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SPECIFIC PROBIOTICS IN THE UPPER RESPIRATORY TRACT:

COLONIZATION, EFFICACY, AND SAFETY WITH A FOCUS ON *LACTOBACILLUS RHAMNOSUS* GG

Laura Tapiovaara

ACADEMIC DISSERTATION

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"It always seems impossible until it's done."
–Nelson Mandela

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I Tapiovaara L, Lehtoranta L, Swanljung E, Mäkivuokko H, Laakso S, Roivainen M, Korpela R, Pitkäranta A. *Lactobacillus rhamnosus* GG in the middle ear after randomized, double-blind, placebo-controlled oral administration. Int J Ped Otorhinolaryngol. 78: 1637-1641, 2014.

II Swanljung E, Tapiovaara L, Lehtoranta L, Mäkivuokko H, Roivainen M, Korpela R, Pitkäranta A. *Lactobacillus rhamnosus* GG in adenoid tissue: double-blind, placebo-controlled, randomized clinical trial. Acta Otolaryngol. 135: 824-830, 2015.*

III Tapiovaara L, Kumpu M, Mäkivuokko H, Waris M, Korpela R, Pitkäranta A, Winther B. Human rhinovirus in experimental infection after per oral *Lactobacillus rhamnosus* GG consumption. Submitted.

IV Tapiovaara L, Lehtoranta L, Poussa T, Mäkivuokko H, Korpela R, Pitkäranta A. Absence of adverse events in healthy individuals using probiotics – analysis of six randomized studies by one study group. Beneficial Microbes, *in press*.**

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ABBREVIATIONS

AE	Adverse event
AOM	Acute otitis media
BB12	*Bifidobacterium lactis* BB-12
BB99	*Bifidobacterium breve* 99
cfu	Colony forming unit
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CTCAE	Common Terminology Criteria for Clinical Adverse Events
dB	Decibel
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
FiRE	Finnish Study Group of Antimicrobial Resistance
EV	Enterovirus
GI	Gastrointestinal
HRV	Human rhinovirus
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
Lc705	*Lactobacillus rhamnosus* Lc705
L. GG	*Lactobacillus rhamnosus* GG (ATCC 53103)
MEE	Middle ear effusion
NSAID	Non-steroidal anti-inflammatory drug
OM	Otitis media
OME	Otitis media with effusion
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PJS	*Propionibacterium freudenreichii* JS
qPCR	Quantitative real-time polymerase chain reaction
QPS	Qualified presumption of safety
rAOM	Recurrent acute otitis media
RNA	Ribonucleic acid
RR	Risk ratio
RSV	Respiratory syncytial virus
$TCID_{50}$	50% Tissue culture infectious dose
TNF	Tumor necrosis factor
URI	Upper respiratory infection

ABSTRACT

Upper respiratory infections are among the most common ailments in humans. Evidence for mechanisms suggests that specific probiotic bacteria could reduce the risk and symptoms of these infections. However, the clinical evidence of probiotics in the upper respiratory tract, especially when colonization and the etiological effects are considered, is sparse. In addition, the safety of probiotics requires constant assessment. This thesis investigated the recovery of probiotic *Lactobacillus rhamnosus* GG (L. GG) from the upper respiratory tract and its effects on pathogens in this tract. In addition, the thesis assessed the adverse events of L. GG alone or in combination with other probiotics (*Bifidobacterium lactis* BB-12 [BB12], or *Lactobacillus rhamnosus* Lc705 [Lc705], *Propionibacterium freudenreichii* JS [PJS], and/or *Bifidobacterium breve* 99 [BB99]).

In a randomized, double-blinded, placebo-controlled study, 40 children consumed per oral L. GG or a placebo (1:1) prior to surgery in which their adenoids were removed and a possible middle ear effusion (MEE) was collected. L. GG was recovered from both the adenoid tissue and MEE, but it did not affect the findings of human rhinovirus (HRV) or enterovirus (EV) in the samples compared to the placebo. In addition, the analysis of the bacterial pathogens in the MEE showed similarities in both intervention groups. No differences between the groups emerged in respiratory or gastrointestinal (GI) symptoms prior to the surgery or in pain or bleeding after the surgery.

In another randomized, double-blinded, placebo-controlled trial, an experimental HRV infection model was used in 59 healthy adult volunteers to investigate the effects of the oral consumption of live, heat-inactivated L. GG on the HRV load in nasopharyngeal lavage samples. The correlation of the HRV load to the subjects' clinical symptom scores was assessed. The use of live or inactivated L. GG did not result in statistical differences in the HRV load, but a tendency to lower loads in the L. GG groups was noted. The HRV load positively correlated with the total symptom scores on day 2 and day 5 after inoculation.

In the fourth study, individual participant data from six randomized placebo-controlled probiotic studies were analyzed for adverse events (AEs), as distributed by the Common Terminology Criteria of Adverse Events (CTCAE). Data on 1,909 healthy subjects, including children, young adults, and elderly participants, revealed no statistical differences in AEs between the groups that consumed L. GG alone, L. GG in combination, or the placebo. A detailed analysis of three specific categories (respiratory diseases, gastrointestinal diseases, and infections) did not yield any statistical differences in AEs between the probiotic and placebo groups.

Based on the results, we concluded that L. GG was able to colonize the upper respiratory tract, but it had no effects on the levels of viral or bacterial pathogens or on the frequency of clinical symptoms in the subjects during either the intervention or the follow-up period. The nasopharyngeal HRV load was positively correlated with the subjects' total symptom score. The use of L. GG alone or in combination did not result in AEs in the population of healthy children, young adults, and elderly participants.

INTRODUCTION

The interest in gut microbiota has emerged in recent decades. Gut microbiota has been associated with the promotion of health, the increased the risk of disease, and the maintenance of some diseases. Upper respiratory infections caused by viruses are among the most common health problems in humans (Fendrick *et al.* 2003). In addition to the misery of sickness, these infections result in a significant burden on society in terms of healthcare visits, absences from work, and reduced school attendance. In addition, unnecessary medical costs are incurred. The careless use of antibiotics during respiratory tract infections has resulted in the constantly growing resistance of microbes to antibiotics (Roca *et al.* 2015). The complications of upper respiratory infections, such as otitis and sinusitis, also result in high expenses and expose patients to potentially harmful operations. If viral upper respiratory infections could be prevented and treated, these outlays would be minimized.

According to a panel of international experts, "probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (WHO/FAO 2011; Hill *et al.* 2014). The strain should be precisely defined (i.e., identified and characterized), the dose should be defined, the health claim should be indicated, and the safety should be assessed. The properties of probiotics vary widely according to the strain. Even the manufacturing process influences the properties of certain strains (Grześkowiak *et al*. 2011).

Lactobacillus rhamnosus GG (L. GG, ATCC 53103) is one of the most-often studied probiotics. This bacterial strain of human origin has been isolated from the human gut. Its benefits in GI disorders have been demonstrated (Vitetta *et al.* 2014), and similar effects have been found in upper respiratory infections (Hojsak et al. 2010a, Hojsak et al. 2010b). Although the colonization of the gut and the fecal recovery of specific probiotics, including L. GG, have been extensively studied, little information is available on the colonization of the upper respiratory epithelium where the lymphatic system is present. Even less is known about the effects of the possible colonization of the related mucosal tissues. Probiotics are widely added to commercial dairy products and food products, and they are increasingly consumed as supplements (Siró *et al.* 2008). The safety of L. GG has been monitored since 1989. A few case reports have describe infections caused by probiotics, such as bacteremia, endocarditis, and internal organ abscesses. However, the incidence of *Lactobacillus* bacteremia has remained stable although the consumption of probiotic products has increased exponentially (Salminen *et al.* 2002). Infections seem to be very sparse and affect mostly immunocompromised or critically ill patients (Boyle *et al*. 2006). Probiotic consumption has been documented as safe in neonates and even in preterm infants (AlFaleh, Anabrees 2013). A report from Finland suggested that L. GG is safe for premature infants based on 12 years of its administration to all premature and very low birth weight infants born in the area around one university hospital (Luoto *et al.* 2010).

In the USA, probiotics are regulated by The Food, Drug, and Cosmetic Act (Degnan 2008). The Food and Drug Administration assigns probiotic products to one of several regulatory categories: food, medical food, dietary supplement, or drug or biological products, and the regulation of the product depends on the category. In Europe, the European Food Safety Authority panel on Dietetic Products, Nutrition and Allergies assesses the scientific evidence of potential probiotic health claims and the safety of novel probiotics.

The safety of such products must be comprehensively studied. However, few previous studies have investigated the possible adverse events of probiotics.

2 LITERATURE REVIEW

2.1 Probiotics in the respiratory tract

2.1.1 Probiotics and their health effects

Probiotics should fulfill the following criteria: they must survive in the gastrointestinal tract and be able to proliferate in the gut; they should benefit the host through growth and/or activity in humans; and they should be non-pathogenic and non-toxic (Wassenaar *et al.* 2008). Probiotic micro-organisms exist in multiple genus, species, and strains. Although recent evidence suggests that they have some common health effects, they have many strain-specific health effects (Hill *et al.* 2014). The most common probiotic organisms are bacteria from the genus *Bifidobacterium* and the genus *Lactobacilli* (Guarner *et al.* 2012). The findings of broad meta-analyses of strain-specific probiotics support that common health benefits are derived from consuming an adequate dose of any safe strain of a species that is already known to be an effective probiotic. For example, a meta-analysis of different strains and 10,351 patients found that probiotics had a positive effect on eight gastrointestinal diseases across the all studied probiotic species (Ritchie, Romanuk 2012). However, the results showed differences in efficacy regarding specific diseases and specific differences in strains.

Professional medical organizations have made clinical recommendations of well-defined specific probiotics for specific clinical conditions. In particular, gastrointestinal conditions have shown health effects: probiotics are recommended in the treatment and prevention of acute gastroenteritis, necrotizing enterocolitis, and antibiotic-associated diarrhea (Ebner *et al.* 2014). They can be supplemented with infant formula to enhance growth and improve clinical outcomes although evidence is still lacking (Braegger *et al.* 2011). Some evidence exists to support the use of probiotics in several conditions: constipation, irritable bowel syndrome, inflammatory bowel diseases, lactose intolerance, allergies, atopic eczema, certain cancers, hepatic diseases, hyperlipidaemia, *Helicobacter pylori* infection, genitourinary tract infections, and oral health (Brown, Valerie 2004, Kopp-Hoolihan 2001). In 2015, the World Allergy Organization (WAO) convened a guideline panel to develop evidence-based recommendations for the use of probiotics in the prevention of allergy (Fiocchi *et al.,* 2015). The European Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) association has also established recommendations for the use of probiotics in the prevention and treatment of acute gastroenteritis in children (Szajewska *et al.,* 2014) ESPGHAN recommends the use of specific, well-studied probiotics to prevent and treat acute gastroenteritis in infants and children and to reduce the side-effects associated with antibiotics (Szajewska *et al.,* 2014). In addition, the meta-analysis of a specific probiotic strain concluded that L. GG was effective in preventing antibiotic-associated diarrhea in children and adults who were treated with antibiotics for any reason (Szajewska and Kołodziej, 2015).

The heat inactivation of probiotic bacteria has some advances over live bacteria. It prolongs shelf life and facilitates storage and transportation. Heat-inactivated probiotics have been studied mainly in animals, where they have shown favorable effects in immune responses (Chen *et al.* 2013, Liu *et al.* 2014, Liu *et al.* 2015, Munoz-Atienza *et al.* 2015).

2.1.2 Mechanisms of action

The mechanisms of the action of probiotics in viral and bacterial infections are not completely understood. Specific probiotics show strain-specific potential for reinforcing the integrity of the intestinal epithelium and regulating immune components. In regulating complex immune responses, the gastrointestinal tract from the oral cavity to the rectum is considered the largest immune interface with the environment (MacDonald *et al.* 2011). The potential mechanisms are studied mainly in the gastrointestinal epithelium. Some postulated mechanisms of probiotic action in intestinal epithelial defense are presented in Figure 1.

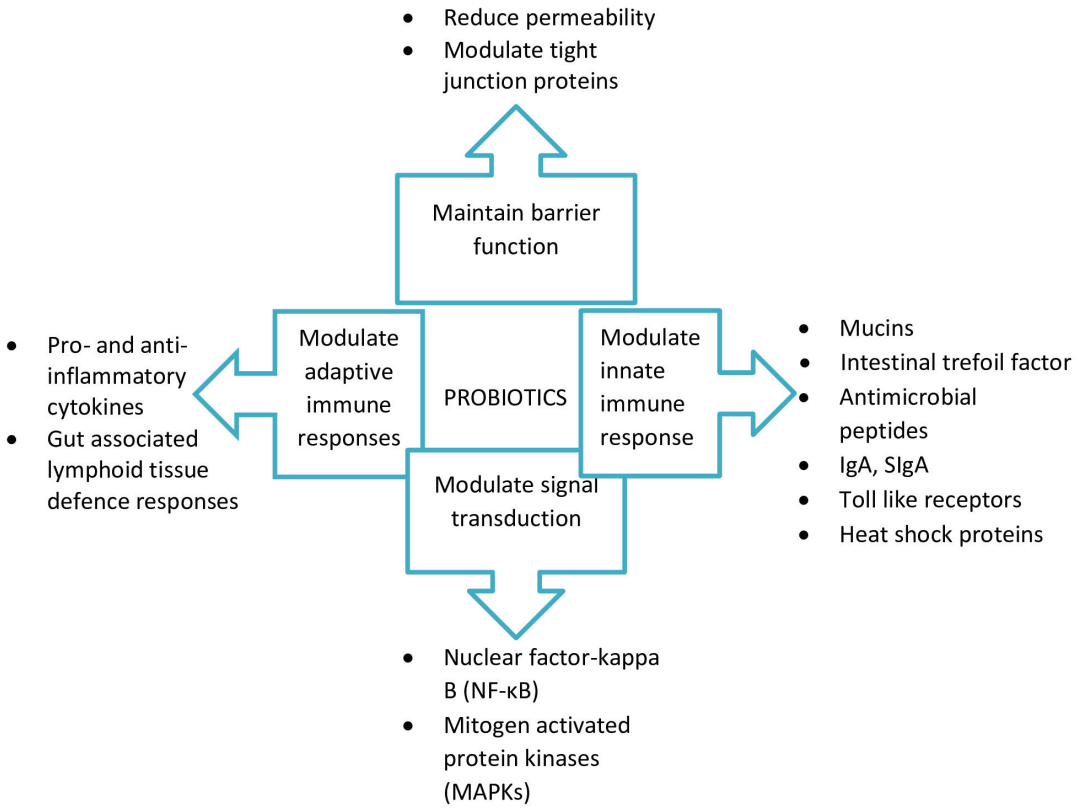

Figure 1. Possible mechanisms by which probiotic bacteria modulate intestinal defense responses. Adapted from Wan et al. (2015)

It is possible that probiotic bacteria could bind to an invading virus, thus inhibiting virus attachment to the host-cell receptor (Salminen *et al.* 2010). Lactic acid bacteria may exert antiviral activity by the following: 1) direct interaction as an adsorptive or trapping mechanism; 2) stimulation of the immune system by interleukin, natural killer cells, Th1 immune response activity, and IgA production; 3) production of antiviral agents (e.g., hydrogen peroxide, lactic acid, and bacteriocins) (Al Kassaa *et al.* 2014).

2.2 Acute upper respiratory infections

2.2.1 Viral infections

Acute viral upper respiratory infections (URI), which are also known as the common cold, are among the most common health problems in humans (Heikkinen, Järvinen 2003). The economic burden on society of the otherwise usually benign disease is enormous because of absences from work, school, and daycare, as well as the utilization of health care providers and treatments. In the USA, 25 million health care visits a year are made because of URI (Gonzales *et al.* 2001), and in Finland, 2.4 million visits are made annually for upper and lower respiratory infections (Lumio *et al.* 1996). On average, children 1-2 years of age experience 3-8 respiratory infections yearly, and children over 5 years of age experience about three respiratory infections yearly (Wald *et al.* 1991, Nokso-Koivisto *et al.* 2006).

More than 200 viruses are known to cause respiratory infections in humans (Eccles 2005). Major pathogens that induce URIs are human rhinoviruses (HRVs) from the family *Picornaviridae,* genus *Enterovirus* (Fendrick 2003). Other common causative agents are respiratory syncytial virus (RSV), parainfluenza virus, enterovirus (EV) from the family *Picornaviridae* and genus *Enterovirus*, coronavirus, influenza virus, and adenovirus (Passioti *et al.* 2014). Of these, influenza virus, RSV, and parainfluenza virus are more frequent causes of lower than upper respiratory infections (Mäkelä *et al.* 1998, Heikkinen, Järvinen 2003, Nokso-Koivisto *et al.* 2006). The symptoms of URI arise after an incubation period that varies depending on the causative agent.

The symptoms of HRV infection include sore throat, sneezing, nasal obstruction and discharge, hoarseness, cough, malaise, myalgia, and chills. HRV can also induce acute otitis media (AOM) and rhinosinusitis (Jacobs *et al.* 2013, Nokso-Koivisto *et al.* 2015). EV is traditionally associated with severe clinical conditions, such as meningitis, encephalitis, and neonatal sepsis, but it is also recognized as a common causative agent in respiratory infections and AOM, inducing 10% of common colds in Finnish children (Ruohola *et al.* 2009).

2.2.2 Experimental rhinovirus infection

The understanding of pathogenetic mechanisms in URIs is mainly derived from experimental HRV inoculation studies in animals and humans. An infectious dose of HRV is small. Viruses are mainly transmitted by small aerosol particles and direct or indirect contact with contaminated secretions to anterior nasal mucosa or eye and eventually to the nasal cavity through the ductus lacrimal. Mucociliary action in the nasal cavity transports viruses to the nasopharynx and adenoid tonsil. The viral load peaks 48 to 72 hours after inoculation and rapidly produces new variants via mutations (Cordey *et al.* 2010). In the adenoid epithelium, HRV binds to the specific intercellular adhesion molecule 1 (ICAM-1) to gain access to the cell and then starts to replicate and spread (Winther *et al.* 2002). Infection of the epithelial cells results in the increased expression of several proinflammatory cytokines, such as interleukins (IL-1β, IL-6, IL-8), tumor necrosis factor (TNF)-α, leukotrienes, histamine, and kinins; in addition, neutrophil and monocyte recruitment is observed (van Kempen *et al.* 1999, Naclerio *et al.* 1988, Winther 2011). In HRV infection, the first symptoms occur 10 to 16 hours after HRV inoculation in the nose and peak after 2 to 3 days (Gwaltney Jr 2002). An average duration of infection is 7.5 days, but a quarter of infections can last as long as 3 weeks. Experimental HRV infection studies have several

advantages over natural observational wild type studies: the easier utilization of controlled clinical settings, selection of a focused population, the use of defined inoculum and challenge dose, uniform timing, and the possible identification of co-infections. The disadvantages include milder infections and the possible disturbance of other virus infections. Several studies have been conducted using an experimental HRV model in a healthy adult population and in a population 60 years and older with chronic pulmonary diseases (del Vecchio *et al.* 2015).

2.2.3 Treatment and prevention of viral upper respiratory infections

Several multidrug cocktails, herbal and natural preparations, and dietary supplements are used in the treatment of URI, but their clinical effects are minimal. Even though antibiotics do not influence viral infections, they are widely used in treatment. In the USA, 30% of patients with URI were prescribed antibiotics at a medical costs of $726 million (Gonzales *et al.* 2001). The inevitable result is the ever-increasing resistance to antibiotics, which is emerging a global threat (Roca *et al.* 2015). Generally, a symptom-relief medication is used in URI, such as topical anesthetics, non-steroidal anti-inflammatory drugs (NSAIDs), antihistamines, anticholinergic nasal compounds, pseudoephedrine, expectorants, mucolytic cough medication, codeine, zinc, vitamin C, and *Echinacea*. However, according to a review based on seven Cochrane reviews (Arroll 2005), most of these are probably not efficacious against the common cold.

Data from experimental HRV infection trials have contributed to the information about possible treatment strategies. Intranasal oxymetazoline (used as a decongestant) reduced viral load significantly day two after inoculation but had no effect on the subjects' symptoms (Winther *et al.* 2010). In another experimental study, a recombinant soluble ICAM-1 (tremacamra) significantly reduced total symptom scores and the proportion of subjects with clinical colds (Turner *et al.* 1999). However, the treatment has not been standardized. The neuraminidase inhibitors oseltamivir and zanamivir are recommended for preventing and treating seasonal and pandemic influenza. According to a recent Cochrane review, these drugs reduced the time to the first alleviation of symptoms by approximately half a day in adults, and they reduced the risk of symptomatic influenza when used as a prophylactic drug (number needed to treat to benefit [NNTB] = 33 for oseltamivir and NNTB = 51 for zanamivir) (Jefferson *et al.* 2014).

