	Yasui et al. 1995
Combination of: <i>B. bifidum</i> ATCC 15696 <i>B. infantis</i> ATCC 15697 with or without prebiotics	Rhesus RV	BALB/c mice, n=3-8/group, 1 dose weekly orally administered up to 7 weeks after infection	Bifidobacteria alone or with prebiotics: Onset of diarrhea ↓ Recovery of diarrhea ↑ RV-specific IgA in feces and in serum ↑	Qiao et al. 2002
Combination of: <i>L. acidophilus</i> strain NCFM <i>L. reuteri</i> strain ATCC 23273	Human RV Wa strain and RV vaccination	Gnotobiotic pigs, n=4-10/group, oral administration in peptone water with gradual concentrations 2x before infection and 3x after infection	Total intestinal IgA cell responses ↑ Total serum IgM, intestinal IgM, IgG ↑ RV shedding or diarrhea ↔ RV-specific IFN-γ producing CD8+ T cell responses in ileum and spleen ↑ IgA and IgG antibody-secreting cell responses in ileum ↑ Serum IgM, IgA and IgG antibody and RV neutralizing antibody titers ↑	Zhang et al. 2008a Zhang et al. 2008b
<i>B. breve</i> YIT4064	IFV A/PR/8/34 (H1N1)	BALB/c mice, n=9-10/group, oral administration in food 15 weeks before infection	Protection against virus infection ↑ Serum anti-IFV IgG ↑	Yasui et al. 1999
<i>L. pentosus</i> b240	IFV A/PR/8/34 (H1N1)	BALB/c/Cr Slc (SPF), n=10/group, administration daily by gavage all experiment (1mo)	Virus titer ↓ Anti-IFV IgA, IgG titers in BALF and plasma on day 7 ↑	Kobayashi et al. 2011
<i>L. delbrueckii ssp. Bulgaricus</i> OLL1073R-1 and its exopolysaccharides	IFV A/PR/8/34 (H1N1)	BALB/c mice, oral administration for 21d prior infection	In both groups: Mice survival ↑ Virus titer ↓ Anti-IFV IgA, IgG1 in BAL ↑ NK cell activity ↑	Nagai et al. 2011

↑ = increase; ↓ = decrease; ↔ = no effect; IFV = influenza virus; BALF = bronchoalveolar lavage fluid

3.3 HEALTH EFFECTS OF PROBIOTICS IN VIRUS INFECTIONS

3.3.1 Animal experiments

Animal experiments provide insight of clinical effects of probiotics against certain virus infections. Studies of respiratory virus infections in mice provide strong evidence that certain strains of lactobacilli and bifidobacteria protect from virus infection by reducing virus titers in the lungs or nasal washings and RTI symptoms, or increasing body weight during infection and mice survival (Ohashi et al., 1989, Yasui et al., 1995, Yasui et al., 1999, Hori et al., 2001, Yasui et al., 2004, Maeda et al., 2009, Harata et al., 2010, Izumo et al., 2010, Kawase et al., 2010, Gabryszewski et al., 2011, Izumo et al., 2011, Harata et al., 2011, Kobayashi et al., 2011, Nagai et al., 2011, Takeda et al., 2011, Kawase et al., 2012, Youn et al., 2012,). There are also few studies concerning gastrointestinal virus infections in animals, which concentrate in RV infections. Specific probiotics have reduced RV diarrhea occurrence, duration, and severity, and reduced RV shedding in the feces or viral load in the intestine (Duffy et al., 1994, Guérin-Danan et al., 2001, Qiao et al., 2002, Pant et al., 2007, Moreno Munoz et al., 2011). In addition, some lactobacilli have reduced RV induced histological changes in the intestine (Guérin-Danan et al., 2001, Pant et al., 2007, Preidis et al., 2012).

3.3.2 Clinical trials

Human intervention studies have demonstrated that specific probiotics may be able to shorten the duration or reduce the risk of certain viral infections. Several trials in children address the effects of probiotics in the prevention of respiratory infections. *L. rhamnosus* GG (Hatakka et al., 2001, Hatakka, 2007), *L. casei* DN114001 (Cobo Sanz et al., 2006), *B. animalis* subsp. *lactis* Bb12 (Taipale et al., 2011), and a combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS (Hatakka, 2007), and a combination of *L. rhamnosus* GG and *Bifidobacterium lactis* Bb12 (Rautava et al., 2009) have reduced the incidence or risk of respiratory infections. Treatment with *L. rhamnosus* GG (Hatakka et al., 2001) or a combination of *L. reuteri* SD112 and *B. lactis* Bb12 (Weizman et al., 2005) resulted in fewer days of absence from day care due to illness. In addition, probiotics such as *Lactobacillus* have reduced the incidence of otitis media in healthy children and in newborns (Niittynen et al. 2012). However, the viral etiology of RTIs was investigated only in one study (Hatakka, 2007).

The strongest health benefits of probiotics in virus infections are demonstrated with *L. rhamnosus* GG in RV gastroenteritis in infants and in children (**Table 7**). These studies show that *L. rhamnosus* GG is able to shorten RV induced diarrheal phase/duration of diarrhea (Isolauri et al., 1991, Kaila et al., 1992, Isolauri et al., 1994, Majamaa et al., 1995, Guarino et al., 1997, Shornikova et al., 1997c, Guandalini et al., 2000), which was also shown in meta-analysis (Szajewska et al., 2007). According to another meta-analysis, *L. rhamnosus* GG reduces the risk of symptomatic RV gastroenteritis (Szajewska et al., 2011). *L. rhamnosus* GG reduces also the number of RV infections (Salazar-Lindo et al., 2004). In a study conducted in India, however, *L. rhamnosus* GG did not have an impact on the duration of RV diarrhea, vomiting, or in the length of hospital stay (Basu et al., 2007). In undernourished Peruvian children, *L. rhamnosus* GG did not reduce the number of RV induced diarrhea. *L. rhamnosus* GG was also ineffective in preventing nosocomial RV infections (Mastretta et al., 2002). Similar effects were seen in a study by Szajewska and others 2001, although in that study the risk of RV gastroenteritis reduced significantly (Szajewska et al., 2001). *L. rhamnosus* GG promotes recovery from RV diarrhea possibly via augmentation of the local immune defense by enhancing nonspecific humoral response (IgG, IgA, and IgM -secreting cell numbers) during the acute phase of RV infection (Kaila et al., 1992). Furthermore *L. rhamnosus* GG promotes the development of RV specific IgA response, which may be relevant in protection against reinfections (Kaila et al., 1992, Majamaa et al., 1995, Kaila et al., 1995). Probiotic properties may alter in production processes, and thus the ability of *L. rhamnosus* GG to counteract diarrhea can be associated with the production method or food matrix as demonstrated by Grześkowiak et al. 2011 (Grześkowiak et al., 2011).

Other *Lactobacillus* and *Bifidobacterium* strains have been studied in the treatment of RV gastroenteritis in children and infants as well (**Table 8**). *L. reuteri* in particular, has been effective in shortening the duration of RV diarrhea and number of days of illness (Shornikova et al., 1997a, Shornikova et al., 1997b). *L. sporogenes* shortened the duration of RV diarrhea (Chandra, 2002) *L. rhamnosus* 35 reduced significantly fecal RV concentration (Fang et al., 2009). Although *L. paracasei* strain ST11 had a clinically significant benefit in the management of non-RV-induced diarrhea, ST11 treatment against severe RV diarrhea was ineffective (Sarker et al., 2005). Children with RV infection received either *B. Bb12* alone or together with *Streptococcus thermophilus* had less RV infections when measured by RV specific IgA concentration in the saliva (Phuapradit et al., 1999). Reduction of diarrhea duration in infants was also seen when *S. boulardii* was given to children within 72 hours after the onset of acute diarrhea although the number of RV infections were similar between groups (Corrêa et al., 2011).

Overall, studies conducted with bacterial combinations are also promising. A combination of *L. rhamnosus* strains, or *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246, or *L. acidophilus*, *L. rhamnosus*, *B. longum* and *S. boulardii* significantly reduced RV diarrhea and parenteral dehydration, consistency of feces, or duration of fever (Rosenfeldt et al., 2002, Szymanski et al., 2006, Grandy et al., 2010). Supplementation of infant formula with *B. bifidum* and *S. thermophilus* reduced significantly the incidence of acute RV diarrhea and RV shedding (Saavedra et al., 1994). Therapy with probiotic mixture VSL#3 (*L. acidophilus*, *L. paracasei*, *L. bulgaricus*, *L. plantarum*, *B. breve*, *B. infantis*, *B. longum*, *S. thermophilus*) resulted in earlier recovery and reduced frequency of ORS administration during RV diarrhea (Dubey et al., 2008). In another study probiotic product containing *S. faecalis* T-110, *Clostridium butyricum* TO-A, *Bacillus mesentericus* TO-A, and *L. sporogenes* reduced the number of RV episodes, mean duration of diarrhea, degree of dehydration, duration and volume of ORS therapy and intravenous fluid therapy, and duration of RV shedding (Narayanappa, 2008). However, therapy of *L. casei* together with *L. acidophilus*, or *S. boulardii* alone did not affect the number of stools between RV negative and positive children, but significantly reduced the number of daily stools, diarrhea duration, and vomiting (Gaón et al., 2003).

In summary, animal experiments have shown that certain strains of probiotics provide protection against respiratory and gastrointestinal viruses. In clinical studies, probiotics seem to be beneficial in the treatment and prevention of respiratory virus infections, although viral etiology has not been investigated. The strongest evidence concerning the treatment of RV gastroenteritis is attributed to L. rhamnosus GG, which seems to promote also humoral RV specific response. Combinations of several bacteria may ameliorate symptoms or reduce virus shedding as well.

Table 7. The reported effects of *L. rhamnosus* GG on RV infections in clinical interventions.

Subjects	Design and duration	Probiotic supplementation	Main findings: probiotic vs. placebo	Reference
Children aged 4-45 mo with acute gastroenteritis (n=71)	RPC	<i>L. casei</i> ssp. GG fermented milk 10^{10-11} colony forming unit (cfu) , or 1x dose freeze dried powder, or placebo 2x daily for 5d on recovery from RV diarrhea	<i>L. casei</i> ssp. GG fermented milk or powder: Diarrhea duration ↓ Number of RV in feces ↔	Isolauri et al. 1991
Children aged 7-33 mo with acute gastroenteritis(n=44)	RDBPC	<i>L. rhamnosus</i> GG (10^{10} - 10^{11} cfu) in fermented milk and placebo, 2x daily for 5d	Diarrhea duration ↓ Nonspecific humoral response during the acute phase infection ↑ IgG, IgA, and IgM Ig-secreting cell numbers ↑ At convalescence, majority in L. GG group developed RV IgA specific cell response	Kaila et al.1992
Children aged 5-28 mo with acute RV diarrhea (n=42)	RPC	<i>L. casei</i> GG (10^{10} cfu) or control group, oral administration 2x daily for 5d	RV diarrhea duration ↓	Isolauri et al. 1994
Children aged <4 years with acute gastroenteritis (n=41)	RDBPC	Inactive or live <i>L. casei</i> GG 2x daily for 5d	Live L.GG: RV serum IgA antibody responses ↑	Kaila et al. 1995
Children aged 6-35 mo (n=49)	RCT	<i>L. casei</i> ssp. <i>casei</i> GG , <i>L. casei</i> ssp. <i>rhamnosus</i> , or combination of <i>Streptococcus thermophilus</i> / <i>L. delbrueckii</i> ssp. <i>bulgaricus</i> 2x daily for 5d	Effects with all bacteria: Mean duration of diarrhea ↓ L.GG therapy: Antibody secreting cell IgA to RV ↑ Serum IgA at convalescent stage ↑	Majamaa et al. 1995
Children aged 3-36 mo with mild diarrhea (n=100)	RPC	ORS or ORS+ <i>L. casei</i> strain GG (3×10^9 cfu) in milk 2x daily for 5d	Diarrhea duration ↓ RV shedding in stools ↓	Guarino et al. 1997
Children aged 1- 36 mo with acute diarrhea (n=123)	RPC	ORS + <i>L. GG</i> 5×10^9 cfu or placebo 2x daily for 5d	RV diarrhea duration ↓ Cumulative number of diarrhea stools ↓	Shornikova et al. 1997c
Undernourished children aged 6-24 mo (n=200)	RDBPC, 15 mo	<i>L. rhamnosus</i> GG (10^{10} cells) or placebo in capsules mixed with liquid gelatin 6d a week	Incidence of diarrhea ↓ (especially in 18- 29-mo age group) ADV in stools ↓ Other pathogens ↔	Oberhelman et al. 1999

Table 7. Continued

Subjects	Design and duration	Probiotic supplementation	Main findings: probiotic vs. placebo	Reference
Children aged 1 mo-3 years with acute-onset diarrhea (n=140)	RDBPC	ORS+ <i>L.rhamnosus</i> GG (10 ¹⁰ cfu) or placebo administered first 4-6h	Diarrhea duration ↓ RV diarrhea duration ↓	Guandalini et al. 2000
Hospitalized children aged 1-36 mo (n=81)	RDBPC	<i>L.rhamnosus</i> GG (6×10 ⁹ cfu) or placebo orally 2x daily for the duration of hospital stay	Risk of RV gastroenteritis ↓ No. of RV in stool ↔	Szajweska et al. 2001
Hospitalized children aged 1-18 mo for common infections (n=220)	RDBPC, 17 mo	Capsules of <i>L.rhamnosus</i> GG (10 ¹⁰) or placebo daily during hospital	No. of RV infections ↔	Mastretta et al. 2002
Infants (male) aged 3-36 mo with acute watery diarrhea (n=89)	RDBPC	<i>L. casei</i> GG (10 ⁹ cfu/ml) or placebo in milk powder in water after ORS therapy	Diarrhea duration ↔ RV in stool ↓	Salazar-Lindo et al. 2004
Young children under 2 years with acute watery diarrhea (n=662)	RDBPC, 12 mo	<i>L.rhamnosus</i> GG powder (10 ⁶ cells) dissolved in ORS or ORS alone 2x daily for at least 7d	Daily frequency or duration of RV diarrhea/vomiting ↔ Length of hospital stay ↔	Basu et al. 2007
Young children (n=988)	Meta-analysis: 8 RCT	<i>L.rhamnosus</i> GG	RV diarrhea duration ↓ Risk of diarrhea ↓ Duration of hospitalization ↓	Szajewska et al. 2007
Hospitalized children aged 1 mo-18 years (n=1902)	Meta-analysis: 3 RCT	<i>L.rhamnosus</i> GG or placebo/or no intervention	Rates of diarrhea ↓ Symptomatic RV gastroenteritis ↓	Szajweska et al. 2011

RDBPC = randomized double-blind placebo-controlled; RPC = randomized placebo-controlled; RCT = randomized clinical trial; mo = months; ↑ = increase; ↓ = decrease; ↔ = no effect

Table 8. The reported effects of other bacteria on RV infections in clinical interventions.

Subjects	Design and duration	Probiotic supplementation	Main findings: probiotic vs. placebo	Reference
Hospitalized infants aged 5-24 mo (n=55)	DBPC, 17 mo	<i>B. bifidum</i> and <i>S. thermophilus</i> (10^8 cfu) in formula or standard infant formula	Acute diarrhea incidence ↓ Stool RV shedding ↓	Saavedra et al. 1994
Children aged 6-36 mo with acute diarrhea (n=66)	RDBPC, 6mo	<i>L. reuteri</i> (10^7 or 10^{10} - 10^{11} cfu) or placebo in capsules mixed with breast milk/infant formula daily up to 5d	Subjects receiving larger dosage: Duration of RV watery diarrhea ↓ Watery diarrhea after second day ↓ Serum RV IgG ↔	Shornikova et al. 1997a
Children aged 6-36 mo with diarrhea (n=40)	RDPC	<i>L. reuteri</i> 10^{10} - 10^{11} cfu, or placebo 1xdaily for 5d	Duration of watery diarrhea ↓ Duration of RV associated diarrhea ↔, Fecal RV IgA ↔	Shornikova et al. 1997b
Children aged 6-36 mo (n=175)	8 mo	<i>B.Bb12</i> alone or together with <i>S.thermophilus</i>	In both groups: Protection against RV ↑	Phuapradit et al. 1999
Healthy newborns (n=94)	RPC, 12 mo	<i>L. sporogenes</i> (10^{10} cells) or placebo mixed in sterile water, dosing daily for 12 mo	Episodes of RV diarrhea ↓ No. of days of illness ↓ Duration of diarrhea episodes ↓	Chandra et al. 2002
Day care children aged 9-44 mo with acute diarrhea (n=33)	RDBPC, 6 mo	Mixture of lyophilized <i>L. rhamnosus</i> 19070-2 and <i>L. reuteri</i> DSM 12246 (10^{10} cfu each) or placebo, dosing 2x daily for 5d	Mean duration of diarrhea after intervention ↓ Recovery from diarrhea after early treatment ↑ Consistency of stools after treatment ↑	Rosenfeldt et al. 2002
Children aged 6-24 mo with persistent diarrhea (n=89)	DBPC	<i>L. casei</i> and <i>L. acidophilus</i> strains CERELA (10^{10} - 10^{12} cfu), <i>S. boulardii</i> in milk, or placebo for 2x daily for 5d	Effects with both bacteria: No. of depositions, diarrheal duration, vomiting ↓ RV infections ↔	Gaón et al. 2003
Boys aged 4-24 mo with acute watery diarrhea (n=230)	RPBPC, 24mo	<i>L. paracasei</i> strain ST11 (5×10^9 cfu) and placebo, dosing 2x daily for 5d + ORS and continued feeding	Severe RV diarrhea ↔ Children with less severe non-RV diarrhea: Cumulative stool output, stool frequency, and ORS intake ↓ Diarrhea resolution after 6d ↑	Sarker et al. 2005

Table 8. Continued

Subjects	Design and duration	Probiotic supplementation	Main findings: probiotic vs. placebo	Reference
Children aged 2 mo-6 y with infectious diarrhea (n=87)	RDBPC	Mixture of 3 <i>L. rhamnosus</i> (1.2x 10 ¹⁰ cfu)(573L/1; 573L/2; 573L/3) or placebo 2x daily, for 5d	RV diarrhea duration ↓	Szymanski et al. 2006
Children aged 6 mo-2 y with acute watery diarrhea (n=224)	RDBPC, 24 mo	ORS with <i>L. acidophilus</i> , <i>L. paracasei</i> , <i>L. bulgaricus</i> , <i>L. plantarum</i> , <i>B. breve</i> , <i>B. infantis</i> , <i>B. longum</i> , <i>S. thermophilus</i> (VSL#3), 9x10 ¹⁰ /sachet or placebo mixed in breast milk, formula milk, ORS or water daily for 4d	Recovery from acute RV diarrhea ↑ Frequency of ORS administration ↓ Stool volume losses during diarrhea ↓	Dubey et al. 2008
Hospitalized children aged 3mo - 3 y (n=80)	RDBPC	ORS+ placebo or ORS + <i>S. faecalis</i> T-110, <i>Clostridium butyricum</i> TO-A, <i>Bacillus mesentericus</i> TO-A, <i>L. sporogenes</i> (Bifilac™) dissolved in water 3x daily up to 14d.	No. of episodes (frequency) of diarrhea in a day ↓ Mean duration of diarrhea (in days) ↓ Degree of dehydration ↓ Duration and volume of ORS and intravenous fluid therapy ↓ Duration of RV shedding ↓	Narayana et al. 2008
Children with acute RV gastroenteritis (n=23)	Open-label randomized trial	Low (2x10 ⁸) or high dose (6x10 ⁸ cfu) of <i>L. rhamnosus</i> 35 or placebo for 3d	In high-dose group: Fecal RV concentration ↓	Fang et al. 2009
Children aged 1-23 mo hospitalized for acute RV diarrhea (n=64)	RDBPC, 8 mo	ORS+ placebo or ORS+ <i>Saccharomyces boulardii</i> (10 ¹⁰ cfu) or ORS+ combination of <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. longum</i> and <i>S. boulardii</i> (10 ⁷ -10 ¹⁰ cfu) 2x daily for 5d	Both supplementations: duration of diarrhea ↓ (significant only for single species product) <i>S. boulardii</i> : duration of fever ↓	Grandy et al. 2010
Infants aged 6-48 mo with acute diarrhea (n=186)	RDBPC	<i>S. boulardii</i> (4 x10 ⁹ cells) or placebo orally 2x daily for 5d	Diarrhea duration ↓ RV diarrhea ↓ RV in stool ↔	Corrêa et al. 2011

RDBPC = randomized double-blind placebo-controlled; DBPC = double-blind placebo-controlled; RPC = randomized placebo-controlled; mo = months; y = years; ↑ = increase; ↓ = decrease; ↔ = no effect

3.4 THE EFFECTS OF NONVIABLE PROBIOTICS ON VIRUS INFECTIONS

Increasing evidence shows that killed/nonviable/inactive bacteria, products derived from bacteria, or end products of bacterial growth could provide some health benefits (Kataria et al., 2009, Lahtinen and Endo, 2012). In addition, nonviable bacteria would serve as a great potential for food industry by providing new product applications, increasing product shelf life, and reducing storage costs. However, because they are not alive when administered they cannot be considered as probiotics (Sanders et al., 2007, Sanders et al., 2010). Probiotics are inactivated with heat-inactivation in 70-120°C, sonication, UV- or γ -radiation, freezing, and acidification. Different inactivation methods may have an impact on the cell wall components of bacteria, which affect the adhesion properties of bacteria in the gut epithelium (Ouwehand et al., 2000). In addition, inactivation may release biological compounds from the cytosol of bacteria, leading possibly to dissimilar effects than effects induced by viable bacteria.

The effects of nonviable bacteria in virus infections have been addressed in several studies. In children, live *L. rhamnosus* GG enhanced RV specific antibody response over inactivated product form (Kaila et al., 1995). In mice, *L. rhamnosus* GG was more effective than inactivated product form in protection against RV diarrhea (Pant et al., 2007). In respiratory virus infections using mice models, orally or intranasally administered boiled or heat killed *L. plantarum* O6CC2 and L-137, *L. gasseri*, or *L. pentosus* strain b240 were effective against influenza virus infection (Hori et al., 2001, Maeda et al., 2009, Izumo et al., 2010, Izumo et al., 2011, Kobayashi et al., 2011, Takeda et al., 2011, Kawase et al., 2012). However, these studies did not include viable strains in comparison. In mice, administration of live or heat-inactivated lactobacilli equally protected against lethal infection with pneumonia virus (Gabryszewski et al., 2011). In addition, in pig alveolar macrophages live or heat-inactivated lactobacilli and bifidobacteria equally increased cell survival against vesicular stomatitis virus infection depending on strain used (Ivec et al., 2007). Another study, however, showed that live *Lactobacillus* strains conferred protection against influenza infection in mice more efficiently than nonviable strains (Youn et al., 2012).

AIMS OF THE STUDY

The aim of this series of studies was to evaluate the effects of probiotic bacteria, especially *Lactobacillus rhamnosus* GG in viral respiratory and gastrointestinal infections in experimental models and in children. A special emphasis was on the questions, whether viability of a strain plays a role in beneficial effects of probiotics, and whether combination of strains is more effective over single strains.

The specific aims were:

1. To develop a novel colorimetric neutralization assay for detection of influenza virus antibodies from human sera for vaccine research (I).
2. To compare the ability of combination of probiotic and potentially probiotic bacteria to induce immune responses over single strains in primary cell culture using human monocyte-derived macrophages (II).
3. To characterize the effects of the viable and nonviable *L. rhamnosus* GG in rotavirus infection in neonatal rats (III).
4. To evaluate whether *L. rhamnosus* GG is effective in reducing common respiratory virus infections in children (IV).
5. To investigate whether a combination of probiotics including *L. rhamnosus* GG reduces nasopharyngeal occurrence of human bocavirus in otitis-prone children (V).

MATERIALS AND METHODS

1 STUDY MATERIAL AND SUBJECTS

1.1 BACTERIAL STRAINS AND PRODUCTS (II-IV)

Five potentially probiotic strains or known probiotic strains used in the experiments were obtained from Valio Ltd (Helsinki, Finland) (**Table 9**). In Study II, bacteria were stored in skimmed milk at -70°C and passaged three times before use in stimulation experiments as described previously (Miettinen et al., 1998, Kekkonen et al., 2008). For stimulation experiments, bacteria were cultured to a late logarithmic phase, and the number of bacteria was determined by counting in a Petroff -Hauser counting chamber. In Study III, viable *L. rhamnosus* GG (GG) stock (10^{11} cfu/ml) was aliquoted in MRS culture medium broth and stored at -20°C prior use. Powdered nonviable GG (the method of inactivation is a trade secret by Valio Ltd) in equivalent cfu number of 10^{11} cfu/g of viable GG was maintained at room temperature. Prior experiments, live GG was thawed and nonviable GG weighed, and both were prepared daily in PBS at a concentration of 3×10^9 cfu/ml. In Study IV, unsweetened Gefilus® milk containing 1 % fat included concentrations of GG ranging from 10^5 - 10^6 cfu/ml depending on shelf life. In Study V, the gelatine capsules contained a mixture of four probiotic strains (GG, *L. rhamnosus* Lc705 (Lc705), *B. breve* 99 (Bb99), *Propionibacterium freudenreichii* JS (PJS)), $8-9 \times 10^9$ cfu/capsule of each strain. Bacterial strains used in this study are presented in more detail in **Table 9**.

Table 9. Bacteria used in the study.