No medical treatment is available for preventing URIs, which can be avoided by interrupting viral transmission by practicing good hand hygiene, cleaning surfaces, and avoiding touching the eyes or nose (Savolainen-Kopra *et al.* 2012, Uhari, Möttönen 1999). Efforts to develop a flu vaccine have not been successful. Indeed, the fact that HRV presents with over 150 serotypes hinders the development of a vaccine (Glanville, Johnston 2015). At present, only the commercially available vaccines against viral URI are administered against influenza.

2.2.4 Acute otitis media

Acute otitis media (AOM) is defined as an acute, short-term, clinically verified infection of the middle ear, where the tympanic membrane appears infected and middle ear effusion exists. At least one general or topical infection-related symptom or finding also needs to exist. AOM is the most common complication of URI, the leading cause of the visits of sick children to the doctor, and the main reason that antibiotics are prescribed for children (Klein 2000). In Finnish children, 63% of AOM cases occurred during the first week of URI (Koivunen *et al.* 1999). Approximately 80% of children experience at least one episode of AOM before the age of three, which peaks in children aged 6 to 15 months (Harmes *et al.* 2013). Viral URI increases the risk of AOM by

promoting the replication of bacteria, and the inflammation of the nasopharynx and eustachian tube facilitates bacterial entry into the middle ear (Uhari *et al.* 2000). However, in addition to bacteria, respiratory viruses can act as a causative agent in AOM, and coinfections are common (Pettigrew *et al.* 2011, Nokso-Koivisto *et al.* 2015). In particular, HRV, EV, and RSV have been found to be present in the nasopharynx and/or middle ear effusion (MEE) in two-thirds of children with AOM (Nokso-Koivisto *et al.* 2004). The bacteria most-often implicated with AOM are *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenzae* (*H. influenzae*), and *Moraxella catarrhalis* (*M. catarrhalis*). After the introduction of the pneumococcal vaccine, *H. influenzae* became the most prevalent pathogen in severe and recurrent AOM (Coker *et al.* 2010, Harmes *et al.* 2013).

Several guidelines exist to aid clinicians in the challenging diagnosis of AOM. Myringotomy is considered the gold standard for diagnosing middle ear fluid. However, the procedure is not practical in daily clinical practice. Therefore, three criteria are used in diagnosing an AOM: 1) evidence of middle ear inflammation; 2) presence of MEE; 3) acute symptoms of infection (Coker *et al.* 2010). The symptoms of AOM include fever, otalgia, irritability, otorrhea, lethargy, anorexia, and vomiting. However, the diagnosis cannot be based on symptoms only (Qureishi *et al.* 2014). Furthermore, indicating MEE is challenging; simple otoscopy is 60 to 70% accurate in diagnosing MEE. In AOM, bulging of the tympanic membrane or new onset otorrhea, not secondary to otitis externa, should be present for the diagnosis. The use of pneumatic otoscopy elevates the sensitivity and specificity for diagnosing MEE by skilled hands up to 70 to 90%. Tympanometry or acoustic reflectometry may be valuable adjuncts in predicting the presence or absence of MEE with sensitivity and specificity of 40 to 90% (Rogers *et al.* 2010, Muderris *et al.* 2013). Tympanometry has also been successfully used in monitoring the disappearance of the effusion (Renko *et al.* 2006).

2.2.5 Treatment and prevention of acute otitis media

Because the origin of AOM may be viral, bacterial, or a combination thereof, not all patients benefit from antibiotic therapy. A meta-analysis demonstrated that 80% of AOM resolved spontaneously in 2 to 7 days and 15 children needed to be treated with antibiotics to prevent one child from having some pain after two days (Glasziou *et al.* 2011). Prompt analgesic medication should be used because antibiotic therapy does not provide symptomatic relief in the first 24 hours. The clinical practice guidelines of the American Academy of Family Physicians and Pediatrics suggest antibiotic therapy for AOM in children 6 months or older with severe signs and symptoms. Antibiotic therapy is also recommended for children younger than 24 months with bilateral AOM and non-severe signs and symptoms. Clinicians should prescribe antibiotic therapy or offer observation with close follow-up in non-severe unilateral AOM in young children and non-severe AOM in older children (Lieberthal *et al.* 2013). However, the increasing prevalence of antibiotic-resistant bacteria makes antibiotic management difficult. Guidelines recommend amoxicillin or penicillin as the first-line treatment (Acute otitis media: Current Care Guidelines Abstract, 2010) (Lieberthal *et al.* 2013). According to the Finnish Study Group of Antimicrobial Resistance (FiRe), the resistance of *H. Influenzae* to amoxicillin and sulfa-trimethoprim is around 25%. The resistance of *S. pneumoniae* to penicillin is around 15%, to erythromycin around 20%, to clindamycin around 11%, and to sulfa-trimethoprim around 14%. *M. catarrhalis* is resistant to amoxicillin (FiRe, National Institute for Health and Welfare 2014).

Ventilation tubes are commonly used in recurrent AOM (rAOM = over three episodes in six months or over four episodes in 12 months) although literature on surgery in rAOM is scant. A Cochrane review of two studies concluded that ventilation tubes reduced AOM episodes in the first 6 months, but further research is warranted (McDonald *et al.* 2008). A Finnish study group found tympanostomy tubes with or without adenoidectomy to be effective in preventing rAOM episodes in children younger than 2 years (Kujala *et al.* 2012). However, surgery did not provide any additional benefit in the quality of life of children with rAOM (Kujala *et al.* 2014). In contrast, adenoidectomy is not recommended as the first-line surgery for rAOM (Koivunen *et al.* 2004).

The best way to prevent AOM is to prevent URI. A recent Cochrane review of influenza vaccines found a modest decrease of AOM in infants and children (Norhayati *et al.* 2015). Pneumococcal conjugate vaccines have reduced AOM caused by pneumococcal serotypes contained in the vaccine (Pelton *et al.* 2013). In addition, in a meta-analysis of five studies on the outcome of AOM, pneumococcal vaccine resulted in a reduction by 29% in all *S. pneumoniae* serotypes among children who received the heptavalent pneumococcal conjugate vaccine (PCV7) before 2 years of age (Pavia *et al.* 2009). In Finland, a ten-valent pneumococcal conjugate vaccine (PCV10) has been implemented in the national vaccination program since 2010, and an influenza vaccine has been implemented since 2007. In addition, xylitol has potential for the prevention of AOM, but not when only used in acute URI (Tapiainen *et al.* 2002, Uhari *et al.* 2000).

2.2.6 Otitis media with effusion

Otitis media with effusion (OME) is defined as a collection of fluid in the middle ear without the signs or symptoms of an acute ear infection. OME is the most common disease of the ear in childhood; approximately 90% of children present with OME before school age (Minovi, Dazert 2014, Rosenfeld *et al.* 2004). It is also the main reason for impaired hearing in children; the majority of children show a conductive hearing loss of 25 dB, and approximately 20% exceed a hearing level of 35 dB (Rosenfeld *et al.* 2004). The effects of hearing loss on children´s receptive and expressive language remain unclear (Lang-Roth 2014). Other possible symptoms of OME are otorrhea, tinnitus, otalgia, and pressure sensation. The pathogenesis of OME consists of the spontaneously impaired function of the Eustachian tube or an inflammatory response after AOM. The majority of the episodes resolve spontaneously within three months, but 30 to 40% of children suffer from recurrent OME, and 5 to 10% might persist for a year (Tos 1984, American Academy of Family Physicians *et al.* 2004). Adults have a considerably lower prevalence of OME, but underlying diseases often emerge: paranasal sinus disease, smoking-induced nasopharyngeal lymphoid hyperplasia, adenoid hypertrophy, and head and neck tumors are identified as inducing factors (Qureishi *et al.* 2014).

The occurrence of *H. influenzae* and HRV in MEE in OME is common (70% and 44%, cultivated and analyzed by PCR, respectively) (Stol *et al.* 2013). The nasopharyngeal carriage of two or three bacterial pathogens was found to be associated with the bacterial findings in MEE. In another study, HRV and EV were found from 32% of MEE samples from children with OME (Rezes *et al.* 2009). Bacterial biofilms have also been recognized as important in the etiology of OME (Hall-Stoodley *et al.* 2006).

The guidelines advise clinicians to use pneumatic otoscopy as the primary diagnostic method for OME, and OME should be distinguished from AOM. Tympanometry is an optional

method for confirming the diagnosis (American Academy of Family Physicians *et al.* 2004, Rosenfeld *et al.* 2004).

2.2.7 Treatment and prevention of otitis media with effusion

Children who are not at risk for speech, language, learning delay, or difficulties should be managed by watchful waiting for three months. The risks include permanent hearing loss independent of OME, speech delay, autism-spectrum disorders, syndromes, craniofacial disorders, visual impairment, or developmental delay. These children should be distinguished and evaluated promptly (Rosenfeld *et al.* 2004).

A Cochrane systematic review of a database concluded that routine antibiotic therapy is not supported in the treatment of OME (van Zon *et al.* 2012). A surgical intervention should be considered if bilateral OME persists over three months and hearing level is 25 to 30 dB at 0.5 to 4 kHz or less and if the child is at risk for speech, language, or learning. Tympanostomy (ventilation tube insertion) is the preferred initial procedure, and adenoidectomy should not be performed unless a distinct indication (nasal obstruction, chronic adenitis) exists (Khanna *et al.* 2008). Adenoidectomy with myringotomy with or without tubes is recommended for repeat surgery in children older than 2 years (Rosenfeld *et al.* 2004).

Vaccines preventing AOM could be considered to prevent OME because the condition commonly persists after AOM. Epidemiological evidence exists that the risk of OME is increased by passive smoking, bottle feeding, low socioeconomic group, and exposure to a large number of other children (Williamson 2011). However, there is no evidence to show whether modifying these risk factors would prevent OME.

2.3 Colonization of upper respiratory tract with probiotics

Colonization of the gut epithelium by probiotics has been extensively studied (Alander *et al.* 1999, Ramakrishna 2009, Bermudez-Brito 2012). Mucosal adhesion is incorrectly taught as essential for both non-immune and mucosal immune defense mechanisms. For example, noncolonizing probiotics, such as *Lactobacillus casei*, may exert their functions in a transient manner or by influencing the existing microbial community (Ohland, MacNaughton 2010). Thus far, few trials have investigated the colonization of upper respiratory tract with probiotics. In a pilot study, probiotic *Lactobacillus plantarum* DSM9843 was cultured from the tonsillar surfaces of 6 subjects up to eight hours after the per oral consumption of fermented oatmeal gruel enriched with this probiotic (Stjernquist-Desatnik *et al.* 2000). Another small population trial investigated the recovery of *Streptococcus salivarius* K12 in the nasopharynx and oral cavity after oral intake (Power *et al.* 2008). In this study, one of 19 nasopharyngeal cultures was reported positive for the probiotic, and it was recovered from three adenoids of the seven examined. Tonsillar recovery of L. GG after per oral consumption was studied in 57 young adults in a placebo-controlled and randomized trial (Kumpu *et al.* 2013a). L. GG was recovered in 40% of the L. GG groups' tonsillar samples and in 30% of the placebo groups' samples. In a recent trial, 20 adults were treated with intranasal *Streptococcus salivarius* 24SMBc for three days (Santagati *et al.* 2015). The results showed that 95% of the subjects were colonized in the nasopharynx with the probiotic at least four hours after spray administration; colonization persisted for at least six days in in 55% of the subjects. These studies are presented in Table 1.

Table 1. Characteristics of the previous studies investigating the colonization of upper respiratory tract with probiotics.

Subjects	Design and duration	Probiotic supplementation	Main findings	Reference
Healthy volunteers, mean age 38 (n = 6)	Swab samples from tonsils after single per oral intake	*L. plantarum* ($2x10^{11}$ cfu)	Colonization remained for 8 h	Stjernquist-Desatnik 2000
Children scheduled for tympanostomy, aged 0.5-5 years (n = 19)	Swab samples from tongue and nasopharynx, 10 days	*S. salivarius* K12 ($1.7x10^{10}$ cfu)	33% colonized	Power 2008
Young adults scheduled for tonsillectomy, mean age 24.5 years (n = 57)	RDBPC Tonsil tissue samples, 3 weeks	L. GG ($2x10^{10}$ cfu) or multispecies L. GG, Lc705, PJS, BB12	30-40% colonized NS in different intervention groups	Kumpu 2012
Healthy adults aged 30-54 (n = 20)	Nasal spray, rhinopharyngeal swabs, 3 days	*S. salivarius* 24SMBc ($8x10^{9}$ cfu)	95% colonized 55% remained for six days	Santagati 2015

2.4 Clinical effects of probiotics in the upper respiratory tract

The prevention of upper respiratory infections by the use of probiotics has been studied in several trials. For instance, L. GG alone or in combination with other probiotics was shown to reduce the incidence or risk of URI in children (Hatakka *et al.* 2001, Rautava *et al.* 2008, Kumpu *et al.* 2012). A recent systematic review found a favorable outcome of the use of probiotics in reducing the episodes of new respiratory infection in children (de Araujo *et al.* 2015). However, further studies are required to confirm these results. A recent Cochrane database review of the use of probiotics in URI found 13 randomized controlled trials with participants in several age groups (see Table 2). (Hao *et al.* 2015). Probiotics were found to be better than the placebo in reducing the number of subjects who experienced acute URI, the mean duration of acute URI, the number of antibiotic prescriptions, and cold-related school absences. However, the quality of evidence was considered low or very low.

Table 2. Characteristics of the included randomized controlled studies in Hao *et al.* 2015

Subjects	Design and duration	Probiotic supplementation	Main findings: Probiotic vs. placebo	Reference
Healthy adults aged 18-65 (n = 318)	RDBPC, 3 mo	*L. plantarum* and *L. paracasei* ($1x10^9$ cfu)	Incidence of common cold episodes ↓ Number of days with respiratory symptoms ↓	Berggren 2011
Day-care children aged 1-5 (n = 398)	RDBC, 3 mo	*L. rhamnosus* HN001 (10^{10} cfu)	Number and duration of URI ↔ Level of secretory IgA ↑	Cáceres 2010
Older volunteers in daycare facilities (n = 154)	RDBPC, 5 mo	*L. casei* strain *Shirota* ($4x10^{10}$ cfu)	Number of acute URI and symptom score ↔	Fujita 2013
Day-care children aged 13-86 mo (n = 281)	RDBPC, 3 mo	*L. rhamnosus* GG (10^9 cfu)	Risk of URI ↓ Days with respiratory symptoms ↓	Hojsak 2010a
Hospitalized in pediatric department, over 1-year-old (n = 742)	RDBPC, duration of hospitalization	*L. rhamnosus* GG (10^9 cfu)	Risk of URI ↓ Episodes of URI >3 days ↓	Hojsak 2010b
Healthy volunteers aged 69-80 (n = 60)	RPC, 2 or 3 mo	*L. bulgaricus* (1.8-$3.2x10^{10}$ cfu) and *S. thermophilus* ($5.7\text{-}7.9x10^{10}$ cfu)	Risk of URI ↓ Natural killer cell activity ↑	Makino 2010
Healthy day-care or school children aged 3-6 (n = 638)	RDBPC, 3 mo	*L. casei* ($2x10^{10}$ cfu), *S. thermophiles* and *L. bulgaricus* (10^9 cfu)	Incidence for common infectious diseases ↓	Merenstein 2010
Infants needing formula aged 0-2 mo (n = 81)	RDBPC, 12 mo	*L. rhamnosus* and *B. lactis* BB-12 ($1x10^{10}$ cfu)	Risk of URI ↓ Risk of AOM and antibiotics ↓	Rautava 2009
Healthy children aged 8-13 (n = 80)	RDBPC, 3 mo	*L. acidophilus* and *B. bifidum* ($1x10^9$ cfu)	Symptoms of URI ↓ Absences from school related to URI ↓	Rerksuppaphol 2012
Children aged 6-25 mo (n = 100)	RPC, 3 mo	*L. acidophilus* and *L. casei* (10^9 -10^{10} cfu)	Episodes of respiratory tract infections ↓	Rio 2002
School children aged 3-12 years (n = 251)	RDBPC, 5 mo	*L. casei*	Duration of lower respiratory infections ↓	Cobo Sanz 2006
College students aged 18-24 (n = 198)	RDBPC, 3 mo	*L. rhamnosus* GG and *B. animalis ssp. lactis* BB-12	Duration or URI ↓ Median severity score ↓ Missed school days ↓	Smith 2013
Healthy adults, average age 38±13 (n = 479)	RDBPC, 8.5 mo	*L. gasseri, B. longum,* and *B. bifidum* ($5x10^7$ cfu)	Duration of URI ↓ Total symptom score ↓ Days with fever during URI ↓	de Vrese 2005

RD = randomized, B = placebo-controlled, P = prospective, C = clinical trial

A meta-analysis of randomized, placebo-controlled trials indicates that L. GG is able to reduce the incidence of AOM and antibiotic prescriptions and decrease the risk of URI in children (Liu *et al.* 2013). However, in otitis-prone children with nasopharyngeal pathogen colonization, L. GG did not reduce the occurrence of AOM (Hatakka *et al.* 2007a). A novel treatment model of intranasal spray bacteriotherapy with *Streptococcus sanguinis* was found to be effective in decreasing MEE in children with prolonged OME (Skovbjerg *et al.* 2009). Statistically significant recovery was achieved with *Streptococcus sanguinis*, and a more modest, yet positive effect was achieved with L. GG. In otitis-prone children, the consumption of L. GG, Lc705, BB99, and PJS significantly reduced the number of positive human bocavirus nasopharyngeal samples (Lehtoranta *et al.* 2012). The colonization of the epithelium of the upper respiratory system with specific probiotics or lactic acid bacteria is not well known. *Lactobacillus plantarum* DSM 9843 was recovered from the tonsillar surface after oral administration, suggesting that the strain may possess the capacity to adhere to tonsillar cells (Stjernquist-Desatnik *et al.* 2000). *Streptococcus salivarius* K12 was cultured from the nasopharynx of infants after the consumption of an oral powder prepared with this probiotic bacterium (Power *et al.* 2008). Recently, L. GG was recovered from tonsil tissue after oral consumption, and prolonged adhesion (over 4 weeks) was suspected (Kumpu *et al.* 2013a). The consequences of colonization are unknown. An *in vitro* experiment indicates that L. GG is able to inhibit the adherence of *S. pneumoniae* to human epithelial cells (Wong *et al.* 2013). Two review studies suggested that specific probiotics interact with pathogens and have the potential to reduce pathogen colonization in the nasopharynx, thus potentially reducing AOM and URI (Salminen *et al.* 2010, John *et al.* 2013).

2.5 Safety of probiotics

2.5.1 Infections caused by probiotics

Because probiotics are live bacteria, and any live bacterium can potentially cause an infection, concerns about their safety have been discussed. Although the use of probiotics is generally recognized as safe, there are some case reports of infections caused by probiotics. In a four-year study in southern Finland, only eight *Lactobacillus* bacteremia were found, none of which was identified as L. GG (Saxelin *et al.* 1996). However, a rapid increase in L. GG consumption occurred in Finland in the 1990s. Nonetheless, increases in *Lactobacillus*-associated bacteremia were not implicated, and the incidence remained stable at 0.3/100,000 inhabitants/year (Salminen *et al.* 2002). In a cohort of 89 cases of *Lactobacillus* bacteremia, the majority of patients suffered from serious underlying conditions, such as malignancies or severe gastrointestinal diseases, and the majority had undergone at least one surgical intervention and several courses of antibiotics (Salminen *et al.* 2004). In this study, among 48 *Lactobacillus* isolates, 11 could not be differentiated from L GG. Furthermore, *Lactobacillus* bacteremia was found to be present with high mortality (26% in one month, 48% in one year).