Bacterial strain	Abbreviation in the study	ATCC/DSM number	Culture medium	Growth conditions	Use in the experiments	Study number
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> ARH74	ARH74	DSM 18891	MRS medium and M17 broth with 20g/L-lactose (2%)	22°C, aerobic	<i>In vitro</i>	II
<i>Lactobacillus rhamnosus</i> GG	GG	ATCC 53103	de Man, Rogosa and Sharpe (MRS) medium	37°C, aerobic	<i>In vitro</i> , PBS supplement, milk, capsules	II-V
<i>Lactobacillus rhamnosus</i> Lc705	Lc705	DSM 7061	MRS medium	37°C, aerobic	<i>In vitro</i> , capsules	II, V
<i>Bifidobacterium breve</i> Bb99	Bb99	DSM 13692	MRS medium with cysteine	37°C, anaerobic	Capsules	V
<i>Propionibacterium freudenreichii</i> JS	PJS	DSM 7067	Propionimedium	30°C, aerobic	Capsules	V

1.2 VIRUS STRAINS AND CULTURES (I, III)

In Study I, influenza A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2) and B/Finland/159/02 virus strains were obtained as egg isolates from the WHO Collaborating Center for Reference and Research on Influenza in London (UK) and adapted to grow in MDCK cells.

In Study II, Influenza virus strain A/Beijing/353/89 (H3N2) egg isolate were obtained from the National Institute for Health and Welfare (Helsinki Finland) and grown as described previously (Ronni et al., 1995).

In Study III, simian RV SA-11 strain was obtained from Department of Food Hygiene and Environmental Health (University of Helsinki, Finland). RV was propagated in a continuous cell line of rhesus monkey kidney cells, MA-104. The cells were cultivated in minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum supplemented with 2 mM L-glutamine, penicillin and streptomycin in roller flasks in a roller apparatus at 37°C. When the cells had a confluency of 70-80%, they were inoculated from a stock containing 10⁸ pfu/ml of plaque-purified RVs. Before inoculation the RV stock was treated with 10-20 µg/ml (final concentration) of trypsin (Sigma, St Louis, USA) for 30 min at 37°C.

RV stock at dilution of 10^{-4} was added to each roller bottle. After incubation for one hour (h), 30 ml of serum-free MEM with $1\mu\text{g}/\text{mL}$ of trypsin was added, and the cultivation was continued for 48h at 37°C . RVs were harvested by freeze-thawing of cells three times, cell debris was removed by low-speed centrifugation, and the supernatant was collected, divided into aliquots, and stored at -70°C until use. The RV titer was determined as 1.4×10^8 pfu/ml.

1.3 CELL CULTURES

1.3.1 *Madin-Darby canine-kidney cells (I)*

Madin-Darby canine kidney (MDCK) cells (CCL-34, ATCC, Manassas, VA, USA) were grown in Eagle's MEM (EMEM) supplemented with non-essential amino acids (NEAA), penicillin (100 000 U/ml), streptomycin (78 000 U/ml) and 0.2% bovine serum albumin (BSA), and maintained at 37°C in a 5% CO_2 humidified atmosphere. The cells were subcultured at a split ratio of 1:3–1:5 twice a week. For the NT, a serum free medium (Optipro SFM™, Gibco, Invitrogen; Carlsbad, CA, USA) supplemented with 2mml-glutamin, antibiotics, and 0.2% BSA was used.

1.3.2 *Differentiation of macrophages from peripheral blood-derived monocytes (II)*

Human peripheral blood mononuclear cell (PBMC) - derived monocytes were purified by density gradient centrifugation over a Ficoll-Paque gradient (Amersham-Pharmacia Biotech, Uppsala Sweden) from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusions Service, Helsinki, Finland) as described (Miettinen et al., 2000). Briefly, after washing the cells were resuspended in RPMI-1640 supplemented with 2 mmol/L L-glutamine (Lonza Walkersville, Inc., Walkersville, MD, USA), 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza), and 20 mmol/l HEPES (Lonza). For monocyte differentiation, PBMC were allowed to adhere to plastic six-wells (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for one hour at $+37^{\circ}\text{C}$ in 5% CO_2 . After incubation nonadherent cells were removed, and adherent cells were then grown for seven days in Macrophage SFM media (Gibco BRL, Paisley, Scotland), supplemented with antibiotics and recombinant GM-CSF at 10 ng/ml (Biosource, Camarillo, Ca, USA).

1.4 EXPERIMENTAL ANIMALS (III)

Pregnant specific pathogen free Lewis rats were obtained from Harlan Laboratories Inc. (Horst, The Netherlands) and allowed to give birth naturally in the test facility. Prior to experiments, each litter was adjusted to six pups per dam. Litters were randomly assigned to four experimental groups (n=6 pups/group): rats infected with RV SA-11 alone (RV control group); nonviable GG treated + RV SA-11 infected rats (nonviable GG group); viable GG treated + RV SA-11 infected rats (viable GG group); and MEM control rats (healthy control group). Control and inoculated groups were housed in the same individually ventilated Scantainer (Scanburg, Denmark), and each RV infected group in their own Scantainer, in normal rat cage (Makrolon III) with Aspen chips bedding (Tapvei Oy, Kaavi, Finland) and nest material (Aspen chips PM90L/R). The temperature was $22\pm 2^{\circ}\text{C}$ with relative humidity 50-95 %. Lighting was artificial, 12h light and 12h dark (18:00-06:00). Food (TEKLAD T.2916 IRR*; Irradiated Global 16%, Rodent Diet for mice and rats, Harlan) and deionized water were autoclaved and provided *ad libitum* from the day of the rats' arrival until the completion of the experiments.

1.5 CHILDREN (IV-V)

In Study IV, nasopharyngeal swab (NPS) samples were obtained from children (aged 2-6 years) attending day care five days a week in Northern Finland (Kumpu et al., 2012). Children with milk allergy, lactose intolerance, congenital heart disease requiring regular medication, malignant diseases, cytostatic treatment, use of biological rheumatic medication, continuous microbial medication, regular use of oral corticosteroids, diabetes, or simultaneous participation in other clinical trials had been excluded from the study.

In Study V, NPS samples were obtained from otitis-prone children (aged 10 months to 6 years) (Hatakka et al., 2007). Children had been classified as otitis-prone if they had ≥ 4 AOM episodes during the preceding 12 months or ≥ 3 episodes during the preceding six months. **Children on regular medication, with chronic illnesses, Down's syndrome, lip or palatal cleft,** or otitis media with effusion had been excluded. Those children who had undergone adenoidectomy or tympanostomy had been included if they had suffered the required number of AOM episodes.

2 STUDY DESIGNS

2.1 *IN VITRO* EXPERIMENTS (I-II)

2.1.1 *Colorimetric neutralization test (I)*

A colorimetric cell proliferation assay was developed for the determination of neutralizing antibodies of 40 healthy adult individuals after immunization with seasonal, trivalent, inactivated influenza vaccine. Prior testing, method was optimized with a different set of variables (e.g. cell number, virus concentration, and different serum dilutions). In the final test protocol (**Figure 5**) a flat-bottom 96-well cell culture plates (Nunc, A.S.Roskilde, Denmark) were first pre-treated with SFM containing 10% FBS (100 μ l/well) for 3h at +37°C 5% CO₂. Second, test sera used in optimization were heat-inactivated for 30 min at +56°C. Dilutions of the influenza stock viruses and the test sera were prepared in SFM containing 4 μ g/ml of TPCK–trypsin (Sigma Chemical Co., St. Louis, MO, USA). Sera were serially diluted in half-logarithmic steps starting from a dilution of 1:16.5. Then, a 100 μ l volume of each serum dilution or medium control was combined with an equal volume of medium containing approximately 40 pfu of influenza virus. Third, the serum-virus mixtures were incubated at +37°C for 90 min. Fourth, each serum–virus mixture was transferred to triplicate wells (50 μ l/well), and 50 μ l of a fresh suspension of MDCK cells was added (10⁴ cells/well). Wells containing serum dilutions 1:33–1:100 without viruses, virus dilutions in the absence of test sera, wells containing only MDCK cells in SFM, and wells containing plain SFM media were included on each assay plate as controls. The plates were centrifuged at 170g for 20 min. Fifth, the plates were incubated at +37°C 5% CO₂ for 72h, after which WST-1-reagent (Roche diagnostics, Basel, Switzerland) (10 μ /well) was added. The plates were further incubated at 37°C for 4h allowing the color reaction development. Optical density was measured with an ELISA-spectrophotometer (Thermo Electron Corporation, Multiscan Ex; Vantaa, Finland) at 450 nm with 650 nm as a reference. To calculate neutralizing antibody titers, mean absorbance value from three parallel wells was calculated, after the mean value from the system-background control wells had been subtracted. Neutralizing activity (percent inhibition) was calculated for each serum dilution and the antibody titers were determined using the following equation:

$$\frac{\text{A450 of serum dilution} - \text{A450 of virus control}}{\text{A450 of serum control} - \text{A450 of virus control}} \times 100$$

The neutralizing antibody titer was expressed as the reciprocal of the highest serum dilution that inhibited virus growth by $\geq 50\%$.

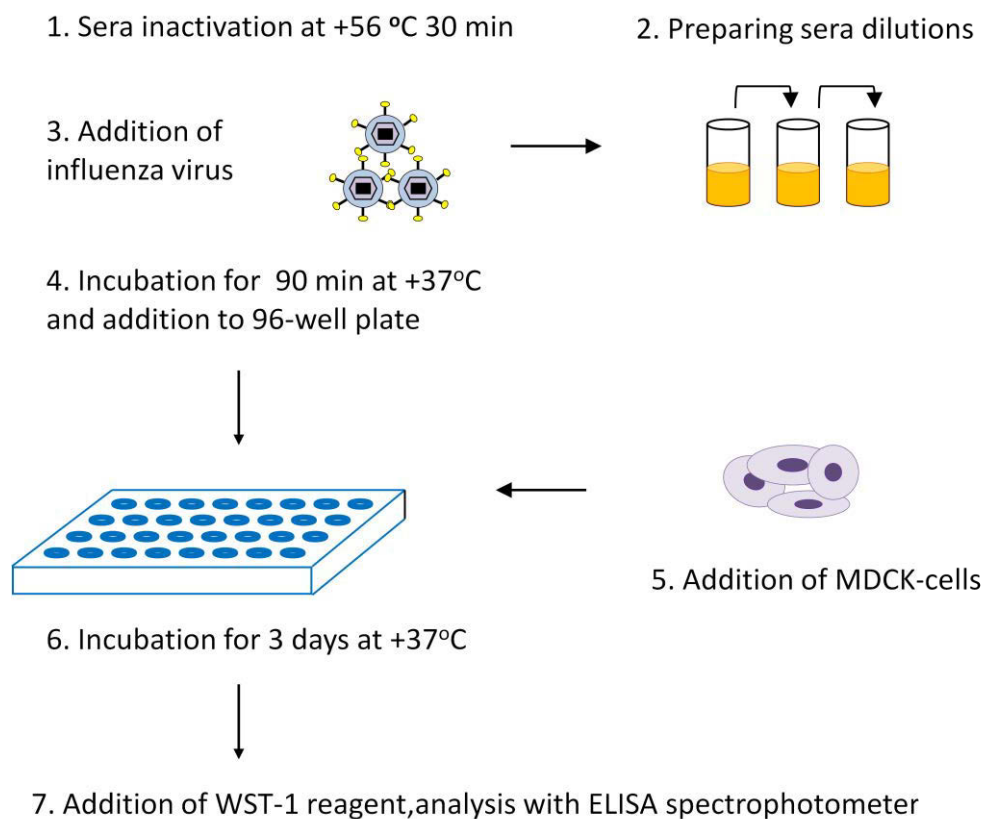


Figure 5. Schematic illustration of colorimetric NT protocol.

2.1.2 Bacterial stimulation experiments (II)

All experiments were performed with cells obtained from four blood donors. Stimulation experiments were performed in RPMI-1640 medium with supplements (**Figure 6**). Macrophages were stimulated with viable probiotic bacteria: host cell ratio of 1:1 for 24h at 37°C in 5% CO₂. When the cells were stimulated with a combination of GG and Lc705, equal

numbers of strains were used and the sum bacterial dose of the combinations was 1:1 of a bacteria: host cell ratio. Cell culture supernatants and cells from different donors were collected 24h after bacterial stimulation and pooled. In the influenza virus experiments, after probiotic stimulation, macrophages were challenged immediately with influenza virus (viral dilutions 1:1000 or 1:5000). After 1h of virus infection in +37°C in 5% CO₂, the cells were washed with PBS and fresh RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Integro Ltd., Zaandam, The Netherlands) was added. Cell culture supernatants and cells from different donors were collected 6h and 24h after virus infection and pooled. All samples were stored at -20°C for cytokine and chemokine measurements.