Lactobacillus has also been implicated as an agent associated with human endocarditis in a few case reports (Husni *et al.* 1997, Mackay *et al.* 1999). A diseased heart valve was identified as a predisposing factor. In extremely rare cases, *Lactobacillus* can cause a liver abscess. In seven described cases, six had a predisposing factor; diabetes mellitus, immunosuppression, or a history of malignancy (Chan *et al.* 2010). However, the overall mortality of subjects with liver abscess was low. Case reports on *Lactobacillus* splenic abscess (Doi *et al.* 2011) and lung abscess (Shoji *et al.* 2010) have been sporadic. Overall, underlying conditions, such as immunosuppression,

major gastrointestinal diseases, other severe illnesses, central venous catheters, or other surgical interventions, seem to be risk factors for infections caused by *Lactobacillus*. However, probiotics are used safely by pregnant women (Elias *et al.* 2011), immunocompromised patients (van den Nieuwboer *et al.* 2015a), and cancer patients (Redman *et al.* 2014). However, a systematic review of people with cancer identified five case reports of probiotic-related bacteremia or fungemia (Redman *et al.* 2014).

2.5.2 Other adverse events

Numerous studies have investigated the efficacy of probiotics in different illnesses or conditions. However, studies focusing on the possible adverse events (AE) are scarce. In most efficacy studies, AEs are not mentioned or they are acknowledged in a sentence or two. The most commonly reported AEs in the consumption of probiotics are of gastrointestinal origin, such as bloating, constipation, diarrhea, or stomachache. A study group reported probiotic and prebiotic safety in infants under 2 years (van den Nieuwboer *et al.* 2014) and children under 18 years (van den Nieuwboer *et al.* 2015b). In the infant study, 65 trials or follow-up studies were evaluated, and the results indicated the safe use of probiotics regarding the evaluated strains. Major safety concerns were not encountered and, in general, AEs were not considered related to the study product, which generally was well tolerated. However, the reporting of AEs was found to be imprecise, inconsistent, and incomplete, thus limiting the generalization of the results. The other study evaluated 74 clinical trials, showing similar results in terms of safety and the poor reporting of AEs. Overall, AEs were encountered more frequently in the placebo group than in the group that received probiotics or prebiotics.

The study also reported the safety of probiotics and prebiotics in immunocompromised adults (van den Nieuwboer *et al.* 2015a). They concluded that AEs occurred more frequently in the placebo group than in the probiotic/prebiotic group, and no serious AEs were related to the products consumed. Inadequate reporting of AEs was indicated, and future studies were strongly recommended to assess a structured method, such as the Common Terminology of Adverse Events (CTCAE), for reporting AEs.

3 AIMS OF THE STUDY

The aims of this thesis were to study the colonization of probiotic *Lactobacillus rhamnosus* GG in the upper respiratory tract, its effects on viral and bacterial pathogens in the upper respiratory tract, and the possible adverse events related to LGG use alone or in combination with other probiotics.

The specific aims are as follows:

1. Characterize how a three-week oral consumption of live *L. rhamnosus* GG influences the colonization of L. GG in the nasopharynx and middle ear of young children (I, II).

2. Determine whether the a three-week use of live *L. rhamnosus* GG prevents the presence of human rhino- and enteroviruses in the nasopharynx or middle ear effusion (I, II) or bacterial pathogens in the middle ear of young children (II).

3. Determine whether live or inactivated *L. rhamnosus* GG have different effects on nasopharyngeal human rhinovirus load in experimental human rhinovirus infection and the correlation of the viral load to total clinical symptom score in adults (III).

4. Analyze the adverse events of *L. rhamnosus* GG alone and in specific combinations (with BB12, or PJS, Lc705, and/or BB99) in 1,909 children, young adults, and elderly in six randomized placebo-controlled studies (IV)

4 MATERIALS AND METHODS

4.1 Subjects, study designs and data collection (I-IV)

4.1.1 *Lactobacillus rhamnosus GG in the middle ear effusion and adenoid tissue (I, II)*

Studies I and II were randomized, double-blind, placebo-controlled clinical intervention studies that used a two parallel-groups design (L. GG and placebo). The two studies were conducted in the same study setting and with the same study population in Helsinki, Finland from January to June 2011. Subjects aged 1 to 5 who were referred for adenotomy and tympanostomy for recurrent otitis media, secretory otitis media, chronic rhinitis and/or recurrent sinusitis were enrolled. Children with snoring as the principal diagnosis, chronic sinusitis, symptomatic allergy, chronic GI diseases, other chronic diseases, continuous use of inhaled asthma medication, immunosuppression, continuous antibiotic prophylaxis, or a course of antibiotics within four weeks prior to the study, milk allergy, lactose intolerance, participation in another study concurrently, and unwillingness to follow the study protocol, were excluded. A computer-generated randomization list was used, and it remained concealed after all data were analyzed.

The study was conducted over a period of nine weeks. The first four weeks were a wash-out period, and any use of probiotic products was prohibited. The following three weeks were the intervention period when the children consumed either capsules containing L. GG (8-9 x 10^9 cfu), or a placebo (crystalline cellulose) twice a day. During the intervention period, parents filled in a daily diary recording signs of respiratory infection (fever, rhinitis, sore throat, and cough), GI symptoms (diarrhea, vomiting, and abdominal pain), other possible symptoms, the use of any medication, and seeking medical care. At the end of the third week, the adenoid was removed, and if there was effusion in the middle ear, the fluid was collected via paracentesis and a tympanostomy tube was inserted. The adenoid tissue and MEE fluid were frozen at -70 °C until the analysis. The final two weeks of the study were a follow-up period. A daily diary was used to record information about respiratory and GI symptoms, postoperative pain or bleeding, medication, and seeking medical care. The follow-up period had no dietary restrictions. The study protocol is presented in Figure 2.

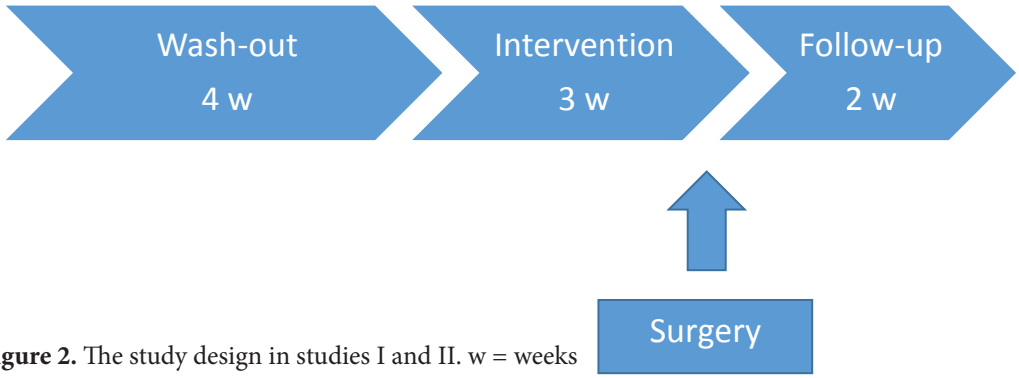

Figure 2. The study design in studies I and II. w = weeks

4.1.2 Lactobacillus rhamnosus GG in experimental rhinovirus infection (III)

Study III was a randomized, placebo-controlled, double-blinded experimental study with a three parallel-group design (live, inactivated L. GG, and placebo). The study extended the analysis performed in a previous study (Kumpu *et al.* 2015). The study was conducted in Charlottesville, Virginia, USA, from August to November 2010. Healthy volunteers between 18 and 65 years were recruited. The exclusion criteria for the recruitment were as follows: significant allergic rhinitis, lower respiratory tract diseases, nasal abnormalities, pregnancy, lactation, history of alcohol abuse, drug abuse during the past year, daily smoking within the past two years, participation in a clinical trial during the past month, previous participation in an experimental study with HRV A39, any surgical or medical condition, or use of any medication or dietary supplement that could disturb the results. A total of 198 volunteers were screened for serum-neutralizing antibody titers of 1:4 or less for the challenge virus, resulting in the selection of 84 subjects. After 24 subjects were excluded, 60 subjects were enrolled and advised not to consume any probiotic products for the three weeks preceding the intervention. They were assigned a study number according to a random code that was generated by a statistician.

The placebo and carrier product for the probiotics was commercially available fruit juice, 100 ml of which was consumed daily for six weeks. Twenty subjects were randomized to receive juice enriched with live L. GG (10^9 cfu), 20 subjects to receive heat-inactivated L. GG (10^9 cfu), and 20 subjects to receive juice with no additives. After consuming the study products for three weeks, every subject received inoculations with a 100–300 tissue culture infectious dose ($TCID_{50}$) of HRV (immunotype 39) in the nasal cavity. After inoculation, the subjects continued using the intervention products for another three weeks. Nasopharyngeal lavage specimens were collected before the inoculation and on days 1 to 5 after the inoculation for quantitative HRV analysis. Specimens were collected by dropping 5 ml of sterile 0.9% saline into each nostril when the subject's head was tilted back. When the subject felt saline running in the naso-oropharynx, the head was bent forward and the fluid was collected in a cup. During the six-week intervention period, the subjects used a daily diary to record URI symptoms (sneezing, runny nose, stopped up nose, sore throat, cough, headache, malaise, and chilliness). Additionally, the severity of the symptoms was recorded on the day of the inoculation and ive days after the inoculation by using a severity score from 0 (none) to 4 (very severe).

4.1.3 Adverse events of probiotics (IV)

In study IV, we included six (Hatakka *et al.* 2001, Hatakka *et al.* 2007a, Hatakka 2007b, Hatakka *et al.* 2007c, Kumpu *et al.* 2012, Lehtoranta *et al.* 2014) of our study groups' previous randomized, double-blinded, placebo-controlled studies to investigate the AEs of L. GG alone or in combination by applying a meta-analysis. The characteristics of the studies are presented in Table 3.

Table 3. Characteristics of the studies included in study IV

Study	Primary endpoints / study population / recording	No of participants[1]	Subject age[2]	Probiotic used	Intervention period[3]
Hatakka 2001	GI and respiratory infections / daycare children / PROM & objective	513 252/261	4.5 (1.3-6.8)	L. GG	7
Hatakka 2007a	Otitis media / otitis-prone children / PROM & objective	269 135/134	2.4 (0.8-6.0)	L. GG + Lc705 + PJS + BB99	6
Hatakka 2007b	GI and respiratory infections / insitutionalised elderly / objective	226 117/109	83 (65-102)	L. GG + Lc705 + PJS + BB99	5
Hatakka 2007c	Oral Candida / independent elderly / objective	192 92/100	78 (68-95)	L. GG + LC705 + PJS	4
Kumpu 2012	Respiratory infections / children / PROM & objective	501 251/250	4.0 (2-6)	L. GG	7
Lehtoranta 2014	GI and respiratory infections / conscripts / PROM & objective	208 100/108	19.3 (18-28)	L. GG + BB12	5 and 3

[1]Total no of subjects (probiotic/placebo) in analysis [2]Mean (range), years [3]Months PROM = patient reported outcome measure

These studies were selected because they used a parallel group design and provided individual follow-up data on primary and secondary variables and possible AEs. The six studies also included a vast cohort of ages (toddlers, daycare children, conscripts, independent and institutionalized elderly), prolonged intervention periods (3–7 months), and no surgical intervention. The child population was healthy because one of the exclusion criteria was suffering from any chronic disease (Hatakka *et al.* 2001, Hatakka *et al.* 2007a, Kumpu *et al.* 2012). In the conscript population, only continuous per oral corticosteroid use or probiotic consumption were exclusion criteria (Lehtoranta *et al.* 2014). The exclusion criteria in the elderly population were moderate to severe dementia, chronic GI diseases (Hatakka 2007b), or the use of oral yeast medication (Hatakka *et al.* 2007c).

In this study, we used the study populations included in the primary analyzes in the individual studies, which yielded 1,909 subjects. The intention-to-treat population (a total of 2,949 subjects) was also analyzed to compare and confirm the results. The individual data on possible AEs were collected from daily diaries, clinical examinations, assigned diagnoses and medications, the AE form, and/or bacterial samples. All these data were assessed, and then all possible AEs were distributed into System Organ Classes I-XXVI, using the Common Terminology Criteria for Clinical Adverse Events 4.0 (CTCAE). AEs in different System Organ Classes were recorded. They then were compared in the probiotic groups and the placebo groups.

Three categories (Gastrointestinal [VII] and Respiratory, thoracic and mediastinal disorders [XXII] and Infections and infestations [XI]) were selected for the detailed analysis of AEs and for comparison between the different probiotic combinations and placebos in the individual studies.

4.2 Bacteriological and virological methods

4.2.1 *Lactobacillus rhamnosus GG extraction (I, II)*

L. GG was investigated in the middle ear effusion (I) and the adenoid tissue (II) samples. Bacterial DNA was extracted from the samples as described by Rinttilä *et al.* (2004). This extraction technique provides a quantitative lysis of all relevant microbial groups, thus ensuring non-biased measurements of microbial profiles. The total bacterial levels were quantified with broad-range primers in Nadkarni *et al.* (2002) and levels of L. GG by primers in Ahlroos and Tynkkynen (2009). The melting curves in the PCR analysis were confirmed to establish qPCR validity.

4.2.2 Bacterial microarray (I)

Bacterial microarray testing was performed using the collected MEE samples (I). DNA was extracted using EasyMAG (EasyMAG bioMérieux) with the Generic 2.0.1 program: 500 µl was added to the lysis of extraction device, and DNA was eluted to the 50 µl elution buffer. The PCR reactions in the extracted DNA were conducted by the Prove-it™ Bone and Joint StripArray assay (Mobidiag, Finland) according to the manufacturers´ instructions. The PCR protocol was carried out using Mastercycler® ep gradient S (Eppendorf, Germany). After the PCR reactions, the amplicons were subjected to hybridization onto StripArray using the Prove-it™ protocol. The detection and analysis of bacteria were conducted with the StripArray Reader and Prove-it™ Advisor software.

4.2.3 Picornavirus PCR (I, II)

Picornaviruses (HRV and EV) were analyzed in MEE (I) and adenoids (II). Buffer RLT (Qiagen, Hilden City, Germany) with Carrier RNA (Qiagen) was added to homogenize the samples. For the lysis, the samples were disrupted by pipetting and vortexing before they were incubated. A tissue lysate was added to a QIAshredder homogenizer (Qiagen) and then centrifuged. After homogenization, the lysate was used for the viral nucleic acid extraction. Viral nucleic acids were purified as described by Kumpu *et al.* (2013b) for HRV and EV PCR assays. Validated real-time RT-PCR methods were used to detect HRV and EV. Amplification curves rising above the threshold were interpreted positively. The assays were run by using the Mx3005P analyzer (Stratagene, La Jolla, CA).

4.2.4 Quantitative human rhinovirus PCR (III)

HRV load was analyzed in the nasopharyngeal lavage samples (III). The quantitative amount of HRV was detected by a real-time PCR, where short double-dye probes with locked nucleic acid analogs were used as described by Österback *et al.* (Österback *et al.* 2013). Wild-type HRV infections were differentiated from the experimental HRV A39 infection by using additional melting curve dsDNA dye BOXTO analysis (Peltola *et al.* 2013, Österback *et al.* 2013). In selected cases, a sequence analysis was performed to confirm the results.

4.3 Statistical methods

Categorical variables were analyzed with Fisher's exact test (I). Comparisons between the intervention groups were made using Levene's test for equality of variances or the Mann–Whitney U-test (I, II). In study III, the three intervention groups were compared by using an analysis of variance or the Kruskal–Wallis non-parametric test. The correlation between the HRV load and the total symptom score was calculated using Spearman's correlation test. In study IV, the individual participant data were analyzed independently in each study by using risk ratios (RRs). A meta-analysis then was conducted using random effect models to estimate the overall RRs. These models incorporated variations both within and between the studies. All RRs presented with 95% confidence intervals. The statistical heterogeneity among the studies was assessed using Cochrane's Q statistic, inconsistency was quantified with the I^2 statistic, and the guidelines were used for low, moderate, and high heterogeneity.

A two-tailed *P*-value less than 0.05 was considered statistically significant. The data were analyzed using IBM SPSS version 22 software (IBM Corp., Armonk, NY, USA) and NCSS 8 (NCSS LLC, Kaysville, Utah, USA).

4.4 Ethics

These studies followed the guidelines of the Declaration of Helsinki and were accepted by the Ethics Committee of Helsinki University Hospital (I, II, IV) or the Human Investigation Committee of the University of Virginia (III). A personal data register was reported to the Finnish confidentiality representative (IV). All subjects participated voluntarily, and a written informed consent was obtained from the study subjects or their legal guardians. The studies were registered at http://clinicaltrials.cov with identifier NCT02110732 (I, II) and NCT01229917 (III).

5 RESULTS

5.1 Baseline characteristics (I, II)

The baseline characteristics of the children in studies I and II are presented in Table 4. There were 13 children in study I and 40 children in study II. The groups were statistically similar in their baseline information, with the exception of OME prevalence in study II (L. GG vs. placebo group $P = 0.02$).

Table 4. Baseline characteristics of the study children

Characteristics	**Study I**	
	L. GG (n = 10)	**placebo (n = 3)**
Age (median, months)	35.0	27.0
Male gender	8	3
MEE samples	19	6
Capsules consumed (mean)	28.6	36.5
Indication for tympanostomy -recurrent AOM -OME -chronic rhinitis	 9 8 4	 2 2 -
Prior use of L. GG	8	2
Mother´s gestational use of probiotics	4	1

Numbers represent the number of children unless otherwise stated.

Characteristics	**Study II**	
	L. GG (n=20)	**placebo (n=20)**
Age (median, months)	40.5	35.0
Male gender	12	11
Adenoid samples	14	17
Capsules consumed (mean)	30.5	27.7
Indication for adenotomy -recurrent AOM -OME -chronic rhinitis	 15 8* 6	 15 2* 4
Prior use of L. GG	14	14
Mother´s gestational use of probiotics	5	7

*$p = 0.02$. Numbers represent the number of children unless otherwise stated.

5.2 Lactobacillus rhamnosus GG in the middle ear effusion and adenoid (I, II)

Twenty-five MEE samples were available for the L. GG analysis. Among these, four of 19 (21%) in the L. GG group presented with L. GG; one of 6 (17%) in the placebo group presented with L. GG (findings in L. GG vs. placebo group, $P = 1.0$). Traces of total bacterial DNA were detected in all 25 MEE samples. When the detection limit for total bacteria was set at > $3x10^6$ 16S copies/g, the percentages of total bacteria were 37% and 50% in the L. GG group and the placebo group, respectively ($P = 0.65$).

Thirty-one adenoid samples were available for the L. GG analysis. Among these, all 14 (100%) in the L. GG group presented with L. GG; 13 of 17 (76%) presented with L. GG in the placebo group (L. GG vs. placebo group $P = 0.07$). Only four samples (24%) were negative for L. GG, all of which were in the placebo group.

5.3 The effect of Lactobacillus rhamnosus GG on picornaviruses and bacterial pathogens, and symptoms in study diaries (I, II)

HRV and EV prevalence was studied in both MEE and adenoid tissue; 25 MEE samples and 30 adenoids were available for analysis. HRV was present in 12 (48%) of the MEE samples and 7 (23%) of the adenoid samples. Of the positive HRV findings, 10 (83%) samples in the MEE and 4 (57%) samples in the adenoid were in the L. GG group (L. GG vs. placebo $P = 0.6$ in MEE and $P = 0.7$ in adenoid). EV was present in 1 (4%) of 25 MEE samples (in the L. GG group, $P = 0.8$) and in 7 (23%) of 30 adenoid samples (4 in the L. GG group, $P = 0.7$) (see Table 5).

Table 5. Human rhinovirus and enterovirus -findings in the middle ear effusion and adenoid tissue, in the L. GG and placebo groups

	HRV (%)	EV (%)
MEE (n=25)	**12 (48)**	**1 (4)**
L. GG (n=19)	10 (83)	1 (100)
Placebo (n=6)	2 (17)	0
P-value	0.6	0.7
Adenoid (n=30)	**7 (23)**	**7 (23)**
L. GG (n=14)	4 (57)	4 (57)
Placebo (n=16)	3 (43)	3 (43)
P-value	0.8	0.7

Pathogenic bacteria were analyzed in all 25 MEE samples; 15 (60%) of the samples presented with at least one bacteria species. The findings between the groups did not differ (12 in L. GG vs. 3 in the placebo group, $P = 0.65$). *H. influenzae* was the most prominent pathogen and was recovered from 12 (80%) samples (10 in the L. GG group, 2 in the placebo group, $P = 0.6$). Multiple bacteria were detected in four samples. The viral and bacterial findings from the MEE samples in both groups are presented in Figure 3.

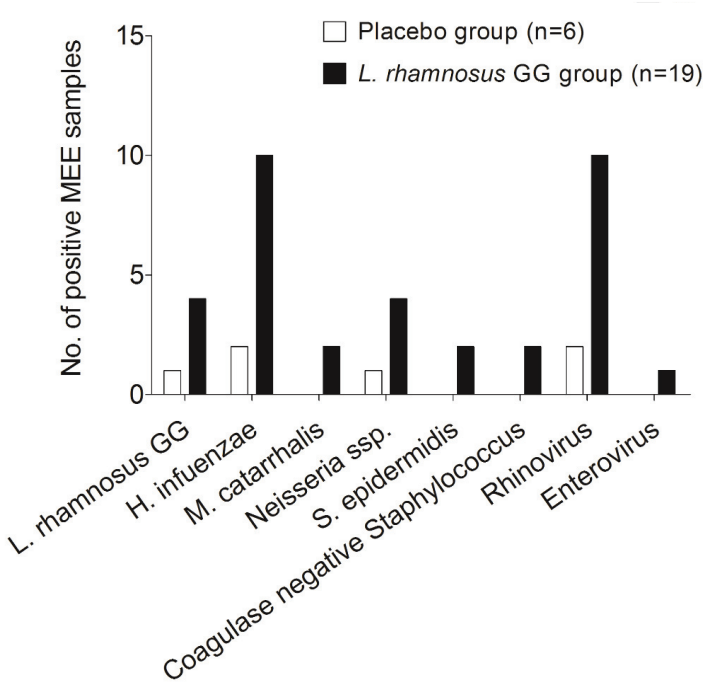

Figure 3. The bacteriological and virological findings from 25 MEE samples in the L. GG and placebo groups The differences between the groups were not statistically significant.

The daily diaries kept in the three-week intervention period and the two-week postoperative period were collected. Data on the symptoms are presented in Table 6. No statistically significant differences were found between the intervention groups. Children with MEE tended to experience respiratory symptoms more frequently compared to those with dry ears but not significantly (median 5.5 vs 0.5 days respectively, $P = 0.16$).

Table 6. Information from the daily diaries in the intervention and postoperative periods. Numbers represent the mean number of symptom days within the observation period. No differences between the L. GG and the placebo group were statistically significant.

INTERVENTION PERIOD	L. GG (n = 17)	PLACEBO (n = 15)
Respiratory symptoms	5.8	4.2
GI symptoms	0.7	1.5
Other symptoms	1.1	0.5
Use of medication	2.8	3.8
Need for medical care	0.6	1.3
POSTOPERATIVE PERIOD	**(n=13)**	**(n=6)**
Respiratory symptoms	3.6	2.5
GI symptoms	1.6	0.2
Bleeding	0.1	0.2
Pain	2.2	0.8
Other symptoms	1.0	1.2
Need for medical care	0.2	0.0

5.4 Effect of Lactobacillus rhamnosus GG on experimental rhinovirus infection (III)

Data on 59 subjects (live L. GG n = 19, inactivated L. GG n = 20, placebo n=20) were available for analysis. Nasopharyngeal lavage samples on day 0 before inoculation, day 2, and day 5 were analyzed in the three intervention groups, resulting in a total of 177 analyzed samples. In nine (15%) subjects, the melting curve analysis and gene sequencing revealed a wild-type infection and thus a different immunotype than the A39 that was used. After excluding the wild-type infections, the analyzed samples were n = 18 in live L. GG, n = 16 in inactivated L. GG, and n = 16 in the placebo group. The results of the HRV load in nasopharyngeal lavage samples in the intervention groups are presented in Figure 4.

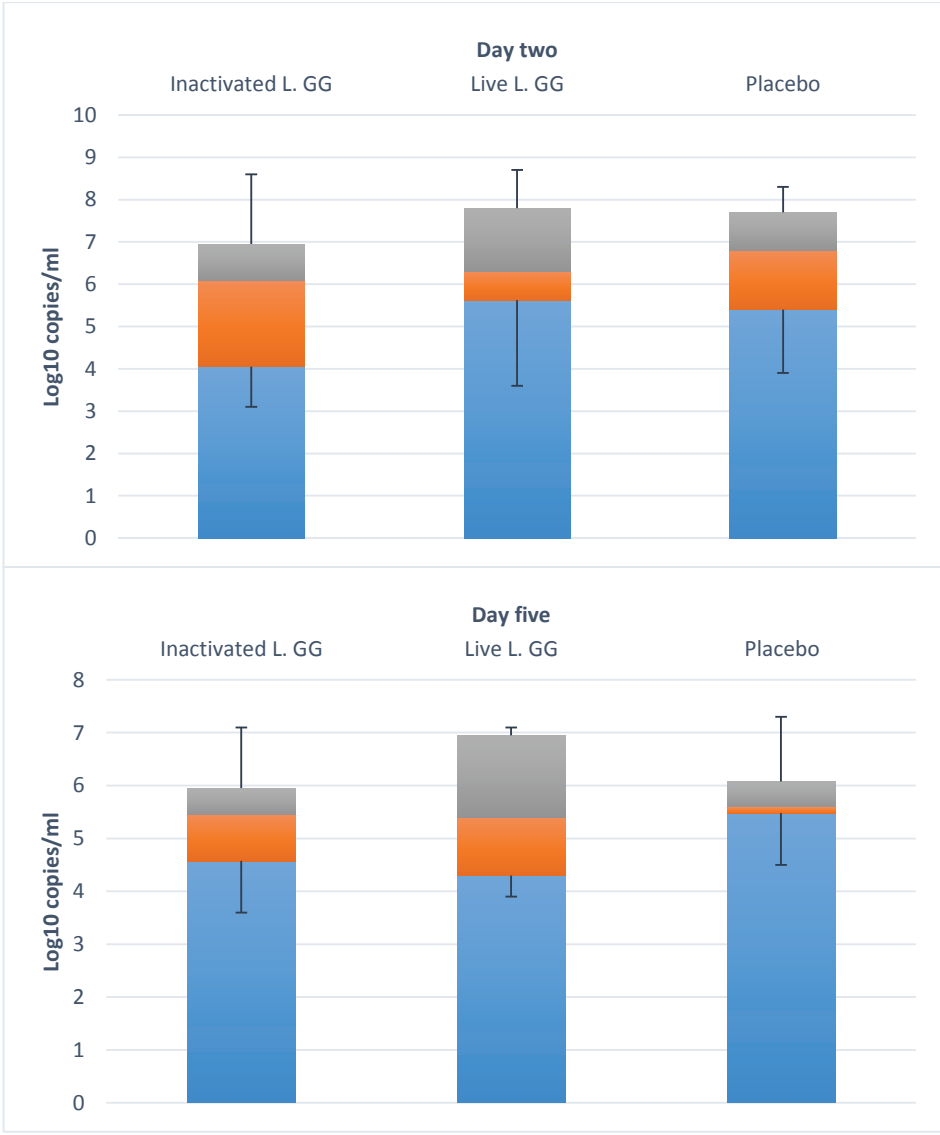

Figure 4. The mean, 95% CI, and range on nasopharyngeal HRV load (log10) in days 2 and 5 in experimental HRV challenge in the three intervention groups. Grey = upper CI, orange = lower CI.

There was a tendency to lower loads in the two L. GG groups (log_{10} copies/ml [95% CI]: 6.20 [5.18-7.40] in live L. GG group, 6.30 [4.91-7.08] in inactivated L. GG group, and 7.25 [5.81-7.52] in the placebo group). However, the difference between the groups was not statistically significant ($P = 0.57$ on day 2 and $P = 0.76$ on day 5 in the excluded wild-type population). In the whole study population, mean (SD) HRV load (log10 copies/ml) was 3.31 (3.58) on day 2, and 3.98 (2.66) on day 5.

The mean (range) total symptom scores were 3.31 (14) and 3.39 (17) on days 2 and 5, respectively. A significant positive correlation between HRV load and total symptom score on days 2 and 5 existed (correlation coefficient 0.61, $P < 0.001$ and correlation coefficient 0.28, $P = 0.034$, respectively). The correlations are presented in Figure 5 and Figure 6.

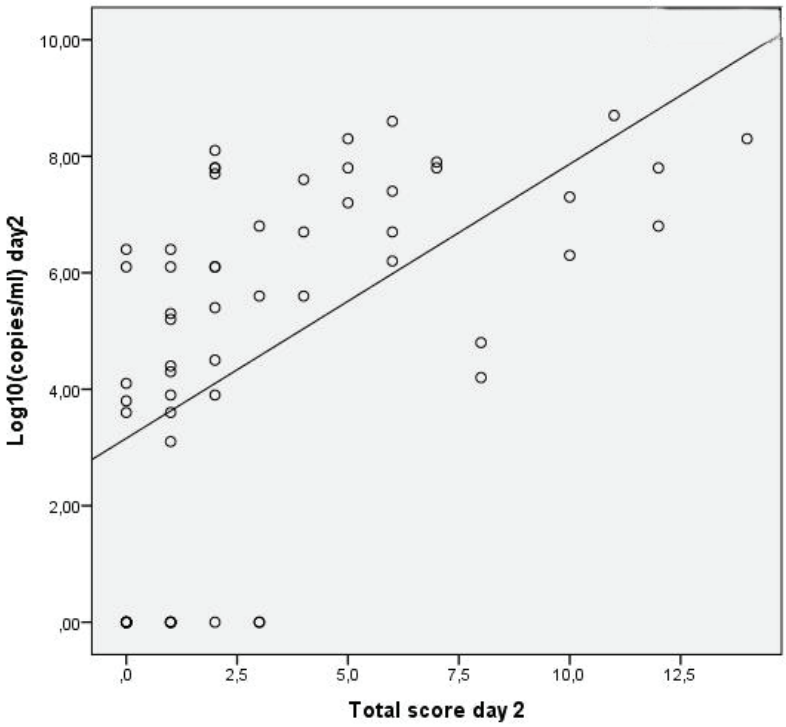

Figure 5. Correlations between human rhinovirus load in nasopharyngeal lavage samples and total symptom scores on day 2 after inoculation in the three intervention groups

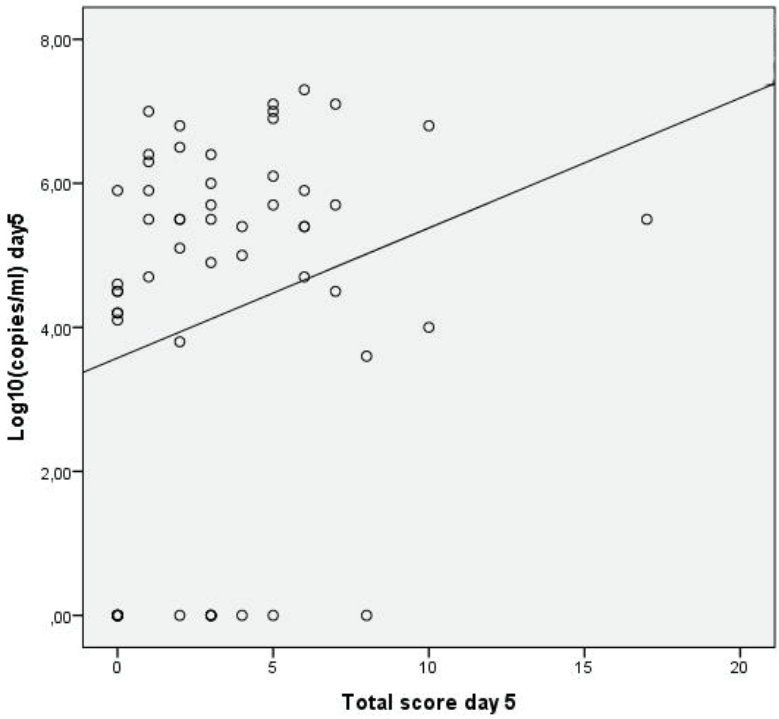

Figure 6. Correlations between human rhinovirus load in nasopharyngeal lavage samples and total symptom scores on day 5 after inoculation in the three intervention groups.

5.5 Adverse events of specific probiotic species (IV)

The individual data on 1,909 study subjects revealed AEs in 20 of the 26 CTCAE groups. The highest incidence (79.2%) was found in the System Organ Class (SOC) of Respiratory, thoracic and mediastinal disorders (XXII). Considerable incidence also occurred in gastrointestinal disorders (VII, 73%), general disorders and administration site conditions (VIII, 62.6%) and infections and infestations (XI, 48.2%). The CTCAE groups and the AE incidences are presented in Table 7.

Table 7. Definition of CTCAE categories and total AE prevalence in the six included studies

	Study						
CTCAE category	**Lehto-ranta 2014 N = 208**	**Kumpu 2012 N = 501**	**Hatakka 2001 N = 513**	**Hatakka 2007a N = 269**	**Hatakka 2007b N = 226**	**Hatakka 2007c N = 192**	**Total N = 1,909**
I Blood and lymphatic system disorders	0.0	0.0		0.0		0.0	0.0
II Cardiac disorders	0.0	0.2		0.0		8.9	0.9
III Congenital, familial and genetic disorders	0.0	0.0		0.0		0.0	0.0
IV Ear and labyrinth disorders	13.0	33.9	40.9	80.7		1.6	32.8
V Endocrine disorders	0.0	0.2		0.0		2.6	0.3
VI Eye disorders	13.0	84.4	18.1	11.9		3.1	30.4
VII Gastrointestinal disorders	29.8	84.4	75.2	93.7	47.8	84.4	73.0
VIII General disorders and adm. site cond.	51.4	83.0	71.0	96.3	16.8	5.7	62.6
IX Hepatobiliary disorders	0.0	0.0		0.0		0.0	0.0
X Immune system disorders	0.0	4.2		1.1		1.0	1.4
XI Infections and infestations	85.1	59.7	23.6	85.5	33.6	9.4	48.2
XII Injury, poisoning and procedural complications	0.0	11.4	0.2	1.9		4.7	3.8
XIII Investigations	0.0	0.0		0.0		38.0	3.8
XIV Metabolism and nutrition disorders	0.0	0.2		0.4		5.2	0.6
XV Musculoskeletal and connective tissue disorders	30.8	9.4		1.5		21.9	8.2
XVI Neoplasm benign, malignant and unspecified	0.0	0.2		0.0		0.5	0.1

Table 7 cont.

	Study						
CTCAE category	**Lehto-ranta 2014 N = 208**	**Kumpu 2012 N = 501**	**Hatakka 2001 N = 513**	**Hatakka 2007a N = 269**	**Hatakka 2007b N = 226**	**Hatakka 2007c N = 192**	**Total N = 1,909**
XVII Nervous system disorders	52.9	14.6		4.5		4.2	10.6
XXIV Pregnancy, puerperium and perinatal cond.	0.0	0.0		0.0		0.0	0.0
XIX Psychiatric disorders	0.0	0.0		1.9		1.0	0.4
XX Renal and urinary disorders	0.0	2.8		2.2		2.6	1.3
XXI Reproductive system and breast disorders	0.0	0.0		0.0		0.0	0.0
XXII Respiratory, thoracic and mediastinal disorders	98.1	95.6	91.0	99.6	39.4	2.6	79.2
XXIII Skin and subcutaneous tissue disorders	7.2	15.4		12.3		0.0	6.5
XXIV Social circumstances	0.0	0.0		0.0		0.0	0.0
XXV Surgical and medical procedures	0.0	79.8		0.4		76.6	28.7
XXVI Vascular disorders	0.0	0.0		0.0		6.8	0.7

The AEs reported in the specific System Organ Classes in the comparison between the probiotic and placebo groups are presented in Figure 7. Risk ratio over one, which favors the placebo, was found in five System Organ Classes: cardiac disorders (II), injury, poisoning and procedural complications (XII), metabolism and nutrition disorders (XIV), nervous system disorders (XVII), and vascular disorders (XXVI).

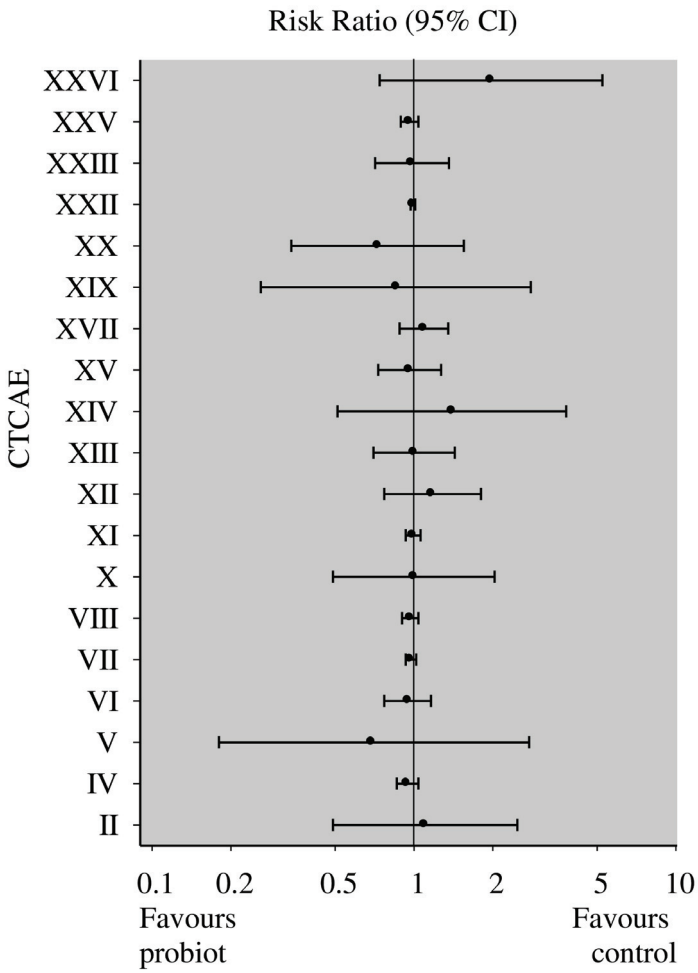

Figure 7. Risk ratios and 95% confidence intervals of AEs in specific CTCAE categories between the probiotic and placebo groups.

The detailed analysis of gastrointestinal disorders (CTCAE category VII), yielded the overall RR of AEs in the probiotic group vs. the placebo group of 0.97 (95% CI 0.93–1.02, $P = 0.30$). The results appeared to favor probiotics in specific studies (RR 0.73-1.08) and in different probiotic combinations (RR 0.99-1.00). The dispersion of respiratory, mediastinal and thoracic disorders (XXII) was greater in different studies, but the corrected weight of the overall RR of AEs in the probiotic group vs. the placebo group was 0.99 (95% CI 0.97–1.01, P=0.35). In the class of infections and infestations (XI), dispersion occurred between the studies, and the overall RR of AEs in the probiotic group vs. the placebo group was 0.99 (95% CI 0.93–1.06, $P = 0.62$).

6 DISCUSSION

The studies conducted in this thesis investigated probiotic *Lactobacillus rhamnosus* GG in the upper respiratory tract and the safety of specific probiotic species. The objectives were to determine whether L. GG colonized the upper respiratory epithelium, whether potential colonization had an effect on subjects' microbiota, whether L. GG affected the nasopharyngeal rhinovirus load, and whether the use of specific probiotics, specifically L. GG, resulted in adverse events.

6.1 Colonization of the upper respiratory tract by Lactobacillus rhamnosus GG

The two prospective, randomized, double-blinded, placebo-controlled studies (I, II) indicated that L. GG is able to colonize upper respiratory tract mucosa after per oral administration in the following locations: the effusion in middle ear cavity and adenoid tonsil. Previously, L. GG (Kumpu *et al.* 2013a) and *Lactobacillus plantarum* DSM9843 (Stjernquist-Desatnik *et al.* 2000) were recovered from tonsillar tissue, and *Streptococcus sanguinis* K12 (Power *et al.* 2008) and *Streptococcus salivarius* 24SMBc (Santagati *et al.* 2015) were recovered from the nasopharynx. The fact that L. GG also was recovered in a portion of the samples in the placebo group may indicate a longer persistence or the accidental consumption of products containing L. GG. In addition, maternal transmission during delivery or breastfeeding is possible.

The mechanism by which L. GG is able to colonize upper respiratory epithelium is unknown. The mechanism of adhesion to gut epithelium is hypothesized to occur partly in the pilus-like structures that coat L. GG bacteria (Kankainen *et al.* 2009). Pili and the surface of L. GG contain mucus-binding proteins, which presumably play a role in epithelial adhesion (von Ossowski *et al.* 2010, von Ossowski *et al.* 2011, Tripathi *et al.* 2013, Nishiyama *et al.* 2015). Interestingly, pili are also implicated in L. GG adhesion to macrophages (Vargas Garcia *et al.* 2015). Pili also seem to have immunomodulatory interactions with the host (Zakaria Gomaa 2013). We can only hypothesize that the adherence of L. GG to the adenoid epithelium occurs in the same mechanism as it does in the gut epithelium. Colonization of the MEE could be explained by the migration of L. GG from the nasopharynx to the middle ear via the eustachion tube, which is thought to be the route for otitis media (OM) pathogens (Sadé 1994, Coticchia *et al.* 2013). In addition, different applications and dosages should be studied because probiotic nasal spray therapy results in the colonization of the nasopharynx (Santagati *et al.* 2015), and different applications and dosages may induce differing effects.