Study II

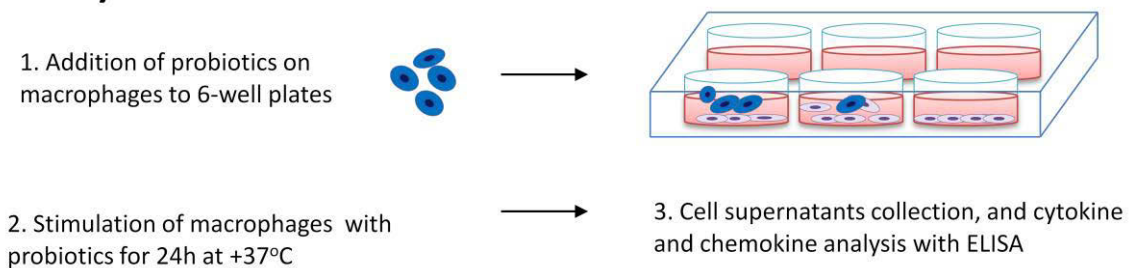


Figure 6. Schematic illustration of bacterial stimulation experiments-protocol.

2.2 ANIMAL EXPERIMENTS (III)

The summary of the study design is presented in **Figure 7**. Briefly, the pups were weighed at fixed times daily before and twice a day after RV infection. At the age of two days, the pups received daily a single dose of 500 μ l of either nonviable or viable GG supplementation (1.5×10^8 cfu/pup). RV (total amount of 10^8 pfu/pup) was inoculated in three separate doses at the age of five days ($2 \times 300 \mu$ l) and six days ($1 \times 120 \mu$ l). Similarly, MEM (100xglutamine, penicillin 100 IU/ml, streptomycin 100 μ g/ml) was administered for healthy controls. The rat pups were randomized for exsanguination from either two or three days post infection at the age of seven and eight days. Blood samples were collected by decapitation into EDTA tubes (Venosafe™), and the plasma was obtained by centrifugation (10 min, 4000 rpm), and frozen at -20°C. After blood sampling, the GI tract was removed for macroscopic observations. The small intestine, colon, and feces were collected and weighed separately, and stored in -80°C. Feces were collected by carefully emptying the colon and rectum. The consistency of feces was classified from 0-3 using a four tier system: 0=normal

feces, 1=slight diarrhea (feces pale but solid), 2=moderate diarrhea (feces pale and semi-solid), 3=strong diarrhea (feces clearly wet).

Study III

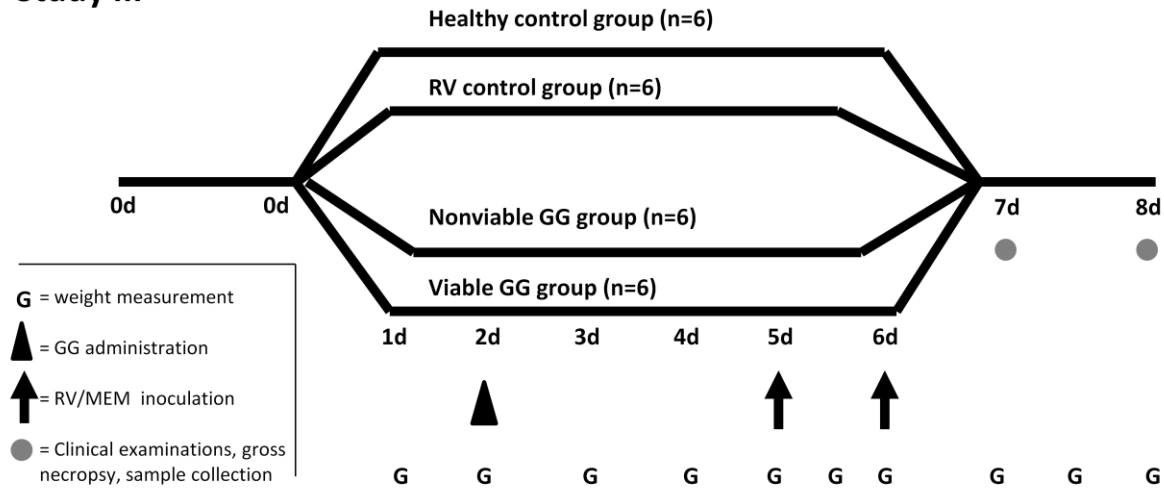


Figure 7. Schematic representation of study design of animal experiments.

2.3 CLINICAL INTERVENTION EXPERIMENTS (IV-V)

The characteristics of the Studies IV and V are presented in **Table 10**. Study IV was a substudy of a randomized, double-blind and placebo-controlled parallel group six months intervention study investigating whether GG can decrease respiratory illness in children attending day care centres in Kainuu and Oulu regions in Finland in October 2009 - April 2010 (Kumpu et al., 2012). A total of 523 children aged 2 to 6 years, who fulfilled the previously reported inclusion criteria, were enrolled in to the study and randomized to receive either control milk (n= 262) or the same milk with GG (n=261) on three daily meals. During the intervention, whenever a subject experienced symptoms of respiratory or **gastrointestinal infection that, according to parents' judgement, required physician's** appointment, parents were advised to take the child to a study physician. In addition to clinical assessment and treatment, study physicians filled out a structured questionnaire regarding information on respiratory symptoms, and collected a NPS sample by a deep nasal swab using flocced-tip nylon swabs. Swabs were immediately submerged into a vial containing 3 ml of universal transport medium (UTM-RT, Copan, Diagnostics Inc., Murrieta, CA), and then stored at -70°C. For the present study, a total of 315 NPS samples were

available from 194 children (97 in the placebo and 97 in the probiotic group). A total of 14 respiratory viruses were analyzed from the NPS samples.

Study V was conducted in conjunction with two other substudies (Blomgren et al., 2004, Pitkäranta et al., 2006) with a study population part of a larger project described by Hatakka and others (Hatakka et al., 2007). Briefly, in a double-blind, placebo-controlled, randomized, 6-month intervention study in September 2000 - April 2001, originally 269 otitis-prone children (from 9 months to 5.6 years old) consumed daily either one capsule of probiotics (GG, Lc705, Bb99, and PJS) (n=135) or placebo (n=134). NPS samples were collected at the scheduled baseline visit in autumn, at the first follow-up visit after three months in winter, and at the final visit after six months in spring. NPS samples were collected from the nasopharynx with a calcium alginate swab through the nostril. The swab was immediately immersed in a tube containing 1 ml of STGG transport medium. The STGG tube with the swab was vortexed immediately, stored in the refrigerator for maximum 8h, and then frozen at -70 °C before analysis. Parents had received advice to avoid days when the child had respiratory symptoms when making scheduled collection visits. Parents had to keep daily diaries, including signs and symptoms of AOM and respiratory infections, such as fever, earache, otorrhoea, rhinitis, cough, sore throat, chest wheezes, or night restlessness, and listing visits to health care authorities, and of the use of any medication. For the present study, all three NPS samples were available from 152 otitis-prone children (105 in the placebo and 47 in the probiotic group). Of these, the presence and persistence of HBoV1-4 were analyzed.

Table 10. Study characteristics of the clinical intervention experiments.

Characteristics	Study IV Children		Study V Otitis-prone children	
	Probiotic	Placebo	Probiotic	Placebo
No. of children included in the analysis	97	97	47	105
No. of collected NPS samples	160	155	141	315
Monthly distribution of collected NPS				
September	0	0	47	105
October	11	24		
November	35	30		
December	23	16	47	105
January	16	18		
February	34	32		
March	26	19	47	105
April	15	16		
Viruses analyzed	HRV, HEV, HBoV1-4, ADV, RSV, influenza A/(H3N2), A(H1N1)pdm09, influenza B, PIV1-3		HBoV1-4	

3 VIROLOGICAL, IMMUNOLOGICAL, AND SEROLOGICAL ANALYSIS (I-V)

3.1 SEROLOGICAL TESTING (I)

For comparing the sensitivity and sensitivity of NT with HI test to measure influenza virus antibodies in serum, 40 pre- and post-vaccination serum pairs collected from army conscripts were tested. These conscripts had been immunized with the seasonal trivalent, inactivated influenza vaccine in autumn 2002, containing the three WHO recommended vaccine viruses A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Hong Kong/330/2001. Prevacination sera (S1) were collected at the time of vaccination, and post-vaccination sera (S2) were drawn approximately one month later. The serum samples were kept frozen at -70°C until testing, and were heat inactivated at +56°C for 30 min prior use. The HI test was performed according to standard procedures (Kendal et al., 1982) with 0.4% suspension of goose red blood cells and two hemagglutinating units of influenza virus antigens. Sera were tested in serial fourfold dilutions starting from 1:10. Next, S1 and S2 sera were analyzed with the newly developed NT as described in the **section “Study designs”**. A threefold or higher titer increase between the pre- and post-vaccination sera was considered significant for NT, a fourfold increase for HI test.

3.2 IMMUNOLOGICAL MEASUREMENTS (II)

Cytokine levels from the cell culture supernatants were analyzed by using ELISA. TNF- α were determined with antibody pairs and standards obtained from BD Pharmingen (San Diego, CA, U/SA) essentially as described (Miettinen et al., 1998). IL-1 β was determined with antibody pairs and standards obtained from R&D Systems (R&D Systems, Inc, Minneapolis, MI, USA). The bead based analyte detection assay for quantitative detection of IL-6, IL-10, IL-12 p70, CCL2/monocyte chemoattractant protein-1 (MCP-1) and CXCL10/IFN-inducible protein 10 (IP-10) were performed with FlowCytomix Human Simplex Kits (Bender MedSystems GmbH, Vienna, Austria) in capillary tubes following the manufacturer's protocol. Flow cytomix assay were run with BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA.)

3.3 VIROLOGICAL DIAGNOSTICS (III-V)

The summary of viral detection methods used in the studies III-V is presented in **Table 11**. In Study III, prior nucleic acid extraction, frozen small intestine, colon, and feces were processed as follows: Colon and the entire small intestine with its contents were homogenized with sterile glass rods, and 30 mg of homogenized tissue was added into 600 μ l of RLT buffer (Qiagen) and incubated for +37°C 10 min in water bath. Prior RNA extraction, the lysate was centrifugated in a QIAshredder (Qiagen) (2 min, 12 000 rpm). Feces were processed on ice in 200 μ l of 10% protease-inhibitor solution containing 1% BSA, 10 mM pefabloc (Roche Applied Science, Mannheim, Germany), 100 μ g/ml aprotinin (Sigma-Aldrich, St. Louis, MO, USA) 100 μ g/ml leupeptin (Sigma-Aldrich) in EMEM I (Gibco, Carlsbad, CA) supplemented with 5% FCS and 20 mM Hepes (pH 7.4). Suspensions were vortexed with sterile glass beads, centrifugated (10 min, 5000 rpm), and viral RNA was extracted from supernatants.

Table 11. The summary of viral detection methods.

Virus	Sample	Nucleic acid extraction	PCR	Study
Rotavirus SA-11	Plasma	BioSprint® 96 One For All Vet-kit (Qiagen)	High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), real time qPCR, modified (Li et al., 2010)	III
	Small intestine	RNeasy Mini Kit (Qiagen),		
	Colon	BioSprint® 96 One For All Vet-kit (Qiagen)		
	Feces	E.Z.N.A.® Total RNA Kit (Omega Bio-Tek)		
Rhinovirus	NPS	BioSprint® 96 One For All Vet-kit	Real-time PCR assays (Taqman Chemistry), multiplex RT PCR-hybridization assays (Blomqvist et al., 1999)	IV, V
Enterovirus	NPS	BioSprint® 96 One For All Vet-kit, QIAamp DNA Mini Kit (Qiagen)	HBoV1 real time qPCR (Allander et al., 2007), multiplex real time qPCR (Kantola et al., 2010)	IV, V
Bocavirus 1-4	NPS			IV, V
Influenza A and B virus	NPS	BioSprint® 96 One For All Vet-kit	cDNA RT, real-time multiplex PCR (Templeton et al., 2004, Rönkkö et al., 2011, Akinloye et al., 2011)	IV
Respiratory syncytial virus	NPS			IV
Parainfluenza virus 1-3	NPS			IV
Adenovirus	NPS		Real-time PCR Quantitect SYBR Green PCR (Qiagen) (Akinloye et al., 2011)	IV

4 STATISTICAL ANALYSES

The statistical analyses in studies I-V were performed using SPSS software versions 15.0-18.0 (SPSS Inc, Chicago, IL, USA). In all studies, P-values <0.05 were considered statistically significant.

In Study I, during optimization of NT, the intra-assay and inter-assay variations of the test results were determined, and the result was given as a coefficient of variation (CV). When comparing the HI and NT test results, HI and NT titers were converted into logarithmic values, and the comparisons between the tests were executed with Student's two-sided t-test. HI and NT titers below the lower limit of detection of the assay were given a value of 5. Antibody titer increases between HI and NT were compared by using the χ^2 -test. Also the correlation coefficients between the HI and NT test results were calculated.

In Study II, data were analyzed by a one-way analysis of variance (ANOVA), followed by **Bonferroni's post hoc test**.

In Study III, ANOVA was applied to compare the groups with respect to weight gain and tissue swelling, and the results are given as means with SEM. In cases of significant global p-values, multiple comparisons were performed and the p-values were Bonferroni corrected. RV diarrhea occurrence and severity between the study groups were analyzed using logistic regression analysis. Statistical differences in the CT-values between SA-11 RV infected groups were analyzed using the Kruskal-Wallis test (global test) and Mann-Whitney U-test (pair-wise comparisons).