The nasopharyngeal and adenoid microbiota is a complex interactive system, and the consequences of changing the proportions, such as by probiotic colonization, remain unknown. The nasopharynx harbors a wide variety of bacteria and viruses, both commensal and pathogenic (Chi *et al.* 2003, Skevaki *et al.* in press). The bacterial composition of nasopharyngeal microbiota differs from other body parts, which was surveyed in healthy Chinese young adults (Ling *et al.* 2013). The nasopharyngeal microbiota presented with potentially invasive bacteria, such as *Streptococcus pneumoniae*, *H. infuenzae*, *Staphylococcus aureus*, *M. catarrhalis*, and *Neisseria meningitidis* in an overall healthy population (Bogaert *et al.* 2011, Hendley *et al.* 2005). In children, the nasopharyngeal microbiota was noted to change according to the season (Bogaert *et al.* 2011). Furthermore, certain commensal taxa were found to be negatively associated with AOM

pathogens, and the proportions of taxa changed depending on the use of antibiotics (Pettigrew *et al.* 2012). Respiratory viruses have been shown to accomplish changes in bacterial adhesion (Avadhanula *et al.* 2006), colonization (Tong *et al.* 2001) and immunological mechanisms (Colamussi *et al.* 1999) in the nasopharynx. Eventually, complex interactions between viruses and bacteria in the nasopharyngeal epithelium can evoke bacterial superinfections (Bakaletz 1995).

The MEE has been extensively studied, but not with regard to the presence of probiotics. The introduction of PCR revealed a larger proportion of pathogens in MEE than previously was thought (Ueyama *et al.* 1995, Hotomi *et al.* 1993). Furthermore, the same pathogens were recognized in childhood OME and rAOM (Stol *et al.* 2013). The sensitivity of PCR also revealed the polymicrobial nature of otitis media (Pettigrew *et al.* 2011, Nokso-Koivisto *et al.* 2004, Holder *et al.* 2012, Marom *et al.* 2012, Nokso-Koivisto *et al.* 2015). The role of nasopharyngeal microbiota in the development of AOM or OME is strongly suggested (Tomonaga *et al.* 1989, Saylam *et al.* 2010, Liu *et al.* 2011, Ruohola *et al.* 2013).

The importance of bacterial biofilms in the middle ear has emerged during the last decade (Post *et al.* 2004, Fergie *et al.* 2004, Post *et al.* 2007). Biofilms are organized heterogeneous bacterial communities that attach to surfaces and produce their own matrix; they have a higher antibiotic resistance than their planktonic (free-floating) counterparts (Macassey, Dawes 2008). One of the first studies to indicate a viable structure in culture-negative OME found that the bacterial messenger RNA in MEE established metabolically active, intact organisms (Rayner *et al.* 1998). It was shown that the naturally existing probiotics *Lactobacilli* and *Bifidobacteria* are able to form biofilm in the gut (Macfarlane *et al.* 2011). Furthermore, *in vitro* experiments indicate that L. GG has the capacity to form biofilm, in contrast to other strains of the *Lactobacillus casei* group (Lebeer *et al.* 2007). Our results did not confirm the possibility of determining whether L. GG presented in the MEE and adenoid samples as a biofilm or a planktonic.

Nevertheless, the ability of probiotics to interfere with biofilm formation *in vivo* has been partly studied (Vuotto *et al.* 2014). Interestingly, *Lactobacillus reuteri* biofilms are capable of modulating the host's immune responses and producing antimicrobial agents (Jones, Versalovic 2009). Furthermore, the biosurfactants produced by Lactobacilli species showed multiple anti-adhesive and antimicrobial activities against several pathogens (Zakaria Gomaa 2013). Probably the best initial evidence of probiotics in biofilm-associated infections is found in dental practice against caries and plaque (Laleman, Teughels 2015, Gungor *et al.* 2015). However, the results are contradictory, and no conclusion on probiotic actions in biofilms can be drawn yet (Ahmed *et al.* 2014, Schwendicke *et al.* 2014).

It has been suggested that nasopharyngeal microbiota could affect subjects' vulnerability to respiratory infections. In an experimental HRV challenge trial, significant differences in two nasopharyngeal genera (*Neisseria* and *Propionibacterium*) were identified between HRV-infected and non-infected subjects (Allen *et al.* 2014). Furthermore, a previous study showed that young children presented with a small number of bacterial taxa in high total numbers in their nasopharynx, contrary to their parents who presented with much more diverse taxa with lower bacterial carriage (Stearns *et al.* 2015). This finding suggests that a greater variety of nasopharyngeal microbiota could protect the subject against URI. It is possible that the maturation of the host-associated microbial community happens similarly in the nasopharyngeal area and in the gut (Adlerberth *et al.* 2009). Interestingly, the development of nasopharyngeal microbiota was studied in 60 healthy infants, and certain microbiota patterns were found to be associated with decreases in URI episodes reported by the parents (Biesbroek *et al.* 2014).

Furthermore, nasopharyngeal microbiota, especially *Streptococcus,* has been implicated to children's risk of developing asthma (Teo *et al.* 2015). These aspects of microbial diversity in the nasopharynx are also considered when breastfeeding is recommended (Biesbroek *et al.* 2014, Berrington *et al.* 2014).

Microbes can adhere directly to each other, but effects can also occur through adhesion on the host's mucosal surfaces. Highly evolved relationships between the upper respiratory microbiota exist, and it is important to understand those interactions, especially when the microbiota is manipulated. In probiotic settings, knowledge of the colonization of the respiratory epithelium is valuable for further research to investigate the effects of probiotics on the natural microbiota.

6.2 Efficacy of Lactobacillus rhamnosus GG to specific upper respiratory infection pathogens

We studied the prevalence of HRV and EV in adenoid and MEE (I, II), and HRV load in nasopharyngeal lavage samples (III) after the oral consumption of L. GG. Interestingly, the percentages of the HRV- and EV- positive samples were higher in the L. GG group compared to the placebo, but the difference was not statistically significant. In contrast, there was a slight tendency to lower HRV loads in the nasopharyngeal lavage samples in the live and inactivated L. GG populations compared to the placebo. However, these studies are not comparable because their settings and objectives are different.

The results of the first study showed a similar prevalence of respiratory symptoms in the L. GG and placebo groups. In previous studies, L. GG alone or in combination reduced the risk of upper respiratory infections in children (Kukkonen *et al.* 2008, Rautava *et al.* 2008, Hojsak *et al.* 2010b, Hojsak *et al.* 2010a, Kumpu *et al.* 2012). Moreover, the risk of HRV infections was significantly lower in preterm infants using L. GG supplementation compared to the placebo (Luoto *et al.* 2013). However, in that study no significant differences were observed between the groups in the clinical symptoms of HRV infection, HRV load, or duration of HRV infection. However, studies have found conflicting results regarding the effects of L. GG on URI in adult subjects. In training marathon runners, L. GG did not have an effect on respiratory infections (Kekkonen *et al.* 2007). In contrast, in college students, the consumption of L. GG and BB12 combined resulted in significantly shorter and less severe URI symptoms compared to the placebo. In addition, the number of absences from school was significantly fewer in the probiotic group (Smith *et al.* 2013).

Many efficacy studies lack information about viral etiology. Virological findings in symptomatic URI patients using L. GG and BB12 combined were studied in military conscripts (Lehtoranta *et al.* 2014). In that trial, a reduction in the total number of HRV- and EV-positive samples was noted after three months of using the probiotic mixture. The knowledge of L. GG's effects on picornavirus (HRV and EV) infections is still insufficient, so further efficacy trials are recommended to investigate the origin of the virus.

No significant differences were found in the presence of pathogenic bacteria in MEE between the L. GG and placebo groups. Our result aligns with a previous study, in which a probiotic mixture with L. GG did not reduce the occurrence or recurrence of AOM in otitis-prone children, and it did not have an effect on the nasopharyngeal carriage of *S. pneumoniae* or *H. influenzae* (Hatakka *et al.* 2007a). In addition, in another study with children at high-risk for

AOM, a probiotic and prebiotic combination did not reduce the incidence of AOM or antibiotic prescriptions; however, the combination did not contain L. GG (Cohen *et al.* 2013). However, there might be a difference in the efficacy of L. GG preventing AOM between otitis-prone and healthy children. The children in our studies (I, II) are the former.

In a meta-analysis, L. GG was associated with lower risk of otitis media and antibiotic prescriptions in children (Liu *et al.* 2013). Infants fed with L. GG and BB12 enriched formula had a significantly lower risk for AOM and antibiotic prescriptions during the first seven months of life compared to the placebo group (Rautava *et al.* 2008). Moreover, in that study, the probiotic-fed infants showed a tendency to the reduced need for tympanostomy. It is possible that efficacy is also dependent on the application form (capsule, powder, dairy product, or nasal spray) and manufacturing process (Grzeskowiak *et al.* 2011).

The mechanism by which L. GG might modulate the risk of AOM has received little attention. Alterations in the nasal or nasopharyngeal microbiota may influence the risk of AOM. Indeed, the consumption of probiotic (L. GG, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium* sp.) yogurt resulted in a significant reduction in potentially pathogenic bacteria (*Staphylococcus aureus*, *S. pneumoniae*, β-hemolytic streptococci, and *H. influenzae*) in the nasal cavity (Glück, Gebbers 2003). *In vitro*, L. GG was able to inhibit the invasion of macrolide-resistant group A streptococci in human epithelial cells (Princivalli *et al.* 2009). L. GG has also been indicated to inhibit the adherence of *S. pneumoniae* to epithelial cells *in vitro* (Wong *et al.* 2013). It is possible that the adhesion of probiotics to epithelium or to mucus forms a protective layer against pathogenic bacteria, and probiotics might compete with pathogenic bacteria for binding sites (Popova *et al.* 2012). The ability of probiotics to aggregate among themselves or with other bacteria in order to form an adequate mass is also needed to promote natural barrier action (Collado *et al.* 2007). In addition, L. GG is capable of immunomodulation through cell-mediated and humoral immunity (Fang *et al.* 2000, Cross 2002, Pohjavuori *et al.* 2004, Viljanen *et al.* 2005, Kukkonen *et al.* 2006, Oksaharju *et al.* 2011, Miettinen *et al.* 2012).

Our results for bacterial DNA and microarray revealed the dominance of *H. influenzae* in MEE. *H. influenzae* was recognized as a major pathogen in OME (Leskinen *et al.* 2002, Pichichero *et al.* 2008, Holder *et al.* 2012, Stol *et al.* 2013). However, new insights were gained as confocal laser scanning microscopy revealed a wide variety of live bacteria in MEE, including biofilm (Daniel *et al.* 2012). Surprisingly, none of our samples presented with *S. pneumoniae*. One explanation might be vaccination; the pneumococcal conjugate vaccine had been implemented in a national vaccination program the year prior to our study. The introduction of the pneumococcal conjugate vaccine has been shown to alter the microbiology of AOM (Block *et al.* 2004, Zhao *et al.* 2014). Similar results in OME have shown decreases in the prevalence of *S. pneumoniae* and increases in the prevalence of *H. influenzae* (Holder *et al.* 2015). Our findings support these previous results.

In the nasopharyngeal lavage sample, we found no significant differences in HRV load between the intervention groups. Moreover, in this sample size, a real effect could not be estimated (III). The 15%-prevalence of wild-type infections was rather high, but the study was conducted during the peak months in the fall. Similar to our results, in a study with preterm infants, no significant differences emerged in HRV load between L. GG and placebo groups (Luoto *et al.* 2013). Furthermore, in that study, the relative HRV copy numbers did not correlate to the severity of symptoms, but they did correlate positively with the duration of the infection. In viral mouse models, L. GG significantly reduced influenza virus titers in the lungs of experimentally infected mice (Kawase *et al.* 2010), and heat-killed lactobacilli showed the same capacity (Maeda

et al. 2009, Kawase *et al.* 2012). Obviously, there is a need for further clinical trials to investigate the viral occurrence and load to confirm or disregard the effects of probiotics.

In our study, there was a positive correlation between HRV load and total symptom scores on day 2 and day 5 after inoculation. Previously, an association was found between higher nasopharyngeal HRV loads and clinical symptoms in upper and lower respiratory infections (Gerna *et al.* 2009). In lung transplant patients, nasopharyngeal HRV load positively correlated with certain symptoms (a sore throat, fever, cough, and sputum production) (Ambrosioni *et al.* 2015). Furthermore, in an experimental study on HRV infection, a correlation between lower respiratory symptoms and nasal viral load was noted in the asthmatic subjects but not in the control subjects (Message *et al.* 2008). Another experimental HRV trial with chronic obstructive pulmonary disease (COPD) patients revealed similar results. The sputum virus load correlated with inflammatory markers in the COPD subjects but not in the control subjects (Mallia *et al.* 2011). On the contrary, in natural viral respiratory infections, a correlation between HRV load and lower respiratory symptoms was not found in the asthmatic or the control subjects (van Elden *et al.* 2008). However, in children aged over 11 months, a strong positive correlation between nasopharyngeal HRV load and disease severity was found but not in younger infants (Takeyama *et al.* 2012). Furthermore, children diagnosed with HRV did not show a correlation between nasopharyngeal viral load and the severity of the disease, but the subjects with HRV viremia had a significantly higher viral load and a more severe disease (Esposito *et al.* 2014). Our results showed a stronger positive correlation 48 h after inoculation when the symptoms (Gwaltney Jr. *et al.* 2003) and virus titers (Hendley *et al.* 2004) were observed to peak.

The dose and duration of the probiotic seem to influence its efficacy. We used moderate dosages of L. GG in the studies: 8-9 x 10^9 in I and II and 1 x 10^9 in III. It was shown that in gnotobiotic pigs, the effects of L. GG in adjuvanticity and protection against rotavirus diarrhea depended on the dosage (Wen *et al.* 2015). Two Japanese studies that investigated *Lactobacillus casei* strain Shirota in URI found better outcomes with a daily dose of 1 x 10^{11} than with a daily dose of 4 x 10^{10} (Fujita *et al.* 2013, Shida *et al. in press*). Moreover, because different strains have different effects, they should be studied separately.

6.3 Safety of probiotics

We utilized the individual participant data on 1,909 patients in prospective randomized placebo-controlled double-blinded studies that were conducted by our study group. These data offered a novel perspective on the analysis of the possible AEs of probiotics. In a healthy population of children, young adults, and the elderly, the consumption of L. GG or L. GG in combination with BB12, or PJS, Lc705 and/or BB99 did not result in AEs that differed from those in the population that used a placebo. The detailed analysis of the categories of respiratory and gastrointestinal diseases and infections, including specific combinations of them, did not show any statistical differences in AEs.

Because the consumption of probiotics is becoming increasingly widespread, clinicians should understand the risks and benefits regarding their use. Probiotics are considered dietary supplements, so they are not subject to the same regulation and safety assessment that drugs are (Boyle *et al.* 2006, Degnan 2008). The general consideration of commercially available probiotics is that they are safe (Ishibashi, Yamazaki 2001). However, although the use of probiotics has raised some concerns about their safety, the topic has not been sufficiently studies (Sanders *et al.* 2010).

Indeed, rare occasions of sepsis (Land *et al.* 2005), fatal infection in an immunocompromised patient (Kalima *et al.* 1996), and increased mortality in patients with severe pancreatitis (Besselink *et al.* 2008) have been implicated in the use of probiotics. However, the European Food Safety Authority (EFSA) has granted the qualified presumption of safety (QPS) to many commercial probiotic strains, as well as to L. GG (EFSA 2013). Probiotic bacteria in food should fulfill the following criteria: they should be non-pathogenic; they should not pose a risk to the host; the absence of virulence genes should be proved; and the absence of antibiotic resistance should be proved (Wassenaar *et al.* 2008). Because different genera and strains have different clinical effects, AEs should be assessed in a strain-specific manner.

Several systematic reviews and meta-analyses of probiotic safety have been conducted (Didari *et al.* 2014, Whelan *et al.* 2010, Hempel *et al.* 2011). However, because the majority of these studies did not investigate the safety of probiotics, there is a major lack of assessment and systematic reporting of AEs. Furthermore, many of these studies have been inconsistent and incomplete in reporting possible AEs. Thus, the evidence that there is no increase in the risk of probiotic consumption must be viewed in a critical light. A Dutch-led study group used the CTCAE as a systematic evaluation tool in three meta-analyses of probiotic safety in different populations: infants under two years of age (van den Nieuwboer *et al.* 2014); children under 18 years of age (van den Nieuwboer *et al.* 2015b); and immunocompromised adults (van den Nieuwboer *et al.* 2015a). In every one of these meta-analyses, relatively more AEs occurred in the control group than in the probiotic group. However, the writers have emphasized limitations in the reporting and classification of AEs, and they therefore caution against the generalization of their conclusions. Because of the precise reporting and classification used in our study, our results contribute to the information about the safety of specific probiotics.

6.4 Strengths and limitations

The strengths of this series of studies are the following. They are prospective, randomized, double-blinded, placebo controlled, and well designed, including the six studies used in study IV. The limitations of the studies are the following. There was a high number of drop-out subjects and a small sample size in studies I, II, and III. In addition, it is confusing that a high number of placebo samples was recovered with L. GG.

The results of study IV could be generalized. This study used a wide range of multiple subjects, and the results were confirmed. We also demonstrated the ability of L. GG to colonize the nasopharynx and the middle ear in children but not in adults. Furthermore, the effects of L. GG on viral and bacterial pathogens could not be confirmed in these studies.

6.5 Future research

Over the past decades, gut microbiota has been increasingly recognized as one of the main factors in the increasing prevalence of immunity-related disorders, such as inflammation, atopy, asthma, musculoskeletal disorders, liver fibrosis, diabetes mellitus type 2, metabolic syndrome, cardiovascular diseases, neurodegenerative diseases, atherosclerosis, and cancer, which is also known as the hygiene hypothesis (Bubnov *et al.* 2015). The research on metagenomics has contributed information on how the microbiota interacts with the host's physiology and has started to provide new therapeutical targets. Finally, by better understanding the role of gut

microbiota, the individual's microbiota could be integrated into personalized healthcare, and the individual's diseases could be targeted and treated more efficiently. However, the complete understanding of the disease process is required to determine whether targeting gut microbiota would be effective or not (Marchesi *et al.* in press).

We showed that, in addition to the gut and the beginning of the GI-tract (oral cavity and oropharynx), probiotics are able to colonize the respiratory tract. Because adenoids are part of the active lymphatic tissue of the Waldeyer's ring, their colonization might have effects that are similar those in the gut epithelium. Commensal and probiotic organisms in the gut are able to trigger the production of host proinflammatory and antimicrobial proteins and peptides (Sassone-Corsi, Raffatellu 2015). These are also implicated in the activation of innate immune responses and are able to promote adaptive immune responses. They also involve the differentiation of T cells and the differentiation and activation of B cells.

Gut microbiota and probiotics have been widely studied and implicated in immunity-related diseases, such as cardiovascular disease (Ryan *et al.* 2015), rheumatoid arthritis (Sandhya *et al.* in press), metabolic diseases (Ojeda *et al.* in press, Le Barz *et al.* 2015), allergies (Vernocchi *et al.* 2015), gynecologic cancers (Chase *et al.* 2015), gestational diabetes (Isolauri *et al.* 2015), and perhaps the most surprisingly, the function of the brain (Liu *et al.* 2015). However, we do not know whether the effects of probiotics in URI are caused by altered gut microbiota or by the local respiratory epithelium. In the coming decades, the research on inflammatory diseases will offer interesting results about the effects of gut microbiota on almost all parts of the human body.

7 CONCLUSIONS

Based on the results presented in this thesis, the following conclusions are drawn regarding the colonization of *Lactobacillus rhamnosus* GG in the upper respiratory tract and its effects on pathogens in the upper respiratory tract, including adverse events of *Lactobacillus rhamnosus* GG and other probiotics:

1. *L. rhamnosus* GG was recovered from 100% of adenoid tonsil samples and 21% of middle ear effusion samples after a three week per oral consumption of the probiotic in children admitted to adenotomy and possible tympanostomy. Because *L. rhamnosus* GG also was recovered in 76% of the adenoid tonsil samples and 17% of the middle ear effusion samples of the placebo group, persistence longer than seven weeks, which was the length of the wash-out plus the intervention period, may occur.