In Study IV, the results are presented as counts with percentages, means with 95 % confidence intervals (95% CI). Number of symptom days per month (incidence rates) and P-value between groups concerning this endpoint was calculated by Poisson regression model. The differences between study groups in viral findings were tested by permutation type probit regressions for which the standard error was adjusted for 194 clusters (number of children). For HBoV virus, only HBoV positive samples with high viral load (>10 000 copies/ml) were included in the analyses. The 95%CI for the number of symptoms were obtained by bias-corrected bootstrapping (2000 replications).

In Study V, HBoV was assessed as positive or negative by four positivity criteria (>100, >1000, >10 000 and >100 000 copies/ml of sample). The association between HBoV DNA-

positivity and respiratory symptoms from 2-week (sampling day \pm 1 week) and 4-week (sampling day \pm 2 weeks) time-periods was analyzed with logistic regression analysis, and GEE (generalized estimating equations) using information on respiratory symptoms provided by parents. The presence of respiratory symptoms in HBoV DNA-positive children were compared to those of HBoV DNA-negative children, with results as odds ratios (OR) with 95% confidence intervals. Logistic regression analysis allowed study of any possible effect of probiotic intervention on HBoV. Results are unadjusted (crude) and baseline-adjusted odds ratios (OR) with 95% confidence intervals. GEE analysis allowed inclusion of 3- and 6- month visits simultaneously and baseline positivity was a categorical covariate.

5 ETHICS

In Study II, the ethical permission to use the freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Transfusion Service, Helsinki, Finland) was given by the ethics committee of the Finnish Red Cross Transfusion Service in Helsinki. Study III was approved by the Animal Care and Use Committee of the State Provincial Office of Southern Finland (license number ESAVI-2010-06221_Ym-23). In Studies IV and V, the study protocol was approved by the ethics committee of Joint Authority of Kainuu Region (IV) (registered to <http://clinicaltrials.gov> with identifier NCT01014676), and by the ethics committee of Helsinki University Central Hospital (V). Before entering to the studies IV and V, the guardians of the children gave their written informed consent.

RESULTS

1 DEVELOPMENT AND EVALUATION OF COLORIMETRIC NEUTRALIZATION TEST (I)

In Study I, a novel colorimetric neutralization test (NT) for the measurement of influenza-specific antibodies in human sera was developed. The assay is based on the colorimetric measurement of formazan salt of metabolic active cells. After parameter optimization, NT test showed high reproducibility evidenced by low intra- and inter-assay variation. In addition, the pattern of significant antibody rises was similar between parallel assays. As a proof of concept, 40 influenza pre- and post-vaccination serum pairs were tested by the newly developed NT, and the results were compared with those obtained by the standard HI test. Overall, there was a good correlation against influenza A/New Caledonia/20/99, A/Panama/2007/99, and B/Fin/159/0 vaccine viruses between the HI and NT pre- and post vaccination antibody titers. Moreover, the neutralizing antibody titers (GMT) to all vaccine viruses were significantly higher than the corresponding HI titers ($P < 0.0001$) (**Figure 8**). In addition, NT revealed low pre-vaccination titers in some sera that remained negative by the HI test. Almost all of the 40 serum pairs exhibited significant titer increases to the H1N1 and the influenza B viruses as determined by both test principles (**Table 12**). More significant antibody increases, particularly against the H3N2 viruses, were detected by the NT, suggesting a higher sensitivity of the assay for detection of influenza virus antibodies.

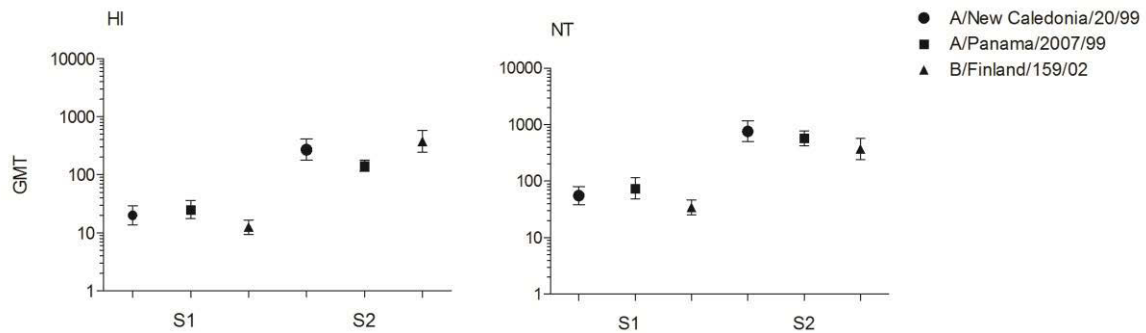


Figure 8. Geometric mean titers (GMT with 95% CI) from pre- (S1) and post-vaccination serum pairs (S2) obtained with HI and NT in 40 subjects immunized with trivalent seasonal influenza vaccine. GMT from all viruses HI vs. NT: $P < 0.0001$ (unpaired t-test).

Table 12. Distribution between pre- and post-vaccination antibody titer increases obtained with HI and NT in 40 subjects immunized with trivalent seasonal influenza vaccine.

Titer increase (fold change)	No. of samples (%)					
	A/New Caledonia/20/99		A/Panama/2007/99		B/Finland/159/02	
	HI	NT	HI	NT	HI	NT
<3-4	3 (8)	1 (3)	14 (35) ^a	3 (8)	3 (8)	1 (3)
3-4	5 (13)	8 (20)	7 (18)	15 (38) ^b	7 (18)	14 (35)
≥8-20	19 (48)	16 (40)	12 (30)	14 (35)	19 (48)	13 (33)
≥30-64	7 (18)	10 (25)	7 (18)	6 (15)	10 (25)	7 (18)
≥100	6 (15)	5 (13)	0 (0)	2 (5)	1 (3)	5 (13)
Total no. of significant antibody rises (%) ^c	37 (93)	39 (98)	26 (65)	37 (93) ^d	37 (93)	39 (98)

^a HI vs. NT: $P = 0.003$

^b HI vs. NT: $P = 0.045$

^c Significant antibody rise for HI ≥ 4 , and for NT ≥ 3 , respectively

^d HI vs. NT: $P = 0.003$

2 IN VITRO SCREENING OF IMMUNOMODULATORY EFFECTS OF PROBIOTICS ON HUMAN MACROPHAGES (II)

The summary of bacteria-induced macrophage cytokine and chemokine responses is shown in **Table 13**. Overall, the combination of GG/Lc705 induced similar or weaker immunomodulatory responses than individual GG or Lc705. However, the combination of GG/Lc705 was effective in enhancing IL-1 β response over Lc705 by 1.5 fold, suggesting that the combination has some synergistic potential over individual strain. In addition, the combination induced TNF- α , IL-1 β , IL-6, and IL-10 production more strongly than ARH74. However, ARH74 was the most potent inducer of chemokines MCP-1 and IP-10. The potential of these bacteria to enhance proinflammatory (TNF- α and IL- β) cytokine production in response to influenza A virus infection was analyzed as well (unpublished results). After 6h of influenza virus infection, GG and Lc705 individually enhanced macrophage TNF- α and IL- β production by 1.3-3.0 fold against viral challenge. The combination of GG/Lc705 or ARH74 had no enhanced effect. However, the study virus alone with used concentrations failed to induce any cytokine production in control cells, and thus did not allow evaluation of probiotic's effects during influenza infection.

Table 13. Cytokine and chemokine production profiles of monocyte-derived macrophages after 24h of bacterial stimulation. The number of + -symbols indicates the order of magnitude of response when comparing the immunomodulatory effects between bacterial strains. 0 indicates no effect.

Cytokine or chemokine response	Bacterial strain			
	GG	Lc705	GG/Lc705	ARH74
TNF- α	+++	++	++	+
IL-6	+++	+++	++	+
IL-1 β	+++	+	++	0
IL-10	++	++	+	0
IL-12	0	+	0	0
MCP-1	++	++	+	+++
IP-10	+	++	+	+++

3 THE EFFECTS OF NONVIABLE PROBIOTIC IN ROTAVIRUS INFECTION IN RATS (III)

3.1 CLINICAL INDICES AFTER ROTAVIRUS INFECTION

The summary of clinical indices of rats following RV infection is presented in **Table 14**. After RV inoculations on days 5 and 6, total body weight increased slightly more effectively in the viable GG group than in the nonviable GG group. On day 7 (after 2 days of RV infection), the viable and nonviable GG group gained significantly more weight than the RV group without probiotics (36% ($P=0.001$) and 28% ($P=0.031$), respectively). In addition, when compared with the RV control group, both viable and nonviable GG reduced the weight ratio of the colon/animal to the same level as in the healthy control group, with reductions of 22% ($P=0.002$) and 28% ($P<0.001$), respectively. Concerning diarrhea incidence, diarrhea increased moderately in both GG groups. However, neither the incidence nor severity of diarrhea in the GG groups was statistically significant ($P>0.05$) as compared with the RV control group. Moreover, observed diarrhea did not provoke weight loss or death.

Table 14. Clinical indices of the study groups of the neonatal rats after RV infection.

Clinical indices	Viable GG	Nonviable GG	RV control	Healthy control	P-value
Weight gain (%) ^a	102.9 (12.4)	92.0 (7.7)	95.2 (9.8)	116.7 (13.8)	ns
Colon weight ^b	2.8 (0.1)	2.5 (0.08)	3.5 (0.18)	2.6 (0.12)	$P<0.05^c$
Diarrhea score (range) ^d	2.5 (1-3)	2.0 (1-3)	1.0 (0-2)	0.0 (0)	ns

^a Total percentual weight gain of the rats after RV inoculations on days 5-6, mean \pm SEM

^b Weight ratio of colon/animal mg/g, mean \pm SEM

^c Viable GG vs. RV control, nonviable GG vs. RV control

^d Median with diarrhea score range

3.2 ROTAVIRUS POSITIVITY IN RAT TISSUES

RV positivity was determined from plasma, small intestine, colon, and feces with RT-qPCR. The number of RV PCR-positive samples in the study groups and their respective CT-values are shown in **Table 15**. Overall, the RV control group had the largest number of RV PCR-positive samples among the RV infected groups, and the viable GG group the smallest. By comparing the CT-values between the groups, rats receiving viable GG had significantly less RV in the colon ($P=0.027$) when compared with the RV control group. Viable GG was also more effective than nonviable GG in reducing the quantity of RV in plasma ($P=0.047$).

Table 15. RV detection from the tissues of neonatal rats.

Tissue sample	Viable GG group		Nonviable GG group		RV control group		Healthy control group	
	No. of positive samples ^a	CT (range) ^b	No. of positive samples	CT (range)	No. of positive samples	CT (range)	No. of positive samples	CT (range)
Plasma	6/6	33 (32-37)	6/6	31 (29-33)	6/6	32 (32-35)	0/5	>45
Small intestine	2/6	44 (37- >45)	4/6	43 (37- >45)	5/6	41 (37- >45)	0/5	>45
Colon	6/6	27 (27-28) ^c	6/6	25 (23-27)	6/6	25 (23-26)	0/5	>45
Feces	3/6	42 (37- >45) ^d	4/6	41 (35- >45)	5/6	39 (35- >45)	0/5	>45

^a Samples with CT-values <45 was regarded as RV PCR positive

^b Mean number of CT values of all tested samples. Samples with CT-value >45 received a random value between 46-50

^c Viable GG vs. RV control: $P=0.027$ (Kruskall-Wallis test)

^d Viable GG vs. nonviable GG: $P=0.047$ (Kruskall-Wallis test)

4 THE EFFECTS OF PROBIOTICS ON RESPIRATORY VIRUSES IN CHILDREN (IV-V)

4.1 THE NASOPHARYNGEAL OCCURRENCE OF VIRUSES

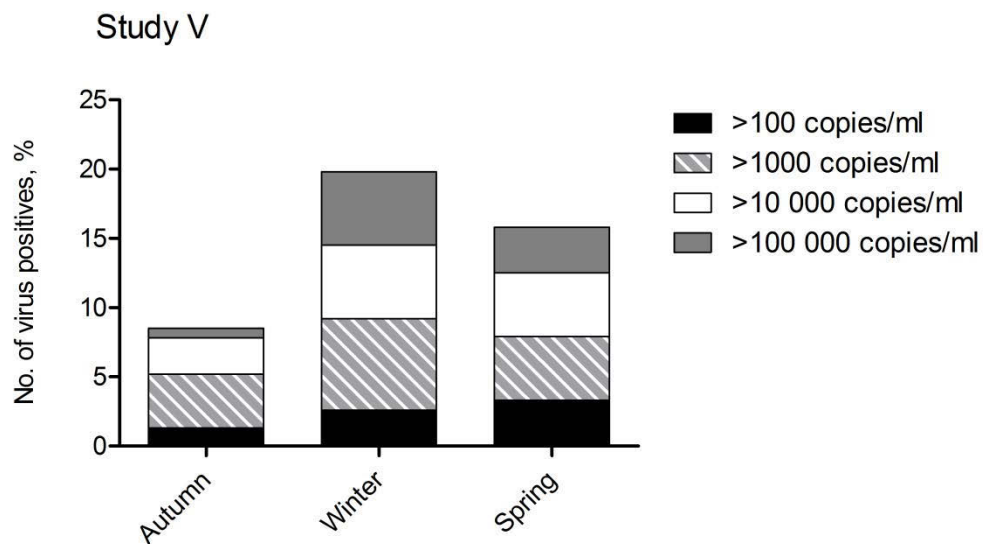
In Study IV, the most commonly identified virus from 194 children was HRV (37.1%), followed by RSV (20.1%), PIV1 (19.6%), HEV (14.4%), influenza A(H1N1)pdm09 (12.9%), HBoV1 (6.2%), PIV2 (5.2%), ADV (4.6%), and influenza A(H3N2) (1.0%). HBoV1 occurred at high load of 2.1% of children. In study V, of 152 otitis-prone children, 43 (28.3%) were HBoV1 DNA positive. Of these, 26 (17.1%) exhibited a high load (>10 000 copies/ml of sample) of HBoV1 DNA. In addition, 16 (10.5%) of 152 children showed prolonged presence of HBoV1 DNA for at least three months, and one child for six months. In addition, two (1.3%) children had one negative sample between the two HBoV DNA-positive samples. Influenza B virus, PIV3, and HBoV2-4 were undetectable either from children in Study IV or HBoV2-4 from otitis-prone children in Study V.

The monthly occurrence of all virus positive NPS samples is presented in **Table 16** (Study IV) and in **Figure 9** (Study V). In Study IV, RSV was most commonly detected in spring (February to March), whereas influenza A(H1N1)pdm09 was found more frequently in autumn (October to November). HRV was found in relatively high numbers throughout the 28-week intervention. Of the more rarely encountered viruses, PIV2 was mainly detected from January to March, HBoV1 from November to April, ADV in November and December, and again between March and April. In study V, HBoV DNA was detected at all three study visits, with the highest occurrence at three months. At the initial study visit, 3.3% of the children carried a high load of HBoV DNA. After three months, the HBoV DNA prevalence among the NPS samples increased to 10.5%, but after three months, decreased to 7.9%.

In Study IV, the majority of RSV and PIV2 were identified from children ≥ 4 years old. HEV predominated in the children under ≤ 3 years. HRV, ADV, influenza A virus subtypes, and PIV1 in Study IV, and HBoV1 in both studies were distributed almost equally between the age groups (unpublished results).

Table 16. The monthly occurrence (%) of respiratory viruses in the NPS samples of children (Study IV).

Virus	October	November	December	January	February	March	April	Total
Rhinovirus	15	25	18	7	7	6	12	90
Enterovirus	8	8	6	0	3	1	2	28
Influenza A(H1N1)pdm09	7	16	0	1	1	0	0	25
Influenza A(H3N2)	0	1	0	0	1	0	0	2
Respiratory syncytial virus	0	0	1	2	18	17	1	39
Parainfluenza virus 1	3	0	2	6	12	8	7	38
Parainfluenza virus 2	0	0	0	0	4	5	1	10
Adenovirus	0	2	1	0	0	2	4	9
Bocavirus 1	0	2	2	3	1	2	2	12

**Figure 9.** The monthly distribution (%) of HBoV1 viral loads in the NPS samples of otitis-prone children (Study V). The number of virus positive samples was compared with the total number of samples collected in each scheduled collection times.

4.2 THE EFFECT OF PROBIOTIC INTERVENTION ON VIROLOGICAL FINDINGS

Distribution of respiratory viruses between the study groups in Study IV and V is presented in **Figure 10**. In Study IV, the number of HRV, RSV, influenza A(H3N2), PIV2 and HBoV1- positive samples distributed almost equally between the study groups. In the GG group, there was a nonsignificant lower risk for HEV ($P=0.083$), influenza A(H1N1)pdm09 ($P=0.12$), and for ADV ($P=0.095$), but a seemingly opposite effect for PIV1 infections ($P=0.035$)

In Study V, probiotic supplementation reduced significantly the number of HBoV1 DNA-positive samples ($>10\ 000$ copies/ml) during the intervention period (probiotic vs. placebo: 6.4% vs. 19.0%, baseline adjusted OR=0.25, 95% CI 0.07–0.94, $P=0.039$). A similar, though not statistically significant, reduction occurred when the results were analyzed by GEE (baseline adjusted OR=0.45, 95% CI=0.12–1.66, $P=0.228$), or when applying another HBoV1 positivity criterion. In addition, to allow time for the intervention to take place, we included only the baseline HBoV1-negative children and analyzed the HBoV-positive children (by first occurrence of HBoV1), and found less HBoV1 in the probiotic group (probiotic vs. placebo: 6.7% vs. 17.6%, OR=0.33, 95% CI=0.09–1.20, $P=0.092$). Probiotic intervention did not, however, reduce the occurrence of prolonged presence of HBoV over three months (probiotic vs. placebo: 8.5% vs. 11.4%, OR=0.72, 95% CI =0.22–2.360, $P=0.589$).

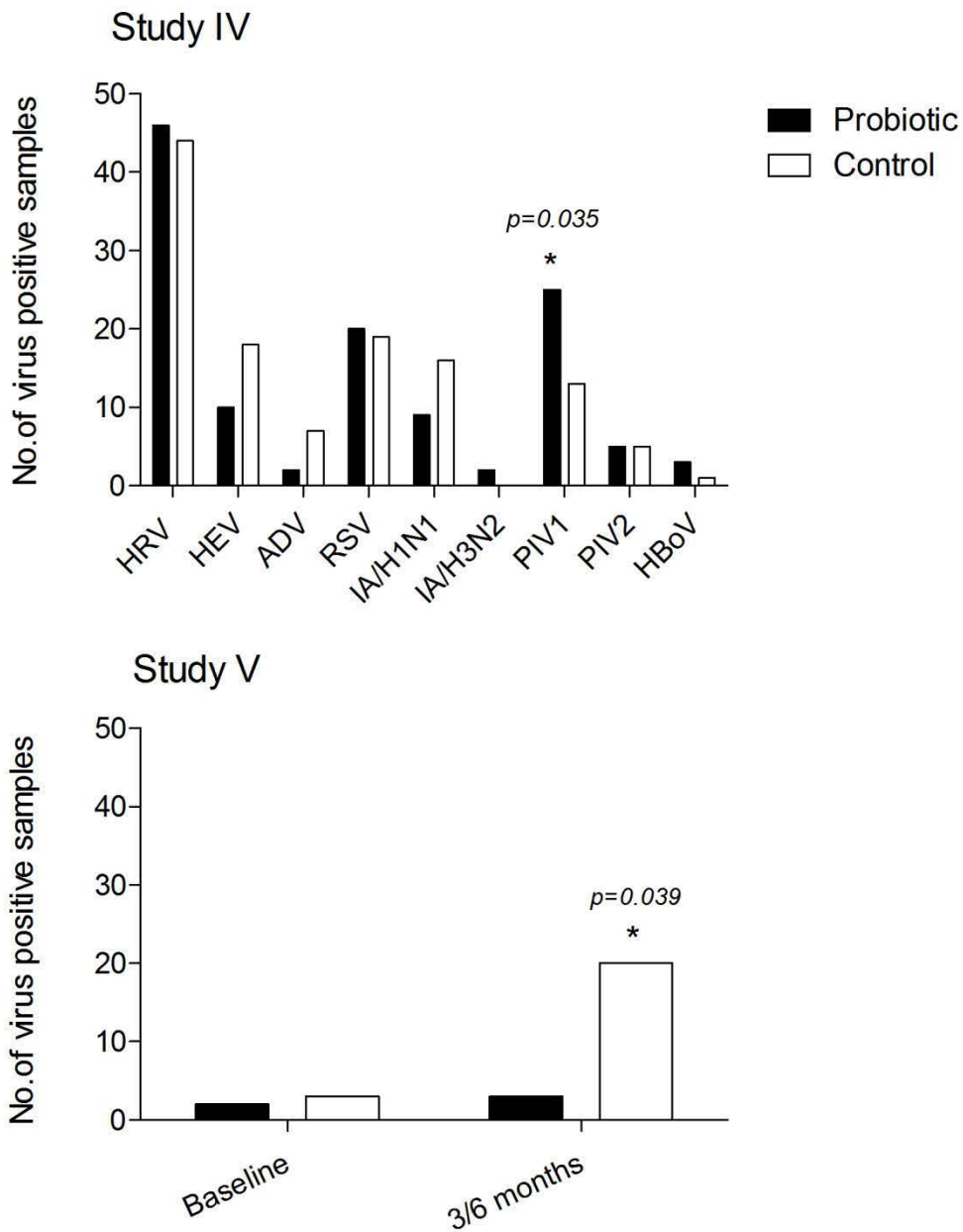


Figure 10. Distribution of respiratory viruses between the study groups in 315 NPS samples of children (Study IV), and distribution of HBoV1 positives in 465 NPS samples of otitis-prone children during intervention (Study V). Only results with HBoV positivity criterion >10 000 copies/ml is shown.

In Study IV, the virus positive samples distributed similarly in the age groups between the study groups (results not shown). In study V, there were more HBoV positive children in the placebo group than in the probiotic group in all age groups (0-2, 2-3, 3-5). However, the differences in HBoV positivities between groups were not statistically significant (**Table 17**).

Table 17. The distribution (%) of HBoV positive children between the age groups in study groups in Study V.

HBoV positivity (copies/ml)	Age (years)					
	0-2		2-3		3-5	
	Placebo (n=51)	Probiotic (n=24)	Placebo (n=37)	Probiotic (n=13)	Placebo (n=17)	Probiotic (n=10)
>1000	18 (35)	7 (29)	9 (24)	3 (23)	5 (29)	1 (10)
>10 000	12 (24)	4 (17)	6 (16)	0	3 (18)	1 (10)
>100 000	6 (11)	1 (4)	3 (8)	0	1 (6)	1 (10)

4.3 THE EFFECT OF PROBIOTIC INTERVENTION ON RESPIRATORY SYMPTOMS ASSOCIATED WITH VIROLOGICAL FINDINGS

In Study IV, during the 28-week intervention period, the children in the GG group had less days with respiratory symptoms per month than the children in the placebo group (6.48 [95 % CI 6.28 to 6.68] vs. 7.19 [95 % CI 6.98 to 7.41], $P < 0.001$). However, probiotic intervention did not have an effect on the mean number of respiratory symptoms observed at the time of the positive viral sample collection.

In Study V, during the 6-month follow-up, there were no statistically significant differences in **children's respiratory symptoms between the** study groups of this substudy (median (IQR) number of days with respiratory symptoms: probiotic vs. placebo; 43 (30-64) vs. 47 (33-70), $P > 0.05$). Overall, no association appeared between HBoV DNA-positive samples (>10 000 copies/ml) and respiratory symptoms, either one or two weeks before and after each sample collection. As HBoV PCR-positivity in the nasopharynx alone is not a reliable marker of acute infection, interactions of HBoV positivity and probiotic treatment on the occurrence of respiratory symptoms were not analyzed.

DISCUSSION

This series of studies investigated the effects of probiotics especially *L. rhamnosus* GG on respiratory and gastrointestinal virus infections in experimental models and in children. A particular focus was on questions, whether viability of a probiotic is an important factor in probiotic-virus interaction, and whether a combination of probiotics is more effective than single strains.

1 METHODOLOGICAL ASPECTS

1.1 HUMAN MACROPHAGE PRIMARY CELL CULTURE MODEL

Human monocyte derived macrophage model was applied for screening of immunomodulatory effects of probiotic combination and individual strains. Cytokine (TNF- α , IL-1 β , IL-6, IL-10, and IL-12) and chemokine (MCP-1, IP-10) responses were analyzed. Macrophages have an important role in innate immune response, as they upon activation produce a large variety of cytokines and chemokines. In addition, macrophages are essential in the pulmonary immune defense against respiratory viruses (Kohlmeier and Woodland, 2009). The interaction with probiotic bacteria and the host takes place most likely on the gut epithelial cells, where probiotics may be ingested by macrophages (Sun et al., 2007), and further initiate innate and adaptive immune responses. *In vitro* cell culture models allow analysis of immunomodulatory effects of probiotics under highly controlled experimental conditions. Lactobacilli and bifidobacteria are known to induce many immunomodulatory effects in human PBMC cells (Miettinen et al., 1998, Drouault-Holowacz et al., 2006, Gackowska et al., 2006, Helwig et al., 2006, Castellazzi et al., 2007, Foligne et al., 2007, Medina et al., 2007, Kekkonen et al., 2008, Dong et al., 2010, Van Hemert et al., 2010).