2. The consumption of *L. rhamnosus* GG did not affect the number of human rhinovirus and enterovirus -positive samples of the adenoid or middle ear effusion, or the bacterial presence in the middle ear effusion in children with recurrent acute otitis, otitis media with effusion, or chronic sinusitis. Based on the study diaries, it did not result in notable differences in clinical symptoms.

3. Live or inactivated *L. rhamnosus* GG did not significantly affect the human rhinovirus load in the nasopharyngeal lavage samples compared to the placebo in an experimental human rhinovirus challenge in healthy adult volunteers. The human rhinovirus load was positively correlated with total clinical symptom scores on days 2 and 5 after inoculation with the experimental human rhinovirus.

4. The use of *L. rhamnosus* GG alone or in combination with BB12, or PJS, Lc705, and/or BB99 did not result in adverse events in healthy child, young adult, or elderly populations according to the individual participant data on 1,909 subjects in six prospective randomized, placebo-controlled, double-blinded studies. No notable adverse events were observed to result from the consumption of these probiotics, which were distributed according an organized classification scale, the Common Terminology Criteria for Adverse Events. A detailed analysis was conducted in three categories: respiratory, gastrointestinal disorders, and infections. The results showed similar adverse events between the probiotic compounds and placebos assigned in the six individual studies and in different probiotic combinations.

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REFERENCES

Acute otitis media (online). Current Care Guidelines. Working group set up by the Finnish Medical Society Duodecim, the Finnish Pediatric Society, the Finnish Otolaryngology Society and the Finnish General Medicine Society. Helsinki: The Finnish Medical Society Duodecim, 2010 (referred Dec 07, 2015). Available online at: www.kaypahoito.fi

Adlerberth, I., Wold, A.E. Establishment of the gut microbiota in Western infants. Acta Paediatr. 98: 229-238, 2009.

Ahlroos, T., Tynkkynen, S. Quantitative strain-specific detection of Lactobacillus rhamnosus GG in human faecal samples by real-time PCR. J Appl Microbiol. 106: 506-514, 2009.

Ahmed, A., Dachang, W., Lei, Z., Jianjun, L., Juanjuan, Q., Yi, X. Effect of Lactobacillus species on Streptococcus mutans biofilm formation. Pak J Pharm Sci. 27: 1523-1528, 2014.

Al Kassaa, I., Hober, D., Hamze, M., Chihib, N.E., Drider, D. Antiviral potential of lactic Acid bacteria and their bacteriocins. Probiotics Antimicrob Proteins 6: 177-185, 2014.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., von Wright, A. Persistence of colonization of human colonic mucosa by a probiotic strain, Lactobacillus rhamnosus GG, after oral consumption. Appl Environ Microbiol. 65: 351-354, 1999.

AlFaleh, K., Anabrees, J. Efficacy and safety of probiotics in preterm infants. J Neonatal Perinatal Med. 6: 1-9, 2013.

Allen, E.K., Koeppel, A.F., Hendley, J.O., Turner, S.D., Winther, B., Sale, M.M. Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge. Microbiome 2: 22, 2014.

Ambrosioni, J., Bridevaux, P.O., Aubert, J.D., Soccal, P., Wagner, G. ,Kaiser, L. Role of rhinovirus load in the upper respiratory tract and severity of symptoms in lung transplant recipients. J Clin Virol. 64: 1-5, 2015.

American Academy of Family Physicians, American Academy of Otolaryngology-Head and Neck Surgery, American Academy of Pediatrics Subcommittee on Otitis Media With Effusion Otitis media with effusion. Pediatrics 113: 1412-1429, 2004.

Arroll, B. Non-antibiotic treatments for upper-respiratory tract infections (common cold). Respir Med. 99: 1477-1484, 2005.

Avadhanula, V., Rodriguez, C.A., Devincenzo, J.P., Wang, Y., Webby, R.J., Ulett, G.C., Adderson, E.E. Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. J Virol. 80: 1629-1636, 2006.

Bakaletz, L.O. Viral potentiation of bacterial superinfection of the respiratory tract. Trends Microbiol. 3: 110-114, 1995.

Berggren, A., Lazou Ahren, I., Larsson, N., Onning, G. Randomised, double-blind and placebo-controlled study using new probiotic lactobacilli for strengthening the body immune defence against viral infections. Eur J Nutr. 50: 203-210, 2011.

Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., Gomez-Llorente, C., Gil, A. Probiotic mechanisms of action. Ann Nutr Metab. 61: 160-174, 2012.

Berrington, J.E., Cummings, S.P., Embleton, N.D. The nasopharyngeal microbiota: an important window of opportunity. Am J Respir Crit Care Med. 190: 246-248, 2014.

Besselink, M.G., van Santvoort, H.C., Buskens, E., Boermeester, M.A., van Goor, H., Timmerman, H.M., Nieuwenhuijs, V.B., Bollen, T.L., van Ramshorst, B., Witteman, B.J., Rosman, C., Ploeg, R.J., Brink, M.A., Schaapherder, A.F., Dejong, C.H., Wahab, P.J., van Laarhoven, C.J., van der Harst, E., van Eijck, C.H., Cuesta, M.A., Akkermans, L.M., Gooszen, H.G. ,Dutch Acute Pancreatitis Study Group Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet 371: 651-659, 2008.

Biesbroek, G., Tsivtsivadze, E., Sanders, E.A., Montijn, R., Veenhoven, R.H., Keijser, B.J., Bogaert, D. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. Am J Respir Crit Care Med. 190: 1283-1292, 2014.

Block, S.L., Hedrick, J., Harrison, C.J., Tyler, R., Smith, A., Findlay, R., Keegan, E. Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. Pediatr Infect Dis.J. 23: 829-833, 2004.

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., Bruin, J., Montijn, R., Bonten, M. ,Sanders, E. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One 6: e17035, 2011.

Boyle, R.J., Robins-Browne, R.M., Tang, M.L. Probiotic use in clinical practice: what are the risks? Am J Clin Nutr. 83: 1256-64; quiz 1446-7, 2006.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., Piescik, M., Puntis, J., Shamir, R., Szajewska, H., Turck, D., van Goudoever, J. ,ESPGHAN Committee on Nutrition Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. J Pediatr Gastroenterol Nutr. 52: 238-250, 2011.

Brown, A.C., Valiere, A. Probiotics and medical nutrition therapy. Nutr Clin Care. 7: 56-68, 2004.

Bubnov, R.V., Spivak, M.Y., Lazarenko, L.M., Bomba, A., Boyko, N.V. Probiotics and immunity: provisional role for personalized diets and disease prevention. EPMA J. 6(1): 14, 2015.

Cáceres, P., Montes, S., Vega, N., Cruchet, S., Brunser, O., Gotteland, M. Effects of HN001 on acute respiratory infections and intestinal secretory IgA in children. J Pediatr Infect Dis 05: 353-362, 2010.

Chan, J.F., Lau, S.K., Woo, P.C., Fan, R.Y., Ip, J.J., Chan, C.F., Luk, J.K. ,Yuen, K.Y. Lactobacillus rhamnosus hepatic abscess associated with Mirizzi syndrome: a case report and review of the literature. Diagn Microbiol Infect Dis. 66: 94-97, 2010.

Chase, D., Goulder, A., Zenhausern, F., Monk, B., Herbst-Kralovetz, M. The vaginal and gastrointestinal microbiomes in gynecologic cancers: a review of applications in etiology, symptoms and treatment. Gynecol Oncol. 138: 190-200, 2015.

Chen, C.Y., Tsen, H.Y., Lin, C.L., Lin, C.K., Chuang, L.T., Chen, C.S. ,Chiang, Y.C. Enhancement of the immune response against Salmonella infection of mice by heat-killed multispecies combinations of lactic acid bacteria. J Med Microbiol. 62: 1657-1664, 2013.

Chi, D.H., Hendley, J.O., French, P., Arango, P., Hayden, F.G. ,Winther, B. Nasopharyngeal reservoir of bacterial otitis media and sinusitis pathogens in adults during wellness and viral respiratory illness. Am J Rhinol. 17: 209-214, 2003.

Cobo Sanz, J.M., Mateos, J.A., Munoz Conejo, A. Effect of Lactobacillus casei on the incidence of infectious conditions in children. Nutr Hosp. 21: 547-551, 2006.

Cohen, R., Martin, E., de La Rocque, F., Thollot, F., Pecquet, S., Werner, A., Boucherat, M., Varon, E., Bingen, E. ,Levy, C. Probiotics and prebiotics in preventing episodes of acute otitis media in high-risk children: a randomized, double-blind, placebo-controlled study. Pediatr Infect Dis J. 32: 810-814, 2013.

Coker, T.R., Chan, L.S., Newberry, S.J., Limbos, M.A., Suttorp, M.J., Shekelle, P.G. ,Takata, G.S. Diagnosis, microbial epidemiology, and antibiotic treatment of acute otitis media in children: a systematic review. JAMA 304: 2161-2169, 2010.

Colamussi, M.L., White, M.R., Crouch, E., Hartshorn, K.L. Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. Blood 93: 2395-2403, 1999.

Collado, M.C., Meriluoto, J., Salminen, S. Measurement of aggregation properties between probiotics and pathogens: in vitro evaluation of different methods. J Microbiol Methods 71: 71-74, 2007.

Cordey, S., Junier, T., Gerlach, D., Gobbini, F., Farinelli, L., Zdobnov, E.M., Winther, B., Tapparel, C., Kaiser, L. Rhinovirus genome evolution during experimental human infection. PLoS One 5(5): e10588, 2010.

Coticchia, J.M., Chen, M., Sachdeva, L., Mutchnick, S. New paradigms in the pathogenesis of otitis media in children. Front Pediatr. 1: 52, 2013.

Cross, M.L. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol Med Microbiol. 34: 245-253, 2002.

Daniel, M., Imtiaz-Umer, S., Fergie, N., Birchall, J.P., Bayston, R. Bacterial involvement in otitis media with effusion. Int J Pediatr Otorhinolaryngol. 76: 1416-1422, 2012.

de Araujo, G.V., de Oliveira Junior, M.H., Peixoto, D.M., Sarinho, E.S. Probiotics for the treatment of upper and lower respiratory-tract infections in children: systematic review based on randomized clinical trials. J Pediatr (Rio J), 91:413-27, 2015.

de Vrese, M., Winkler, P., Rautenberg, P., Harder, T., Noah, C., Laue, C., Ott, S., Hampe, J., Schreiber, S., Heller, K. ,Schrezenmeir, J. Effect of Lactobacillus gasseri PA 16/8, Bifidobacterium longum SP 07/3, B. bifidum MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. Clin Nutr. 24: 481-491, 2005.

Degnan, F.H. The US Food and Drug Administration and probiotics: regulatory categorization. Clin Infect Dis. 46: 133-6, 2008.

Del Vecchio, A.M., Branigan, P.J., Barnathan, E.S., Flavin, S.K., Silkoff, P.E., Turner, R.B. Utility of animal and in vivo experimental infection of humans with rhinoviruses in the development of therapeutic agents for viral exacerbations of asthma and chronic obstructive pulmonary disease. Pulm Pharmacol Ther. 30: 32-43, 2015.

Didari, T., Solki, S., Mozaffari, S., Nikfar, S., Abdollahi, M. A systematic review of the safety of probiotics. Expert Opin Drug Saf. 13: 227-239, 2014.

Doi, A., Nakajo, K., Kamiya, T., Ohkusu, K. Splenic abscess caused by Lactobacillus paracasei. J Infect Chemother. 17: 122-125, 2011.

Ebner, S., Smug, L.N., Kneifel, W., Salminen, S.J., Sanders, M.E. Probiotics in dietary guidelines and clinical recommendations outside the European Union. World J Gastroenterol. 20: 16095-16100, 2014.

Eccles, R. Understanding the symptoms of the common cold and influenza. Lancet Infect Dis. 5: 718-725, 2005.

EFSA European Food Safety Authority (EFSA) (2013) Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA J. 11: 3449, 2013.

Elias, J., Bozzo, P., Einarson, A. Are probiotics safe for use during pregnancy and lactation? Can Fam Physician 57: 299-301, 2011.

Esposito, S., Daleno, C., Scala, A., Castellazzi, L., Terranova, L., Sferrazza Papa, S., Longo, M.R., Pelucchi, C., Principi, N. Impact of rhinovirus nasopharyngeal viral load and viremia on severity of respiratory infections in children. Eur J Clin Microbiol Infect Dis. 33: 41-48, 2014.

Fang, H., Elina, T., Heikki, A., Seppo, S. Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol. 29: 47-52, 2000.

Fendrick, A.M., Monto, A.S., Nightengale, B., Sarnes, M. The economic burden of non-influenza-related viral respiratory tract infection in the United States. Arch Intern Med. 163: 487-494, 2003.

Fendrick, A.M. Viral Respiratory Infections Due to Rhinoviruses: Current Knowledge, New Developments. Am J Ther. 10: 193-202, 2003.

Fergie, N., Bayston, R., Pearson, J.P., Birchall, J.P. Is otitis media with effusion a biofilm infection? Clin Otolaryngol Allied Sci. 29: 38-46, 2004.

Finnish Study Group for Antimictobial Resistence, National Institute for Health and Welfare 2014, date accessed Sept. 2015 http://www.julkari.fi/bitstream/handle/10024/116945/URN_ISBN_978-952-302-359-8.pdf?sequence=1.

Fiocchi, A., Pawankar, R., Cuello-Garcia, C., Ahn, K., Al-Hammadi, S., Agarwal, A., Beyer, K., Burks, W., Canonica, G.W., Ebisawa, M., Gandhi, S., Kamenwa, R., Lee, B.W., Li, H., Prescott, S., Riva, J.J., Rosenwasser, L., Sampson, H., Spigler, M., Terracciano, L., Vereda-Ortiz, A., Waserman, S., Yepes-Nunez, J.J., Brozek, J.L. ,Schunemann, H.J. World Allergy Organization-McMaster University Guidelines for Allergic Disease Prevention (GLAD-P): Probiotics. World Allergy Organ J. 8(1):4, 2015.

Fujita, R., Iimuro, S., Shinozaki, T., Sakamaki, K., Uemura, Y., Takeuchi, A., Matsuyama, Y. ,Ohashi, Y. Decreased duration of acute upper respiratory tract infections with daily intake of fermented milk: a multicenter, double-blinded, randomized comparative study in users of day care facilities for the elderly population. Am J Infect Control 41: 1231-1235, 2013.

Gerna, G., Piralla, A., Rovida, F., Rognoni, V., Marchi, A., Locatelli, F., Meloni, F. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. J Med Virol. 81: 1498-1507, 2009.

Glanville, N., Johnston, S.L. Challenges in developing a cross-serotype rhinovirus vaccine. Curr Opin Virol. 11: 83-88, 2015.

Glasziou, P., Del Mar, C., Rovers, M. Antibiotics and acute otitis media in children. JAMA 305: 997-8, 2011.

Glück, U., Gebbers, J.-. Ingested probiotics reduce nasal colonization with pathogenic bacteria (Staphylococcus aureus, Streptococcus pneumoniae, and ß-hemolytic streptococci). Am J Clin Nutr. 77: 517, 2003.

Gonzales, R., Malone, D.C., Maselli, J.H., Sande, M.A. Excessive antibiotic use for acute respiratory infections in the United States. Clin Infect Dis. 33: 757-762, 2001.

Grzeskowiak, L., Isolauri, E., Salminen, S. ,Gueimonde, M. Manufacturing process influences properties of probiotic bacteria. Br J Nutr. 105: 887-894, 2011.

Guarner, F., Khan, A.G., Garisch, J., Eliakim, R., Gangl, A., Thomson, A., Krabshuis, J., Lemair, T., Kaufmann, P., de Paula, J.A., Fedorak, R., Shanahan, F., Sanders, M.E., Szajewska, H., Ramakrishna, B.S., Karakan, T., Kim, N., World Gastroenterology Organization World Gastroenterology Organisation Global Guidelines: probiotics and prebiotics October 2011. J Clin Gastroenterol. 46: 468-481, 2012.

Gungor, O.E., Kirzioglu, Z., Kivanc, M. Probiotics: can they be used to improve oral health? Benef Microbes 6:647-56, 2015.

Gwaltney Jr, J.M. Clinical significance and pathogenesis of viral respiratory infections. Am J Med. 112: 13-18, 2002.

Gwaltney Jr, J.M.,, Hendley, J.O. ,Patrie, J.T. Symptom severity patterns in experimental common colds and their usefulness in timing onset of illness in natural colds. Clin Infect Dis. 36: 714-723, 2003.

Hall-Stoodley, L., Hu, F.Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J., Forbes, M., Greenberg, D.P., Dice, B., Burrows, A., Wackym, P.A., Stoodley, P., Post, J.C., Ehrlich, G.D. ,Kerschner, J.E. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA 296: 202-211, 2006.

Hao, Q., Dong, B.R., Wu, T. Probiotics for preventing acute upper respiratory tract infections. Cochrane Database Syst Rev. 3;2:CD006895, 2015.

Harmes, K.M., Blackwood, R.A., Burrows, H.L., Cooke, J.M., Harrison, R.V., Passamani, P.P. Otitis media: diagnosis and treatment. Am Fam Physician 88: 435-440, 2013.

Hatakka, K., Ahola, A.J., Yli-Knuuttila, H., Richardson, M., Poussa, T., Meurman, J.H., Korpela, R. Probiotics reduce the prevalence of oral candida in the elderly--a randomized controlled trial. J Dent Res. 86: 125-130, 2007c.

Hatakka, K., Blomgren, K., Pohjavuori, S., Kaijalainen, T., Poussa, T., Leinonen, M., Korpela, R., Pitkäranta, A. Treatment of acute otitis media with probiotics in otitis-prone children-a double-blind, placebo-controlled randomised study. Clin Nutr. 26: 314-321, 2007a.

Hatakka, K. Probiotics in the prevention of clinical manifestations of common infectious diseases in children and in the elderly. Academic dissertation, University of Helsinki, 2007b.

Hatakka, K., Savilahti, E., Pönkä, A., Meurman, J.H., Poussa, T., Näse, L., Saxelin, M., Korpela, R. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. BMJ 322: 1327, 2001.

Heikkinen, T., Järvinen, A. The common cold. Lancet 361: 51-59, 2003.

Hempel, S., Newberry, S., Ruelaz, A., Wang, Z., Miles, J.N., Suttorp, M.J., Johnsen, B., Shanman, R., Slusser, W., Fu, N., Smith, A., Roth, B., Polak, J., Motala, A., Perry, T., Shekelle, P.G. Safety of probiotics used to reduce risk and prevent or treat disease. Evid Rep Technol Assess 200: 1-645, 2011.

Hendley, J.O., Hayden, F.G., Winther, B. Weekly point prevalence of Streptococcus pneumoniae, Hemophilus influenzae and Moraxella catarrhalis in the upper airways of normal young children: effect of respiratory illness and season. APMIS 113: 213-220, 2005.

Hendley, J.O., Gwaltney Jr., J.M. Viral titers in nasal lining fluid compared to viral titers in nasal washes during experimental rhinovirus infection. J Clin Virol. 30: 326-328, 2004.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 11: 506-514, 2014.

Hojsak, I., Snovak, N., Abdovic, S., Szajewska, H., Misak, Z., Kolacek, S. Lactobacillus GG in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers: a randomized, double-blind, placebo-controlled trial. Clin Nutr. 29: 312-316, 2010a.

Hojsak, I., Abdovic, S., Szajewska, H., Milosevic, M., Krznaric, Z., Kolacek, S. Lactobacillus GG in the prevention of nosocomial gastrointestinal and respiratory tract infections. Pediatrics 125: e1171-7, 2010b.

Holder, R.C., Kirse, D.J., Evans, A.K., Peters, T.R., Poehling, K.A., Swords, W.E. ,Reid, S.D. One third of middle ear effusions from children undergoing tympanostomy tube placement had multiple bacterial pathogens. BMC Pediatr. 12: 87-2431-12-87, 2012.

Holder, R.C., Kirse, D.J., Evans, A.K., Whigham, A.S., Peters, T.R., Poehling, K.A., Swords, W.E., Reid, S.D. Otopathogens Detected in Middle Ear Fluid Obtained during Tympanostomy Tube Insertion: Contrasting Purulent and Non-Purulent Effusions. PLoS One 10: 6, e0128606, 2015.

Hotomi, M., Tabata, T., Kakiuchi, H., Kunimoto, M. Detection of Haemophilus influenzae in middle ear of otitis media with effusion by polymerase chain reaction. Int J Pediatr Otorhinolaryngol. 27: 119-126, 1993.

Husni, R.N., Gordon, S.M., Washington, J.A., Longworth, D.L. Lactobacillus bacteremia and endocarditis: review of 45 cases. Clin Infect Dis. 25: 1048-1055, 1997.

Ishibashi, N., Yamazaki, S. Probiotics and safety. Am J Clin Nutr. 73: 465S-470S, 2001.

Isolauri, E., Rautava, S., Collado, M.C., Salminen, S. Role of probiotics in reducing the risk of gestational diabetes. Diabetes Obes Metab. 17: 713-719, 2015.

Jacobs, S.E., Lamson, D.M., St George, K., Walsh, T.J. Human rhinoviruses. Clin Microbiol Rev. 26: 135-162, 2013.

Jefferson, T., Jones, M.A., Doshi, P., Del Mar, C.B., Hama, R., Thompson, M.J., Spencer, E.A., Onakpoya, I., Mahtani, K.R., Nunan, D., Howick, J. ,Heneghan, C.J. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. Cochrane Database Syst Rev. 4: CD008965, 2014.

John, M., Dunne, E.M., Licciardi, P.V., Satzke, C., Wijburg, O., Robins-Browne, R.M., O'Leary, S. Otitis media among high-risk populations: can probiotics inhibit Streptococcus pneumoniae colonisation and the risk of disease? Eur J Clin Microbiol Infect Dis. 32: 1101-1110, 2013.

Jones, S.E., Versalovic, J. Probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol. 9: 35, 2009.

Kalima, P., Masterton, R.G., Roddie, P.H., Thomas, A.E. Lactobacillus rhamnosus infection in a child following bone marrow transplant. J Infect. 32: 165-167, 1996.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P., Lebeer, S., De Keersmaecker, S.C., Vanderleyden, J., Hämälainen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjarvi, T., Auvinen, P., de Vos, W.M. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci U.S.A. 106: 17193-17198, 2009.

Kawase, M., He, F., Kubota, A., Harata, G., Hiramatsu, M. Oral administration of lactobacilli from human intestinal tract protects mice against influenza virus infection. Lett Appl Microbiol. 51: 6-10, 2010.

Kawase, M., He, F., Kubota, A., Yoda, K., Miyazawa, K., Hiramatsu, M. Heat-killed Lactobacillus gasseri TMC0356 protects mice against influenza virus infection by stimulating gut and respiratory immune responses. FEMS Immunol Med Microbiol. 64: 280-288, 2012.

Kekkonen, R.A., Vasankari, T.J., Vuorimaa, T., Haahtela, T., Julkunen, I., Korpela, R. The effect of probiotics on respiratory infections and gastrointestinal symptoms during training in marathon runners. Int J Sport Nutr Exerc Metab. 17: 352-363, 2007.

Khanna, R., Lakhanpaul, M., Bull, P.D., Guideline Development Group Surgical management of otitis media with effusion in children: summary of NICE guidance. Clin Otolaryngol. 33: 600-605, 2008.

Klein, J.O. The burden of otitis media. Vaccine. 19, Suppl 1: S2-S8, 2000.

Koivunen, P., Kontiokari, T., Niemela, M., Pokka, T. ,Uhari, M. Time to development of acute otitis media during an upper respiratory tract infection in children. Pediatr.Infect.Dis.J. 18: 303-305, 1999.

Koivunen, P., Uhari, M., Luotonen, J., Kristo, A., Raski, R., Pokka, T., Alho, O.P. Adenoidectomy versus chemoprophylaxis and placebo for recurrent acute otitis media in children aged under 2 years: randomised controlled trial. BMJ 328: 487, 2004.

Kopp-Hoolihan, L. Prophylactic and therapeutic uses of probiotics: a review. J Am Diet Assoc. 101: 229, 2001.

Kujala, T., Alho, O.P., Kristo, A., Uhari, M., Renko, M., Pokka, T. ,Koivunen, P. Quality of life after surgery for recurrent otitis media in a randomized controlled trial. Pediatr.Infect.Dis.J. 33: 715-719, 2014.

Kujala, T., Alho, O.P., Luotonen, J., Kristo, A., Uhari, M., Renko, M., Kontiokari, T., Pokka, T. ,Koivunen, P. Tympanostomy with and without adenoidectomy for the prevention of recurrences of acute otitis media: a randomized controlled trial. Pediatr.Infect.Dis.J. 31: 565-569, 2012.

Kukkonen, K., Nieminen, T., Poussa, T., Savilahti, E., Kuitunen, M. Effect of probiotics on vaccine antibody responses in infancy--a randomized placebo-controlled double-blind trial. Pediatr Allergy Immunol. 17: 416-421, 2006.

Kukkonen, K., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., Kuitunen, M. Long-term safety and impact on infection rates of postnatal probiotic and prebiotic (synbiotic) treatment: randomized, double-blind, placebo-controlled trial. Pediatrics 122: 8-12, 2008.

Kumpu, M., Kekkonen, R.A., Kautiainen, H., Järvenpää, S., Kristo, A., Huovinen, P., Pitkäranta, A., Korpela, R., Hatakka, K. Milk containing probiotic Lactobacillus rhamnosus GG and respiratory illness in children: a randomized, double-blind, placebo-controlled trial. Eur J Clin Nutr. 66: 1020-1023, 2012.

Kumpu, M., Kekkonen, R.A., Korpela, R., Tynkkynen, S., Järvenpää, S., Kautiainen, H., Allen, E.K., Hendley, J.O., Pitkäranta, A. ,Winther, B. Effect of live and inactivated Lactobacillus rhamnosus GG on experimentally induced rhinovirus colds: randomised, double blind, placebo-controlled pilot trial. Benef Microbes 6:631-639, 2015.

Kumpu, M., Swanljung, E., Tynkkynen, S., Hatakka, K., Kekkonen, R.A., Järvenpaa, S., Korpela, R., Pitkäranta, A. Recovery of probiotic Lactobacillus rhamnosus GG in tonsil tissue after oral administration: randomised, placebo-controlled, double-blind clinical trial. Br J Nutr. 109: 2240-2246, 2013a.

Kumpu, M., Lehtoranta, L., Roivainen, M., Rönkkö, E., Ziegler, T., Söderlund-Venermo, M., Kautiainen, H., Järvenpää, S., Kekkonen, R., Hatakka, K., Korpela, R., Pitkäranta, A. The use of the probiotic Lactobacillus rhamnosus GG and viral findings in the nasopharynx of children attending day care. J Med Virol. 85: 1632-1638, 2013b.

Laleman, I., Teughels, W. Probiotics in the dental practice: a review. Quintessence Int. 46: 255-264, 2015.

Land, M.H., Rouster-Stevens, K., Woods, C.R., Cannon, M.L., Cnota, J., Shetty, A.K. Lactobacillus sepsis associated with probiotic therapy. Pediatrics 115: 178-181, 2005.

Lang-Roth, R. Hearing impairment and language delay in infants: Diagnostics and genetics. GMS Curr Top Otorhinolaryngol Head Neck Surg. 13: Doc05, 2014.

Le Barz, M., Anhe, F.F., Varin, T.V., Desjardins, Y., Levy, E., Roy, D., Urdaci, M.C., Marette, A. Probiotics as Complementary Treatment for Metabolic Disorders. Diabetes Metab J. 39: 291-303, 2015.

Lebeer, S., Claes, I., Tytgat, H.L., Verhoeven, T.L., Marien, E., von Ossowski, I., Reunanen, J., Palva, A., Vos, W.M., Keersmaecker, S.C. ,Vanderleyden, J. Functional analysis of Lactobacillus rhamnosus GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. Appl Environ Microbiol. 78: 185-193, 2012.

Lehtoranta, L., Kalima, K., He, L., Lappalainen, M., Roivainen, M., Närkiö, M., Mäkelä, M., Siitonen, S., Korpela, R., Pitkäranta, A. Specific probiotics and virological findings in symptomatic conscripts attending military service in Finland. J Clin Virol. 60: 276-281, 2014.

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. 76: 206-211, 2012.

Leskinen, K., Hendolin, P., Virolainen-Julkunen, A., Ylikoski, J., Jero, J. The clinical role of Alloiococcus otitidis in otitis media with effusion. Int J Pediatr Otorhinolaryngol. 66: 41-48, 2002.

Lieberthal, A.S., Carroll, A.E., Chonmaitree, T., Ganiats, T.G., Hoberman, A., Jackson, M.A., Joffe, M.D., Miller, D.T., Rosenfeld, R.M., Sevilla, X.D., Schwartz, R.H., Thomas, P.A. ,Tunkel, D.E. The diagnosis and management of acute otitis media. Pediatrics 131: e964-99, 2013.

Ling, Z., Liu, X., Luo, Y., Yuan, L., Nelson, K.E., Wang, Y., Xiang, C. ,Li, L. Pyrosequencing analysis of the human microbiota of healthy Chinese undergraduates. BMC Genomics 14: 390-2164-14-390, 2013.

Liu, X., Cao, S., Zhang, X. Modulation of Gut Microbiota-Brain Axis by Probiotics, Prebiotics, and Diet. J Agric Food Chem. 63: 7885-7895, 2015.

Liu, C.M., Cosetti, M.K., Aziz, M., Buchhagen, J.L., Contente-Cuomo, T.L., Price, L.B., Keim, P.S., Lalwani, A.K. The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. Arch Otolaryngol Head Neck Surg. 137: 664-668, 2011.

Liu, Y.W., Fu, T.Y., Peng, W.S., Chen, Y.H., Cao, Y.M., Chen, C.C., Hung, W.L. ,Tsai, Y.C. Evaluation of the potential anti-allergic effects of heat-inactivated Lactobacillus paracasei V0151 in vitro, ex vivo, and in vivo. Benef Microbes 6: 697-705, 2015.

Liu, S., Hu, P., Du, X., Zhou, T., Pei, X. Lactobacillus rhamnosus GG supplementation for preventing respiratory infections in children: a meta-analysis of randomized, placebo-controlled trials. Indian Pediatr. 50: 377-381, 2013.

Liu, Y.W., Liao, T.W., Chen, Y.H., Chiang, Y.C. ,Tsai, Y.C. Oral administration of heat-inactivated Lactobacillus plantarum K37 modulated airway hyperresponsiveness in ovalbumin-sensitized BALB/c mice. PLoS One 9(6):e100105, 2014.

Lumio J., Rautakorpi U-M., Vuento R. Management of patient with acute infection | Infektiopotilaan tutkiminen ja hoito. Duodecim 112: 495-508, 1996.

Luoto R, Isolauri E, Lehtonen L. Safety of Lactobacillus GG probiotic in infants with very low birth weight: twelve years of experience. Clin Infect Dis 50:1327-8, 2010.

Luoto, R., Ruuskanen, O., Waris, M., Kalliomäki, M., Salminen, S., Isolauri, E. Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: a randomized, placebo-controlled trial. J Allergy Clin Immunol. 133: 405-413, 2014.

Macassey, E., Dawes, P. Biofilms and their role in otorhinolaryngological disease. J Laryngol Otol. 122: 1273-1278, 2008.

MacDonald, T.T., Monteleone, I., Fantini, M.C., Monteleone, G. Regulation of homeostasis and inflammation in the intestine. Gastroenterology 140: 1768-1775, 2011.

Macfarlane, S., Bahrami, B., Macfarlane, G.T. Mucosal biofilm communities in the human intestinal tract. Adv Appl Microbiol. 75: 111-143, 2011.

Mackay, A.D., Taylor, M.B., Kibbler, C.C., Hamilton-Miller, J.M. Lactobacillus endocarditis caused by a probiotic organism. Clin Microbiol Infect. 5: 290-292, 1999.

Maeda, N., Nakamura, R., Hirose, Y., Murosaki, S., Yamamoto, Y., Kase, T. ,Yoshikai, Y. Oral administration of heat-killed Lactobacillus plantarum L-137 enhances protection against influenza virus infection by stimulation of type I interferon production in mice. Int Immunopharmacol. 9: 1122-1125, 2009.

Makino, S., Ikegami, S., Kume, A., Horiuchi, H., Sasaki, H. ,Orii, N. Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with Lactobacillus delbrueckii ssp. bulgaricus OLL1073R-1. Br J Nutr. 104: 998-1006, 2010.

Mallia, P., Message, S.D., Gielen, V., Contoli, M., Gray, K., Kebadze, T., Aniscenko, J., Laza-Stanca, V., Edwards, M.R., Slater, L., Papi, A., Stanciu, L.A., Kon, O.M., Johnson, M. ,Johnston, S.L. Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation. Am J Respir Crit Care Med. 183: 734-742, 2011.

Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D., Hirschfield, G.M., Hold, G., Quraishi, M.N., Kinross, J., Smidt, H., Tuohy, K.M., Thomas, L.V., Zoetendal, E.G. ,Hart, A. The gut microbiota and host health: a new clinical frontier. Gut, in press.

Marom, T., Alvarez-Fernandez, P.E., Jennings, K., Patel, J.A., McCormick, D.P. ,Chonmaitree, T. Acute bacterial sinusitis complicating viral upper respiratory tract infection in young children. Pediatr Infect Dis J. 33: 803-808, 2014.

McDonald, S., Langton Hewer, C.D., Nunez, D.A. Grommets (ventilation tubes) for recurrent acute otitis media in children. Cochrane Database Syst Rev. 8;(4):CD004741, 2008.

Merenstein, D., Murphy, M., Fokar, A., Hernandez, R.K., Park, H., Nsouli, H., Sanders, M.E., Davis, B.A., Niborski, V., Tondu, F. ,Shara, N.M. Use of a fermented dairy probiotic drink containing Lactobacillus casei (DN-114 001) to decrease the rate of illness in kids: the DRINK study. A patient-oriented, double-blind, cluster-randomized, placebo-controlled, clinical trial. Eur J Clin Nutr. 64: 669-677, 2010.

Message, S.D., Laza-Stanca, V., Mallia, P., Parker, H.L., Zhu, J., Kebadze, T., Contoli, M., Sanderson, G., Kon, O.M., Papi, A., Jeffery, P.K., Stanciu, L.A. ,Johnston, S.L. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. Proc Natl Acad Sci U.S.A. 105: 13562-13567, 2008.

Miettinen, M., Pietilä, T.E., Kekkonen, R.A., Kankainen, M., Latvala, S., Pirhonen, J., Osterlund, P., Korpela, R., Julkunen, I. Nonpathogenic Lactobacillus rhamnosus activates the inflammasome and antiviral responses in human macrophages. Gut Microbes 3: 510-522, 2012.

Minovi, A., Dazert, S. Diseases of the middle ear in childhood. Laryngorhinootologie. 93 Suppl 1: S1-23, 2014.

Muderris, T., Yazici, A., Bercin, S., Yalciner, G., Sevil, E., Kiris, M. Consumer acoustic reflectometry: accuracy in diagnosis of otitis media with effusion in children. Int J Pediatr Otorhinolaryngol. 77: 1771-1774, 2013.

Munoz-Atienza, E., Araujo, C., Lluch, N., Hernandez, P.E., Herranz, C., Cintas, L.M. ,Magadan, S. Different impact of heat-inactivated and viable lactic acid bacteria of aquatic origin on turbot (Scophthalmus maximus L.) head-kidney leucocytes. Fish Shellfish Immunol. 44: 214-223, 2015.

Mäkelä, M.J., Puhakka, T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimäki, M., Blomqvist, S., Hyypiä, T., Arstila, P. Viruses and bacteria in the etiology of the common cold. J Clin Microbiol. 36: 539-542, 1998.

Naclerio, R.M., Proud, D., Lichenstein, L.M., Kagey-Sobotka, A., Hendley, J.O., Sorrentino, J., Gwaltney, J.M. Kinins are generated during experimental rhinovirus colds. J Infect Dis. 157: 133-142, 1988.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148: 257-266, 2002.

Nishiyama, K., Nakamata, K., Ueno, S., Terao, A., Aryantini, N.P., Sujaya, I.N., Fukuda, K., Urashima, T., Yamamoto, Y., Mukai, T. Adhesion properties of Lactobacillus rhamnosus mucus-binding factor to mucin and extracellular matrix proteins. Biosci Biotechnol Biochem. 79: 271-279, 2015.

Nokso-Koivisto, J., Marom, T., Chonmaitree, T. Importance of viruses in acute otitis media. Curr Opin Pediatr. 27: 110-115, 2015.

Nokso-Koivisto, J., Räty, R., Blomqvist, S., Kleemola, M., Syrjänen, R., Pitkäranta, A., Kilpi, T., Hovi, T. Presence of specific viruses in the middle ear fluids and respiratory secretions of young children with acute otitis media. J Med Virol. 72: 241-248, 2004.

Nokso-Koivisto, J., Hovi, T., Pitkäranta, A. Viral upper respiratory tract infections in young children with emphasis on acute otitis media. Int J Pediatr Otorhinolaryngol. 70: 1333-1342, 2006.

Norhayati, M.N., Ho, J.J., Azman, M.Y. Influenza vaccines for preventing acute otitis media in infants and children. Cochrane Database Syst Rev. 3: CD010089, 2015.

Ohland, C.L., MacNaughton, W.K. Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol. 298: G807-19, 2010.

Ojeda, P., Bobe, A., Dolan, K., Leone, V., Martinez, K. Nutritional modulation of gut microbiota - the impact on metabolic disease pathophysiology. J Nutr Biochem. in press.

Oksaharju, A., Kankainen, M., Kekkonen, R.A., Lindstedt, K.A., Kovanen, P.T., Korpela, R., Miettinen, M. Probiotic Lactobacillus rhamnosus downregulates FCER1 and HRH4 expression in human mast cells. World J Gastroenterol. 17: 750-759, 2011.

Passioti, M., Maggina, P., Megremis, S., Papadopoulos, N.G. The common cold: potential for future prevention or cure. Curr Allergy Asthma Rep. 14: 413, 2014.

Pavia, M., Bianco, A., Nobile, C.G., Marinelli, P., Angelillo, I.F. Efficacy of pneumococcal vaccination in children younger than 24 months: a meta-analysis. Pediatrics 123: e1103-10, 2009.

Peltola, V., Waris, M., Kainulainen, L., Kero, J., Ruuskanen, O. Virus shedding after human rhinovirus infection in children, adults and patients with hypogammaglobulinaemia. Clin Microbiol Infect. 19: E322-7, 2013.

Pelton, S.I., Pettigrew, M.M., Barenkamp, S.J., Godfroid, F., Grijalva, C.G., Leach, A., Patel, J., Murphy, T.F., Selak, S., Bakaletz, L.O. Panel 6: Vaccines. Otolaryngol Head Neck Surg. 148: E90-101, 2013.

Pettigrew, M.M., Gent, J.F., Pyles, R.B., Miller, A.L., Nokso-Koivisto, J., Chonmaitree, T. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. J Clin Microbiol. 49: 3750-3755, 2011.

Pettigrew, M.M., Laufer, A.S., Gent, J.F., Kong, Y., Fennie, K.P. ,Metlay, J.P. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. Appl Environ Microbiol. 78: 6262-6270, 2012.

Pichichero, M.E., Casey, J.R., Hoberman, A. ,Schwartz, R. Pathogens causing recurrent and difficult-to-treat acute otitis media, 2003-2006. Clin Pediatr. 47: 901-906, 2008.

Pohjavuori, E., Viljanen, M., Korpela, R., Kuitunen, M., Tiittanen, M., Vaarala, O., Savilahti, E. Lactobacillus GG effect in increasing IFN-γ production in infants with cow's milk allergy. J Allergy Clin Immunol. 114: 131-136, 2004.

Popova, M., Molimard, P., Courau, S., Crociani, J., Dufour, C., Le Vacon, F., Carton, T. Beneficial effects of probiotics in upper respiratory tract infections and their mechanical actions to antagonize pathogens. J Appl Microbiol. 113: 1305-1318, 2012.

Post, J.C., Hiller, N.L., Nistico, L., Stoodley, P., Ehrlich, G.D. The role of biofilms in otolaryngologic infections: update 2007. Curr Opin Otolaryngol Head Neck Surg. 15: 347-351, 2007.

Post, J.C., Stoodley, P., Hall-Stoodley, L., Ehrlich, G.D. The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg. 12: 185-190, 2004.

Power, D.A., Burton, J.P., Chilcott, C.N., Dawes, P.J. ,Tagg, J.R. Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral probiotic Streptococcus salivarius K12. Eur J Clin Microbiol Infect Dis. 27: 1261-1263, 2008.