However, only few studies have characterized probiotic induced immune responses in human macrophages (Miettinen et al., 2000, Veckman et al., 2003, Miettinen et al., 2008, Latvala et al., 2011). In addition, this model would allow interaction studies between probiotics and influenza viruses (Pirhonen et al., 1999, Matikainen et al., 2000, Miettinen et al., 2000).

Optimization of the cell culture conditions is a significant factor, which affects the reliability of the results. For example, the variation between blood donors should be minimized by using at least four donors on each experiment. Of note, health status of the blood donors is controlled for potential infections with a health questionnaire of Finnish Red Cross Transfusion Service. In addition, the optimal bacteria: host cell ratio is critical. In monocyte/macrophage cultures, the bacteria: host ratio 1:1 is widely used, as it induces submaximal cytokine gene expression (Miettinen et al., 1998, Miettinen et al., 2000, Veckman et al., 2003). Moreover, the stimulation period of probiotics is an important factor for the magnitude of immune response. In the present study macrophages were stimulated with bacteria for 24h according to other studies for obtaining maximal immunomodulatory effect (Veckman et al., 2003, Miettinen et al., 2008). However, it is possible that synergistic effects of bacterial combination are more pronounced when stimulation is extended over 24h.

Considering virus-bacteria-interaction studies, influenza virus alone failed to induce TNF- α or IL-1 β production in our experiments possibly due to insufficient virus concentration. There is, however, evidence that influenza A virus is capable of inducing inflammatory response in this cell culture (Pirhonen et al., 1999). In order to obtain measurable effect, the virus dose must be increased. However, increased infection due to larger virus dose may weaken the cells and interfere with probiotic-host cell interaction, and lead further to false results.

1.2 ROTAVIRUS INFECTION MODEL IN NEONATAL RATS

In Study III, we examined whether a viability of a bacterial strain is an important factor in exerting the beneficial health effects of probiotics in virus infections. Probiotics affect most likely through the gut mucosal system, where also the possible interactions with gastrointestinal viruses occur. As the strongest evidence of health effects of *L. rhamnosus* GG (GG) in virus infections is the reduction of duration and severity of rotavirus (RV) induced diarrhea, we chose widely investigated RV rat model for probiotic viability evaluation and virus-host interactions studies. These interactions have been investigated

only in few studies in animals (Duffy et al., 1994, Guérin-Danan et al., 2001, Pant et al., 2007), and no reports exist on the effects of viability of GG in RV infection in a rat model. Heterologous simian RV SA-11 infection model in Lewis rats is considered highly valid for group A RV studies as these virus types replicate most efficiently in rats. Moreover, the size of the GI tract of neonatal rats allows pathophysiological studies (Guérin-Danan et al., 1998, Ciarlet et al., 2002). In the present study, RV SA-11 was effective in inducing diarrhea 2-3 days of post-infection to the rat pups, which is in accordance with other studies (Guérin-Danan et al., 2001, Pérez-Cano et al., 2007). Moreover, although RV diarrhea may last up to 12 days, the onset of disease of the animals inoculated with RV SA11 occurs at three days of post-infection (Ciarlet et al., 2002). The experimental protocol lacked GG control without RV, and thus we cannot confirm whether increased diarrhea observed in both GG groups were partly due to relatively large dose of GG (1.5×10^8 cfu/pup) with respect to the animal size. However, other rat and mice studies have observed decrease in diarrhea with equivalent bacterial concentrations (Guérin-Danan et al., 2001, Pant et al., 2007). In the present study, the rats were clinically healthy and their weight gain increased similarly as in the healthy control group. In contrast, in another study *L.casei* DN-114 001 failed to induce weight gain (Guérin-Danan et al., 2001), highlighting strain specific effects of probiotics in virus infections. Determination of histological differences would be valuable for examining this aspect further.

1.3 VIROLOGICAL DETECTION METHODS

In studies III, IV and V, viral occurrence was determined from different sample matrices with validated sensitive and specific PCR-methods, where positive and negative controls were included in each assay. In RV studies RV antigens are often analyzed in fecal samples with ELISA (Guérin-Danan et al., 1998, Guérin-Danan et al., 2001, Ciarlet et al., 2002, Pérez-Cano et al., 2007). However, in Study III the quantity of fecal samples was insufficient for reliable ELISA RV antigen analysis. Thus, RV quantity in the rat tissues was detected with modified semi-quantitative RT-PCR (Li et al., 2010). In addition, quantitative detection of RV allowed detection of virus positive samples even at low levels, which could have been otherwise missed. In respiratory virus diagnostics, the addition of multiplex PCR enabled detection of multiple viruses from one sample.

Drawbacks of PCR methods include factors such as specimen collection, transport, and the influence of nucleic acid extraction on the ability of amplification techniques to detect viral

nucleic acid. In Studies IV-V, no human bocavirus (HBoV) types 2-4 DNA was detectable from NPS samples. However, HBoV2 rarely occurs in respiratory secretions (Arthur et al., 2009, Chieochansin et al., 2009, Kapoor et al., 2009, Kantola et al., 2010), and HBoV3 and HBoV4 have been identified only in stool samples (Arthur et al., 2009, Kantola et al., 2010, Kapoor et al., 2010). In Study IV, the overall positivity rate of the detected viruses was surprisingly low, as compared with other respiratory virus epidemiology studies conducted in Finland (Nokso-Koivisto et al., 2006, Ruohola et al., 2009). However, NPS samples in our study were collected in widely used transport media (UTM-RT), which is acknowledged for long stability and preservation of viability for viral nucleic acid containing samples (Walsh et al., 2008, Luinstra et al., 2011). In addition, the analytic processes were executed with validated nucleic acid and PCR procedures. Some viral infections were probably missed, given that NPS samples were only collected during visits to the study physician; in some cases, parents may have considered a physician's **visit unnecessary, or parents may have** taken their child to physicians who were not involved in the study. Respiratory viruses are also found in asymptomatic children (Jartti et al., 2004, Jansen et al., 2011). Moreover in Study V, HBoV1 was commonly present in the nasopharynx of otitis-prone children during the cold period even when they were free of respiratory symptoms.

In Study V, quantitative PCR detection of HBoV may offer insight into the clinical impact of HBoV, because HBoV at a high viral load (>10 000 copies/ml) has been associated with RTIs (Allander et al., 2007, Jartti et al., 2102). However, recent publications show that HBoV PCR positivity of NPS alone is not a trustworthy marker for detection of acute HBoV infection (Söderlund-Venermo et al., 2009, Christensen et al., 2010, Martin et al., 2010, Don et al., 2011). Serological verification of an actual infection is needed to circumvent the PCR-related problems of virus shedding and mucosal contamination, and to accurately diagnose an acute HBoV infection. Although HBoV respiratory infections can be diagnosed with moderate accuracy by qPCR of nasopharyngeal samples, the most reliable methods for diagnosis of acute symptomatic HBoV infection are PCR of serum samples and serologic analysis for IgM and IgG (Christensen et al., 2010).

2 DEVELOPMENT OF COLORIMETRIC NEUTRALIZATION TEST

A novel colorimetric neutralization test (NT) was developed for the measurement of influenza virus antibodies. NT was applied to study the antibody response after the administration of a seasonal, inactivated, trivalent influenza vaccine. Antibody titers determined by the NT in pre- and post-vaccination serum pairs were compared with those obtained by the traditional hemagglutination inhibition (HI) assay. The results obtained by both assay methods correlated well. Moreover, the NT yielded higher pre- and post-vaccination titers, and a larger number of significant increases in post-vaccination antibody titer than the HI test, indicating a higher sensitivity of this test principle. Similar observations have been reported in other studies (Harmon et al., 1988, Rowe et al., 1999). With the introduction of new vaccines against seasonal and potentially pandemic influenza, and with unusual subtypes of influenza A viruses occasionally causing disease in humans, there is an increased need for sensitive, specific and reproducible serological methods to study the antibody response to vaccines and to infection with wild-type viruses (Leroux-Roels et al., 2007, Bright et al., 2008, Ehrlich et al., 2008). Moreover, standard HI tests have proven to be suboptimal for measuring antibodies to H5 viruses and other influenza A viruses of avian origin (Rowe et al., 1999, Nicholson et al., 2001), although the use of horse red blood cells appears to improve their sensitivity (Stephenson et al., 2004, Kayali et al., 2008, Ducatez et al., 2011).

The advantage of colorimetric NT is that the colorimetric protocol does not involve any washing steps allowing attached and floating cells to be available for viability assessment. This resulted in higher reliability and reproducibility of the assay evidenced by low intra- and inter-assay variation. Other traditional influenza NT protocols quantify remaining infectious virus after the neutralization reaction and an appropriate period of incubation (Harmon et al., 1988, Rowe et al., 1999). Results of such assays can be skewed by several factors. Virus-infected, weakened cells can detach from the growth surface and may be lost during the washing process before the cells are fixed. Trypsin added to the cell culture medium may also enhance such cell-loss. Reducing the number of infected cells may yield in too high titers. Furthermore, commonly used NTs involve several washing steps, fixation of the cells, incubation with specific antibodies, conjugates and substrates, i.e., processes which generate aerosols, produce infective and/or toxic waste, and usually last several hours (Harmon et al., 1988, Tannock et al., 1989, Crawford-Miksza and Schnurr, 1994, Rowe et al., 1999). The single working step required in the colorimetric assay in order to quantify neutralizing antibodies after the incubation period is the addition of the tetrazolium salt-containing

reagent. Under biosafety level-3 laboratory working conditions, where NTs are performed with wild-type H5N1 and other potentially pandemic viruses, the minimization of number of hands-on manipulations in an assay is highly desirable. The only disadvantage of colorimetric NT protocol is that assay procedure involves incubation period for 48-72h, while other NT formats often require only 24h (Harmon et al., 1988, Rowe et al., 1999). However, poorly replicating viruses may not cause complete cell destruction during a short incubation time. Moreover, with well replicating viruses and a slightly higher virus input (e.g. 100 pfu/assay) the incubation period can be shortened without affecting the quality of assay results.

Considering probiotic research applications, colorimetric NT would be highly valid assay for studying the immune adjuvant effects of probiotics on serum influenza antibody titers. This method was recently applied for studying the antibody responses elicited by chicken anemia virus vaccine using *L. acidophilus* as a live delivery vehicle (Moeini et al., 2011). In an influenza vaccination study in healthy adults performed with HI test, *L. rhamnosus* GG failed to improve significantly the efficacy of influenza vaccine to influenza A/H1N1 and B viruses (Davidson et al., 2011). One may speculate whether significant differences would have been observed with more sensitive and specific NT, which is able to detect lower antibody titers and more significant titer increases than the HI test.

3 IMMUNOMODULATORY EFFECTS OF PROBIOTIC COMBINATION IN MACROPHAGES *IN VITRO*

In macrophages, combination of *L. rhamnosus* GG (GG) and *L. rhamnosus* Lc705 (Lc705) was able to induce similar or weaker proinflammatory (TNF- α , IL-6), anti-inflammatory (IL-10), or chemokine (MCP-1, IP-10) responses as individual GG or Lc705. However, the cytokine responses induced by this combination were stronger than responses induced by *Lc. lactis* ARH74 (ARH74). Only few studies have characterized, whether combination of different strains enhances the function of the immune system synergistically *in vitro* compared with individual strains (Drouault-Holowacz et al., 2006, Castellazzi et al., 2007, Kekkonen et al., 2008, Li et al., 2011). Our results are in concordance with studies conducted in human PBMC cells, where GG and Lc705 together with PJS was inefficient in enhancing cytokine responses IL-10, IFN- γ , IL-12, and TNF- α over individual strains (Kekkonen et al., 2008). Bacteria in a combination may inhibit the immunomodulatory action of one another possibly by production of antagonistic agents, by competing with binding to the same receptor, or adhesion to the epithelium. Indeed, a recently discovered unique pilus structure of GG provides better adherence over Lc705 to the epithelial cells in

the GI tract (Kankainen et al., 2009). However, in the present study we found that combination of GG/Lc705 induced IL-1 β 1.5 times more effectively than Lc705, suggesting that when including only lactobacilli genera in the combination, synergistic immunomodulatory effects could be achieved. Nevertheless, the use of probiotic multispecies of GG, Lc705, Bb99 and PJS or VSL#3 in clinical trials has shown great promise in the prevention of irritable bowel syndrome, *Helicobacter pylori* infection, or atopic eczema (Kim and Hodinka, 1998, Kajander et al., 2005, Kim et al., 2005, Myllyluoma et al., 2005, Kukkonen et al., 2007). Interestingly, all bacteria studied in the present study induced MCP-1 and IP-10 production, and ARH74 was the strongest inducer. Thus far only GG is known to induce the expression of these chemokines in human macrophages (Veckman et al., 2003). In human DCs, ARH74 also efficiently induced IP-10 when compared with GG and Lc705 (Latvala et al., 2008).

Cytokine and chemokine profiles may provide mechanistic insight for the observed clinical effects of probiotics. As in other reports (Miettinen et al., 2000, Veckman et al., 2003, Miettinen et al., 2008), in the present study *L. rhamnosus* GG was able to induce low grade inflammation in human macrophages. Moreover, *L. rhamnosus* GG was effective in enhancing chemokine production, which is involved in promoting chemotaxis. As macrophages play an important role in the innate response against virus infections, probiotic induced low grade inflammation may enhance immune system, and recruit leukocytes to the infection site further facilitating viral elimination. Indeed, meta-analyses show that *L. rhamnosus* GG reduces the incidence and duration of RV diarrhea (Szajewska et al., 2007, Szajewska et al., 2011), and reduces the risk of RTIs in children (Hatakka et al., 2001, Hojsak et al., 2010b). In addition, by stimulating the production of IP-10 and MCP-1 chemokines in human macrophages, lactobacilli and lactococci could be efficient in promoting chemotaxis of leukocytes, and further enhancing the clearance of invading pathogens. In mice, *L. rhamnosus* GG has been effective in reducing RV load from the small intestine (Pant et al., 2007). Similarly, in influenza virus infection, lactobacilli have promoted viral clearance from the lungs possibly by activating NK-cells (Yasui et al., 2004, Izumo et al., 2010, Nagai et al., 2011, Takeda et al., 2011).

To conclude, the ability of probiotic combination to induce immune responses in human macrophages differ from that of individual strains. Systematic evaluations of probiotic-interactions *in vitro* are necessary prior selecting and testing the therapeutic efficacy of probiotic combinations in various disease conditions and in human immune status in general in human intervention studies.

4 THE EFFECTS OF NONVIABLE PROBIOTIC IN ROTAVIRUS INFECTION

In RV infection nonviable GG had comparable beneficial effects as viable GG. Although, viable or nonviable GG did not relieve RV diarrhea, both inhibited RV infection induced weight reduction and RV induced colon swelling in our animal model. Moreover, both animal groups receiving GG had less frequently RV in plasma, intestinal tissues, and feces than the RV control group. However, only viable GG was effective in reducing significantly the amount of RV in the colon. Only one clinical study has addressed the effects of nonviable /inactivated probiotics in RV diarrhea (Kaila et al., 1995). Moreover, this study did not include untreated control group allowing comparison between the effects of probiotic product forms and RV. Our results are in concordance with another study in mice (Pant et al., 2007), where only viable GG supplementation in combination with antibodies significantly reduced rhesus RV load in the small intestine (Pant et al., 2007). In the present study, viable GG reduced RV occurrence in plasma possibly by inducing neutralizing antibody production against RV. In children only live GG enhanced IgA antibody response to RV (Kaila et al., 1995).

It is likely that multiple mechanisms are involved in the probiotic-virus interaction in the host, and viability of a bacterium may have a role in the mechanism of action. The host-dependent factors may have an impact on the microbiological and viral interactions as well. In the intestine probiotics may protect from RV by competing adhesion sites and inhibiting viral attachment, which in the present study was possibly seen by reduction of RV from the intestine by both product forms. Other studies confirm that both viable and nonviable lactobacilli are able to adhere to human intestinal cells (Ouwehand et al., 2000), and heat killed *L. acidophilus* LB is able to inhibit adhesion of diarrheagenic bacteria (Coconnier et al., 1993). In macrophages and human T84 intestinal epithelial cells both viable and heat-killed GG are able to induce NO synthesis as well (Korhonen et al., 2001). In RV infection, infected enterocytes release NO (Rodríguez-Díaz et al., 2006), which may stimulate the enteric nervous system, and induce water secretion into a luminal space further causing diarrhea (Izzo et al., 1998). In the present study both GG groups suffered from diarrhea, but less RV was detected from their tissues. It would be interesting to speculate that by enhancing NO-production and diarrhea, GG may inhibit RV adhesion and increase RV clearance by **“flushing” the virus from the body. Thus, by this mechanism GG may shorten the duration,** and enhance the recovery from RV diarrhea, as seen in several clinical studies (Kaila et al., 1992, Isolauri et al., 1994, Majamaa et al., 1995, Guarino et al., 1997, Guandalini et al., 2000). Alleviation of RV infection may also be due to anti-inflammatory effect. In the present study,

RV infection increased colon weight probably by enhancing inflammation and promoting tissue swelling by activating cytokine and chemokine responses (IFN- α , IL-8, IP-10) of intestinal epithelial cells (Rollo et al., 1999). Both viable and nonviable GG, however, reduced **colon weight, which may result from the GG's** ability to decrease proinflammatory mediators (TNF- α , IL-1 β), and increase anti-inflammatory mediators (IL-10) (Zhang et al., 2005, Li et al., 2009). In Study II, GG also induced anti-inflammatory IL-10 production in macrophages. Probiotics may also elicit anti-RV effects via stimulating adaptive immune responses. In the present study, viable GG was more effective than nonviable GG in reducing RV quantity in plasma. Moreover in children, viable GG has been more effective in stimulating RV IgA responses (Kaila et al., 1995). The effects of nonviable bacteria may depend further on the method of inactivation. For instance, inactivation by heat or irradiation may disrupt the surface protein conformation of the bacterium thereby inhibiting the ability of a bacterium to adhere to the epithelial cell (Ouwehand et al., 2000). However, if anti-RV effects are due to secreted bioactive or antimicrobial peptides (Yan et al., 2007, Lu et al., 2009), GG needs to be viable.

In the future, the potential effect of probiotic induced NO production on RV infection clearly deserves further attention. In addition, histological examination of the rat intestines treated with GG would provide more information on the mechanisms underneath (Pant et al., 2007, Preidis et al., 2012). Furthermore, identification of the key factors defining probiotic viability is necessary in order to evaluate the value of nonviable probiotics in virus infections. In a similar manner, it may be important to assess the impact of probiotic processing methods on **the pathogen probiotic interaction (Grześkowiak et al., 2011)**. Finally, it would be of importance to verify the effectiveness of nonviable probiotics in human intervention studies. As noroviruses are second to RVs as causative agents of acute gastroenteritis in children (Puustinen et al., 2011, Räsänen et al., 2011b), future studies could focus on specific probiotics and their ability to alleviate the symptoms of these infections as well.

5 PROBIOTIC'S EFFECTS ON RESPIRATORY VIRUS INFECTIONS

In the present study, we evaluated whether probiotic intervention is effective in reducing the occurrence of common respiratory viruses in the nasopharynx of children. In children in Study IV, *L. rhamnosus* GG intervention did not have significant diminishing effect on the occurrence of respiratory viruses (HRV, HEV, ADV, influenza A and B virus, PIV1-2, RSV, or HBoV). In contrast, in otitis-prone children (Study V), probiotic combination (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* 99, and *P. freudenreichii* JS) was able to decrease the nasopharyngeal presence of HBoV at high viral load three to six months after intervention. Many clinical trials in children have only investigated the effectiveness of probiotics on respiratory infections (Hatakka et al., 2001, Cobo Sanz et al., 2006, Hatakka et al., 2007, Rautava et al., 2009, Hojsak et al., 2010a, Hojsak et al., 2010b, Taipale et al., 2011). Only one, at least to our knowledge, has characterized the viral etiology of these infections to some extent as well (Hatakka, 2007). However, in that study no significant differences appeared between the study groups in HRV and HEV positivity. Our results imply that multispecies of probiotics may have some advantage over single strain in reducing viral occurrence in the **children's respiratory tract. In the present study, a single administration of the *L. rhamnosus* GG** may not have been sufficient to induce changes into the viral occurrence. In Study IV, the consumption of *L. rhamnosus* GG in milk was on average 10^8 cfu (Kumpu et al., 2012) with 95% of recovery of GG in the fecal samples in the probiotic group. In Study V, the concentration of each strain of probiotics in combination was $8-9 \times 10^9$ cfu/capsule with 95% compliance. However, in this study the concentration of probiotics was not analyzed in fecal samples. Studies in children showed that *L. rhamnosus* GG in a concentration of $1-2 \times 10^8$ cfu **slightly reduced children's RTIs** (Hatakka et al., 2001), whereas concentration 10^9 cfu of GG was able to significantly reduce the risk of RTIs (Hojsak et al., 2010a, Hojsak et al., 2010b). Moreover, GG in combination with *B. lactis* Bb12 in capsules with a concentration of 1×10^{10} cfu reduced the risk of early AOM and incidence of recurrent respiratory infections (Rautava et al., 2009). As the immune system undergoes a process of functional maturation through childhood, exposure to multiple probiotics may also accelerate the maturation of immune system. In addition, in children prone to infections, which have impaired microbiota due to treatment with numerous antimicrobials, the effect may be more pronounced.

Considering viral associated respiratory infections, *L. rhamnosus* GG was unable to reduce the number of respiratory symptoms observed at the time of a viral finding in children in Study IV. However, the children in the probiotic group had fewer days with respiratory symptoms per month than the children in the placebo group. In the present study

population, children who visited the study physician due to symptoms of respiratory infection were possibly experiencing illnesses more frequently or more severe infections than the main study population (Kumpu et al., 2012). One may speculate that probiotics could have more pronounced effect in children more susceptible to infections. In otitis-prone children (Hatakka et al., 2007), who are frequently ill, supplementation of *L. rhamnosus* GG in a combination tended to reduce recurrent upper RTI. Interestingly, in these otitis-prone children, probiotic combination reduced the recurrence of RTI episodes more if the children were negative to both HRV and HEV than if they were positive (Hatakka, 2007). As HRV and HEV were the only viruses investigated at that time, it may have been that those virus negative children were actually HBoV positive. Thus, it could be hypothesized that reduction of recurrent RTIs was due to probiotic combination's ability to reduce HBoV occurrence. Indeed, increasing evidence suggests that HBoV is associated with upper and lower RTIs in children (Allander et al., 2007, Söderlund-Venermo et al., 2009, Christensen et al., 2010, Meriluoto et al., 2012).

Oral administration of probiotics may reduce nasopharyngeal viral occurrence (Study V) and shorten days with respiratory symptoms (Study IV) by augmenting local and systemic immune response through colonization of GI tract, or through colonization of tonsil tissue as well (Kumpu and Tikkanen et al., submitted 2012). Few studies highlight these plausible effect mechanisms. In healthy adults, *L. rhamnosus* GG was effective immunoadjuvant for live-attenuated influenza vaccine H3N2 component by increasing seroprotection (Davidson et al., 2011). In mice, *L. rhamnosus* GG protected from H1N1 influenza virus infection by reducing viral titers, accumulated symptom rate, and increasing mice survival rates possibly by regulating respiratory immune responses such as pulmonary IL-1 β , TNF, and MCP-1 mRNA expression (Harata et al., 2010, Kawase et al., 2010). Interestingly, intranasal administration of *L. rhamnosus* GG has been effective in activating immune responses against influenza infection in respiratory epithelia (Harata et al., 2010). Moreover, *L. rhamnosus* GG is able to activate innate defense mechanisms by nasal spray in humans (Skovbjerg et al., 2009). It would be tempting to speculate whether nasal administration of probiotics would have more pronounced effect also on viral occurrence.

In the future, more studies conducted with bacterial combinations in the prevention of respiratory infections in larger number of patients are warranted. In addition, these studies need to uncover the specific patient populations and the specific probiotic strains which could elicit beneficial effects. Moreover, nasal bacteriotherapy would be worth considering approach. **Probiotic's ability to enhance local and systemic innate immunity during virus infection** is a plausible, yet unverified, effect mechanism behind beneficial effects, and an interesting area of future research. Inclusion of serological and immunological diagnostics in research experiments would have clear benefits in providing valuable information on the effects of probiotics in respiratory virus infections.

CONCLUSIONS

The present series of studies investigated the effects of probiotics on respiratory and gastrointestinal virus infections. The main findings are as follows:

1. Colorimetric neutralization test was a sensitive and specific serological method for measuring influenza virus antibodies. This method was valid and suitable for influenza virus vaccine research, and could be also applied for studying immune adjuvant effects of probiotics on serum influenza antibody titers.
2. In human macrophages combination of *L. rhamnosus* GG and *L. rhamnosus* Lc705 induced similar or weaker immune responses than individual *L. rhamnosus* bacteria. However, the combination was more effective in inducing proinflammatory responses over traditional starter culture bacterium *Lc. lactis* ARH74. Probiotics and their combinations differ in their ability to elicit immunomodulatory effects *in vitro*. When applying new probiotic combinations into clinical studies, their interactions should be carefully evaluated.
3. Nonviable *L. rhamnosus* GG was able to elicit comparable beneficial effects as viable product form in rotavirus infection. However, only viable *L. rhamnosus* GG reduced significantly rotavirus load in the colon, highlighting that viability is an important factor in virus infections.
4. In children *L. rhamnosus* GG reduced days with respiratory tract symptoms. However, *L. rhamnosus* GG was not effective in reducing viral occurrence in the nasopharynx, suggesting that *L. rhamnosus* GG may be able to reduce viral respiratory symptoms through augmentation of systemic and local immunity.
5. In otitis-prone children, a specific combination of probiotics including *L. rhamnosus* GG reduced the nasopharyngeal presence of human bocavirus.

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