Princivalli, M.S., Paoletti, C., Magi, G., Palmieri, C., Ferrante, L. ,Facinelli, B. Lactobacillus rhamnosus GG inhibits invasion of cultured human respiratory cells by prtF1-positive macrolide-resistant group A streptococci. Lett Appl Microbiol. 48: 368-372, 2009.

Qureishi, A., Lee, Y., Belfield, K., Birchall, J.P., Daniel, M. Update on otitis media - prevention and treatment. Infect Drug Resist 7: 15-24, 2014.

Ramakrishna, B.S. Probiotic-induced changes in the intestinal epithelium: implications in gastrointestinal disease. Trop Gastroenterol. 30: 76-85, 2009.

Rautava, S., Salminen, S., Isolauri, E. Specific probiotics in reducing the risk of acute infections in infancy--a randomised, double-blind, placebo-controlled study. Br J Nutr. 101: 1722-1726, 2009.

Rayner, M.G., Zhang, Y., Gorry, M.C., Chen, Y., Post, J.C. ,Ehrlich, G.D. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. JAMA 279: 296-299, 1998.

Redman, M.G., Ward, E.J., Phillips, R.S. The efficacy and safety of probiotics in people with cancer: a systematic review. Ann Oncol. 25: 1919-1929, 2014.

Renko, M., Kontiokari, T., Jounio-Ervasti, K., Rantala, H. ,Uhari, M. Disappearance of middle ear effusion in acute otitis media monitored daily with tympanometry. Acta Paediatr. 95: 359-363, 2006.

Rerksuppaphol, S., Rerksuppaphol, L. Randomized controlled trial of probiotics to reduce common cold in schoolchildren. Pediatr Int. 54: 682-687, 2012.

Rezes, S., Söderlund-Venermo, M., Roivainen, M., Kemppainen, K., Szabo, Z., Sziklai, I., Pitkäranta, A. Human bocavirus and rhino-enteroviruses in childhood otitis media with effusion. J Clin Virol. 46: 234-237, 2009.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., Palva, A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 97: 1166-1177, 2004.

Rio, M.E., Zago Beatriz, L., Garcia, H., Winter, L. The nutritional status change the effectiveness of a dietary supplement of lactic bacteria on the emerging of respiratory tract diseases in children. Arch Latinoam Nutr. 52: 29-34, 2002.

Ritchie, M.L., Romanuk, T.N. A meta-analysis of probiotic efficacy for gastrointestinal diseases. PLoS One 7: e34938, 2012.

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O.E., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liebana, E., Lopez-Cerero, L., MacGowan, A., Martins, M., Rodriguez-Bano, J., Rolain, J.M., Segovia, C., Sigauque, B., Taconelli, E., Wellington, E. ,Vila, J. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect. 6: 22-29, 2015.

Rogers, D.J., Boseley, M.E., Adams, M.T., Makowski, R.L. ,Hohman, M.H. Prospective comparison of handheld pneumatic otoscopy, binocular microscopy, and tympanometry in identifying middle ear effusions in children. Int J Pediatr Otorhinolaryngol. 74: 1140-1143, 2010.

Rosenfeld, R.M., Culpepper, L., Doyle, K.J., Grundfast, K.M., Hoberman, A., Kenna, M.A., Lieberthal, A.S., Mahoney, M., Wahl, R.A., Woods, C.R.,Jr, Yawn, B., American Academy of Pediatrics Subcommittee on Otitis Media with Effusion, American Academy of Family Physicians ,American Academy of Otolaryngology--Head and Neck Surgery Clinical practice guideline: Otitis media with effusion. Otolaryngol Head Neck Surg. 130: S95-118, 2004.

Ruohola, A., Pettigrew, M.M., Lindholm, L., Jalava, J., Räisänen, K.S., Vainionpää, R., Waris, M., Tähtinen, P.A., Laine, M.K., Lahti, E., Ruuskanen, O., Huovinen, P. Bacterial and viral interactions within the nasopharynx contribute to the risk of acute otitis media. J Infect. 66: 247-254, 2013.

Ruohola, A., Waris, M., Allander, T., Ziegler, T., Heikkinen, T., Ruuskanen, O. Viral etiology of common cold in children, Finland. Emerg Infect Dis. 15: 344-346, 2009.

Ryan, P.M., Ross, R.P., Fitzgerald, G.F., Caplice, N.M., Stanton, C. Functional food addressing heart health: do we have to target the gut microbiota? Curr Opin Clin Nutr Metab Care 18:566-71, 2015.

Sadé, J. The nasopharynx, eustachian tube and otitis media. J Laryngol Otol. 108: 95-100, 1994.

Salminen S, Nybom S, Meriluoto J, Collado MC, Vesterlund S, El-Nezami H. Interaction of probiotics and pathogens--benefits to human health? Curr Opin Biotechnol. 21:157-67, 2010.

Salminen, M.K., Rautelin, H., Tynkkynen, S., Poussa, T., Saxelin, M., Valtonen, V., Järvinen, A. Lactobacillus bacteremia, clinical significance, and patient outcome, with special focus on probiotic L. rhamnosus GG. Clin Infect Dis. 38: 62-69, 2004.

Salminen, M.K., Tynkkynen, S., Rautelin, H., Saxelin, M., Vaara, M., Ruutu, P., Sarna, S., Valtonen, V., Järvinen, A. Lactobacillus bacteremia during a rapid increase in probiotic use of Lactobacillus rhamnosus GG in Finland. Clin Infect Dis. 35: 1155-1160, 2002.

Salminen, S., Nybom, S., Meriluoto, J., Collado, M.C., Vesterlund, S., El-Nezami, H. Interaction of probiotics and pathogens—benefits to human health? Curr Opin Biotechnol. 21: 157-167, 2010.

Sanders, M.E., Akkermans, L.M., Haller, D., Hammerman, C., Heimbach, J., Hormannsperger, G., Huys, G., Levy, D.D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G. ,Vaughan, E. Safety assessment of probiotics for human use. Gut Microbes 1: 164-185, 2010.

Sandhya, P., Danda, D., Sharma, D., Scaria, V. Does the buck stop with the bugs?: an overview of microbial dysbiosis in rheumatoid arthritis. Int J Rheum Dis. in press.

Santagati, M., Scillato, M., Muscaridola, N., Metoldo, V., La Mantia, I., Stefani, S. Colonization, safety, and tolerability study of the Streptococcus salivarius 24SMBc nasal spray for its application in upper respiratory tract infections. Eur J Clin Microbiol Infect Dis. 2015.

Sassone-Corsi, M., Raffatellu, M. No vacancy: how beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. J Immunol. 194: 4081-4087, 2015.

Savolainen-Kopra, C., Haapakoski, J., Peltola, P.A., Ziegler, T., Korpela, T., Anttila, P., Amiryousefi, A., Huovinen, P., Huvinen, M., Noronen, H., Riikkala, P., Roivainen, M., Ruutu, P., Teirilä, J., Vartiainen, E. ,Hovi, T. Hand washing with soap and water together with behavioural recommendations prevents infections in common work environment: an open cluster-randomized trial. Trials 13: 10-6215-13-10, 2012.

Saxelin, M., Chuang, N.H., Chassy, B., Rautelin, H., Makela, P.H., Salminen, S., Gorbach, S.L. Lactobacilli and bacteremia in southern Finland, 1989-1992. Clin Infect Dis. 22: 564-566, 1996.

Saylam, G., Tatar, E.C., Tatar, I., Ozdek, A., Korkmaz, H. Association of adenoid surface biofilm formation and chronic otitis media with effusion. Arch Otolaryngol Head Neck Surg. 136: 550-555, 2010.

Schwendicke, F., Dorfer, C., Kneist, S., Meyer-Lueckel, H., Paris, S. Cariogenic effects of probiotic Lactobacillus rhamnosus GG in a dental biofilm model. Caries Res. 48: 186-192, 2014.

Shida, K., Sato, T., Iizuka, R., Hoshi, R., Watanabe, O., Igarashi, T., Miyazaki, K., Nanno, M. ,Ishikawa, F. Daily intake of fermented milk with Lactobacillus casei strain Shirota reduces the incidence and duration of upper respiratory tract infections in healthy middle-aged office workers. Eur J Nutr. in press.

Shoji, H., Yoshida, K. ,Niki, Y. Lung abscess and pleuritis caused by Lactobacillus rhamnosus in an immunocompetent patient. J Infect Chemother. 16: 45-48, 2010.

Siró, I., Kápolna, E., Kápolna, B., Lugasi, A. Functional food. Product development, marketing and consumer acceptance—A review. Appetite 51: 456-467, 2008.

Skevaki, C.L., Tsialta, P., Trochoutsou, A.I., Logotheti, I., Makrinioti, H., Taka, S., Lebessi, E., Paraskakis, I., Papadopoulos, N.G., Tsolia, M.N. Associations between Viral and Bacterial Potential Pathogens in the Nasopharynx of Children with and Without Respiratory Symptoms. Pediatr Infect Dis J. in press.

Skovbjerg, S., Roos, K., Holm, S.E., Grahn Hakansson, E., Nowrouzian, F., Ivarsson, M., Adlerberth, I., Wold, A.E. Spray bacteriotherapy decreases middle ear fluid in children with secretory otitis media. Arch Dis Child. 94: 92-98, 2009.

Smith, T.J., Rigassio-Radler, D., Denmark, R., Haley, T. ,Touger-Decker, R. Effect of Lactobacillus rhamnosus LGG(R) and Bifidobacterium animalis ssp. lactis BB-12(R) on health-related quality of life in college students affected by upper respiratory infections. Br J Nutr. 109: 1999-2007, 2013.

Stearns, J.C., Davidson, C.J., McKeon, S., Whelan, F.J., Fontes, M.E., Schryvers, A.B., Bowdish, D.M., Kellner, J.D. ,Surette, M.G. Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age. ISME J. 9: 1246-1259, 2015.

Stjernquist-Desatnik, A., Warfving, H., Johansson, M.L. Persistence of Lactobacillus plantarum DSM 9843 on human tonsillar surface after oral administration in fermented oatmeal gruel. A pilot study. Acta Otolaryngol Suppl. 543: 215-219, 2000.

Stol, K., Verhaegh, S.J., Graamans, K., Engel, J.A., Sturm, P.D., Melchers, W.J., Meis, J.F., Warris, A., Hays, J.P. ,Hermans, P.W. Microbial profiling does not differentiate between childhood recurrent acute otitis media and chronic otitis media with effusion. Int J Pediatr Otorhinolaryngol. 77: 488-493, 2013.

Szajewska, H., Guarino, A., Hojsak, I., Indrio, F., Kolacek, S., Shamir, R., Vandenplas, Y., Weizman, Z. ,European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Use of probiotics for management of acute gastroenteritis: a position paper by the ESPGHAN Working Group for Probiotics and Prebiotics. J Pediatr Gastroenterol Nutr. 58: 531-539, 2014.Szajewska, H. , Kolodziej, M. Systematic review with meta-analysis: Lactobacillus rhamnosus GG in the prevention of antibiotic-associated diarrhoea in children and adults. Aliment Pharmacol Ther. 42: 1149-1157, 2015.

Takeyama, A., Hashimoto, K., Sato, M., Sato, T., Kanno, S., Takano, K., Ito, M., Katayose, M., Nishimura, H., Kawasaki, Y., Hosoya, M. Rhinovirus load and disease severity in children with lower respiratory tract infections. J Med Virol. 84: 1135-1142, 2012.

Tapiainen, T., Luotonen, L., Kontiokari, T., Renko, M. ,Uhari, M. Xylitol administered only during respiratory infections failed to prevent acute otitis media. Pediatrics 109: E19, 2002.

Teo, S.M., Mok, D., Pham, K., Kusel, M., Serralha, M., Troy, N., Holt, B.J., Hales, B.J., Walker, M.L., Hollams, E., Bochkov, Y.A., Grindle, K., Johnston, S.L., Gern, J.E., Sly, P.D., Holt, P.G., Holt, K.E. ,Inouye, M. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. Cell Host Microbe 17: 704-715, 2015.

Tomonaga, K., Kurono, Y., Chaen, T., Mogi, G. Adenoids and otitis media with effusion: nasopharyngeal flora. Am J Otolaryngol. 10: 204-207, 1989.

Tong, H.H., Weiser, J.N., James, M.A. ,DeMaria, T.F. Effect of influenza A virus infection on nasopharyngeal colonization and otitis media induced by transparent or opaque phenotype variants of Streptococcus pneumoniae in the chinchilla model. Infect Immun. 69: 602-606, 2001.

Tos, M. Epidemiology and natural history of secretory otitis. Am J Otol. 5: 459-462, 1984.

Tripathi, P., Beaussart, A., Alsteens, D., Dupres, V., Claes, I., von Ossowski, I., de Vos, W.M., Palva, A., Lebeer, S., Vanderleyden, J. ,Dufrene, Y.F. Adhesion and nanomechanics of pili from the probiotic Lactobacillus rhamnosus GG. ACS Nano 7: 3685-3697, 2013.

Turner, R.B., Wecker, M.T., Pohl, G., Witek, T.J., McNally, E., St George, R., Winther, B. ,Hayden, F.G. Efficacy of tremacamra, a soluble intercellular adhesion molecule 1, for experimental rhinovirus infection: a randomized clinical trial. JAMA 281: 1797-1804, 1999.

Ueyama, T., Kurono, Y., Shirabe, K., Takeshita, M., Mogi, G. High incidence of Haemophilus influenzae in nasopharyngeal secretions and middle ear effusions as detected by PCR. J Clin Microbiol. 33: 1835-1838, 1995.

Uhari, M., Möttönen, M. An open randomized controlled trial of infection prevention in child day-care centers. Pediatr Infect Dis J. 18: 672-677, 1999.

Uhari, M., Tapiainen, T. ,Kontiokari, T. Xylitol in preventing acute otitis media. Vaccine 19 Suppl 1: S144-7, 2000.

van den Nieuwboer, M., Brummer, R.J., Guarner, F., Morelli, L., Cabana, M., Claasen, E. The administration of probiotics and synbiotics in immune compromised adults: is it safe? Benef Microbes 6: 3-17, 2015a.

van den Nieuwboer, M., Brummer, R.J., Guarner, F., Morelli, L., Cabana, M., Claassen, E. Safety of probiotics and synbiotics in children under 18 years of age. Benef Microbes, 6:615-630, 2015b.

van den Nieuwboer, M., Claassen, E., Morelli, L., Guarner, F. ,Brummer, R.J. Probiotic and synbiotic safety in infants under two years of age. Benef Microbes 5: 45-60, 2014.

van Elden, L.J., Sachs, A.P., van Loon, A.M., Haarman, M., van de Vijver, D.A., Kimman, T.G., Zuithoff, P., Schipper, P.J., Verheij, T.J., Nijhuis, M. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. J Clin Virol. 41: 116-121, 2008.

van Kempen, M., Bachert, C., Van Cauwenberge, P. An update on the pathophysiology of rhinovirus upper respiratory tract infections. Rhinology 37: 97-103, 1999.

van Zon, A., van der Heijden, G.J., van Dongen, T.M., Burton, M.J. ,Schilder, A.G. Antibiotics for otitis media with effusion in children. Cochrane Database Syst Rev. 9: CD009163, 2012.

Vargas Garcia, C.E., Petrova, M., Claes, I.J., De Boeck, I., Verhoeven, T.L., Dilissen, E., von Ossowski, I., Palva, A., Bullens, D.M., Vanderleyden, J., Lebeer, S. Piliation of Lactobacillus rhamnosus GG promotes adhesion, phagocytosis, and cytokine modulation in macrophages. Appl Environ Microbiol. 81: 2050-2062, 2015.

Vernocchi, P., Del Chierico, F., Fiocchi, A.G., El Hachem, M., Dallapiccola, B., Rossi, P., Putignani, L. Understanding probiotics' role in allergic children: the clue of gut microbiota profiling. Curr Opin Allergy Clin Immunol. 15: 495-503, 2015.

Viljanen, M., Pohjavuori, E., Haahtela, T., Korpela, R., Kuitunen, M., Sarnesto, A., Vaarala, O., Savilahti, E. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema–dermatitis syndrome. J Allergy Clin Immunol. 115: 1254-1259, 2005.

Vitetta, L., Briskey, D., Alford, H., Hall, S., Coulson, S. Probiotics, prebiotics and the gastrointestinal tract in health and disease. Inflammopharmacology 22: 135-154, 2014.

von Ossowski, I., Reunanen, J., Satokari, R., Vesterlund, S., Kankainen, M., Huhtinen, H., Tynkkynen, S., Salminen, S., de Vos, W.M., Palva, A. Mucosal adhesion properties of the probiotic Lactobacillus rhamnosus GG SpaCBA and SpaFED pilin subunits. Appl Environ Microbiol. 76: 2049-2057, 2010.

von Ossowski, I., Satokari, R., Reunanen, J., Lebeer, S., De Keersmaecker, S.C., Vanderleyden, J., de Vos, W.M., Palva, A. Functional characterization of a mucus-specific LPXTG surface adhesin from probiotic Lactobacillus rhamnosus GG. Appl Environ Microbiol. 77: 4465-4472, 2011.

Vuotto, C., Longo, F., Donelli, G. Probiotics to counteract biofilm-associated infections: promising and conflicting data. Int J Oral Sci. 6: 189-194, 2014.

Wald, E.R., Guerra, N., Byers, C. Upper respiratory tract infections in young children: duration of and frequency of complications. Pediatrics 87: 129-133, 1991.

Wan, L.Y., Chen, Z.J., Shah, N.P., El-Nezami, H. Modulation of Intestinal Epithelial Defense Responses by Probiotic Bacteria. Crit Rev Food Sci Nutr. 0, 2015.

Wassenaar, T.M., Klein, G. Safety aspects and implications of regulation of probiotic bacteria in food and food supplements. J Food Prot. 71: 1734-1741, 2008.

Wen, K., Liu, F., Li, G., Bai, M., Kocher, J., Yang, X., Wang, H., Clark-Deener, S. ,Yuan, L. Lactobacillus rhamnosus GG Dosage Affects the Adjuvanticity and Protection Against Rotavirus Diarrhea in Gnotobiotic Pigs. J Pediatr Gastroenterol Nutr. 60: 834-843, 2015.

Whelan, K., Myers, C.E. Safety of probiotics in patients receiving nutritional support: a systematic review of case reports, randomized controlled trials, and nonrandomized trials. Am J Clin Nutr. 91: 687-703, 2010.

WHO/FAO 2001, *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina, 1-4 October 2001* .

Williamson, I. Otitis media with effusion in children. BMJ Clin Evid 2011: 0502, 2011.

Winther, B., Arruda, E., Witek, T.J., Marlin, S.D., Tsianco, M.M., Innes, D.J., Hayden, F.G. Expression of ICAM-1 in nasal epithelium and levels of soluble ICAM-1 in nasal lavage fluid during human experimental rhinovirus infection. Arch Otolaryngol Head Neck Surg. 128: 131-136, 2002.

Winther, B., Buchert, D., Turner, R.B., Hendley, J.O., Tschaikin, M. Decreased rhinovirus shedding after intranasal oxymetazoline application in adults with induced colds compared with intranasal saline. Am J Rhinol Allergy 24: 374-377, 2010.

Winther, B. Rhinovirus infections in the upper airway. Proc Am Thorac Soc. 8: 79-89, 2011.

Wong, S.S., Quan Toh, Z., Dunne, E.M., Mulholland, E.K., Tang, M.L., Robins-Browne, R.M., Licciardi, P.V. ,Satzke, C. Inhibition of Streptococcus pneumoniae adherence to human epithelial cells in vitro by the probiotic Lactobacillus rhamnosus GG. BMC Res Notes 6: 135-0500-6-135, 2013.

Zakaria Gomaa, E. Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability. J Gen Appl Microbiol. 59: 425-436, 2013.

Zhao, A.S., Boyle, S., Butrymowicz, A., Engle, R.D., Roberts, J.M., Mouzakes, J. Impact of 13-valent pneumococcal conjugate vaccine on otitis media bacteriology. Int J Pediatr Otorhinolaryngol. 78: 499-503, 2014.

Österback, R., Tevaluoto, T., Ylinen, T., Peltola, V., Susi, P., Hyypiä, T.,Waris, M. Simultaneous detection and differentiation of human rhino- and enteroviruses in clinical specimens by real-time PCR with locked nucleic Acid probes. J Clin Microbiol. 51: 3960-3967, 2013.