LACTOBACILLUS RHAMNOSUS GG IN RESPIRATORY TRACT INFECTIONS: Randomized controlled trials

Minna Kumpu

ACADEMIC DISSERTATION

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Helsinki 2016
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ABSTRACT

Respiratory tract infections account for a significant part of total illness episodes, physician consultations and days off from day care, school or work, thus incurring a significant personal and socio-economic burden. Viruses cause most of the acute infections of the respiratory tract, and there is a lack of effective preventive and treatment options available against almost all of the over 200 different viral pathogens. Nutritional interventions are an increasingly researched option for reducing the infection burden, and of these probiotics are among the ones shown most promise. The aim of this work, consisting of three double-blind randomized controlled clinical trials in children and adults, was to investigate whether probiotic *Lactobacillus rhamnosus* GG would reduce the symptoms of respiratory tract infections and nasopharyngeal presence of respiratory viruses, as well as to explore factors hypothesized to have an impact on probiotic efficacy: the pharyngeal colonization of *L. rhamnosus* GG and the viability of the probiotic strain.

In young adult tonsillectomy patients, *L. rhamnosus* GG was analysed from palatine tonsil samples and results compared to the faecal recovery of GG after three weeks’ daily consumption of placebo or *L. rhamnosus* GG as a single strain or as a part of a multispecies combination. *L. rhamnosus* GG was recovered in the tonsil tissue of 40% of the subjects in the GG group, 41% in the multispecies group and 30% in the placebo group. The results suggest that individual variation exists in the ability of the probiotic to adhere to the tonsil tissue, as the compliance of study product consumption was confirmed by the analysis of the faecal recovery of the strain and counting of leftover study products. Most of the subjects in the control group with *L. rhamnosus* GG harboured from the tonsil tissue had the strain recovered from the faecal sample already at the start of the intervention, indicating that persistent colonization could be a factor behind positive tonsillar recoveries in the control group.

In a 28-week prospective trial on children aged 2–6 years attending day care in Finland, milk supplemented with *L. rhamnosus* GG was not able to reduce respiratory tract infections compared to the control group. Analysis on the completed cases subgroup, excluding subjects with intestinal *L. rhamnosus* GG colonization originating from outside the trial, suggested that children in the probiotic group had one day less per month with respiratory symptoms compared to the control group, but this exploratory finding warrants further research for confirmation. In a subgroup of children who visited the study physician due to an infection during the trial, the presence of 14 respiratory viruses was analysed from nasopharyngeal swab samples. *L. rhamnosus* GG did not reduce the occurrence of respiratory viruses, or the number of
infection symptoms observed at the time of the viral findings. In this subgroup of more symptomatic children, subjects in the probiotic group had fewer days with respiratory symptoms than children in the control group, but number of study physician visits was not different between the groups, thus suggesting that *L. rhamnosus* GG might be able to reduce only the symptoms of the less severe infections treated at home in the more symptomatic subjects.

The potential of the experimental rhinovirus challenge model in studying probiotic efficacy in viral infections was tested in a 6-week trial on live and inactivated *L. rhamnosus* GG in adults. In this pilot study, the subjects were intranasally inoculated with experimental rhinovirus mid-intervention. Occurrence and severity of cold symptoms and number of subjects with positive rhinovirus culture and rhinovirus infection were the lowest in the group receiving live *L. rhamnosus* GG, but differences were not statistically significant.

Taken together, *L. rhamnosus* GG was overall not effective in reducing the symptoms of respiratory tract infections, or occurrence of respiratory viruses in the nasopharynx of symptomatic children, but appeared to reduce symptoms in specific subgroups within the study cohort. It was demonstrated that *L. rhamnosus* GG can be recovered from the tonsil tissue of some but not all subjects after oral consumption, thus warranting future research on the role of the probiotic recovery in infection outcomes. The experimental rhinovirus model was demonstrated a potential controlled approach to studying the efficacy of probiotics, and the pilot study on the model indicated that live *L. rhamnosus* GG was more promising than the inactivated strain in reducing respiratory infections, but further research is needed to confirm the preliminary findings.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


In the text, the publications are referred to by their Roman numerals. The original articles are reprinted with the kind permission of their copyright holders. Publication III was included earlier in the PhD thesis of Liisa Lehtoranta (Lehtoranta 2012).
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AOM</td>
<td>Acute otitis media</td>
</tr>
<tr>
<td>AAD</td>
<td>Antibiotic-associated diarrhoea</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CoV</td>
<td>Coronavirus</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EV</td>
<td>Enterovirus</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>GG</td>
<td><em>Lactobacillus rhamnosus</em> GG</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HBoV</td>
<td>Human bocavirus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFV</td>
<td>Influenza virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>IRR</td>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>LRI</td>
<td>Lower respiratory tract infection</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MPV</td>
<td>Metapneumovirus</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPS</td>
<td>Nasopharyngeal swab</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza virus</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>RTI</td>
<td>Respiratory tract infection</td>
</tr>
<tr>
<td>RV</td>
<td>Rhinovirus</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>subsp.</td>
<td>Subspecies</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>URI</td>
<td>Upper respiratory tract infection</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1 INTRODUCTION

Acute infections of the respiratory tract place an enormous social and economic burden on families and societies. This is due to their frequency as well as mostly viral origin, meaning that there is lack of effective treatment options available. Of healthy population, children are most susceptible to respiratory tract infections (RTIs): they have two to five times as many episodes compared to adults per year. Viruses causing RTIs spread from person to person by direct contact with contagious secretions or by air in small particles. The symptoms start a few days after the infection. Common symptoms include runny and stuffy nose, sore throat, cough and fever. The severity and type of symptoms vary among different infective agents, individuals and population groups, for example fever being common in children but rare and mild in adults. In children, day care attendance is a well-identified risk factor (Grüber et al. 2008), whereas is adults stress (Stover 2010), smoking (Bilello 2005) and lack of sleep (Prather et al. 2015) can have an impact on infection occurrence.

Majority of respiratory tract infections are mild and affect the upper respiratory tract, whereas infections in the lower respiratory tract are less frequent but often more severe. Viruses are the most common aetiological agents in respiratory tract infections. Even though most cases are mild and self-limiting, they can also have serious consequences if the viral infection spreads outside the upper respiratory tract, or due to viral infection paving the way for other pathogens to enter the body, causing more severe illnesses (Heikkinen and Järvinen 2003). There are simple and effective preventive behavioural measures available, such as hand washing, to hinder the pathogens from spreading. For the viral infections, antiviral drugs have been successfully developed only against a few viruses. Unfortunately, many viral RTIs are still treated with antibiotics, even though positive progress has been made in recent years towards reducing this especially in paediatric populations (Donnelly et al. 2014; Lee et al. 2014). Antibiotic resistance, in which excessive and unnecessary use of antibiotics is a major causative factor, presents one of the biggest medical threats of the near future (Roca et al. 2015).

As all individuals are susceptible to respiratory tract infections, nutritional strategies, that would have a low risk of adverse events and an easy incorporation into daily life, are appealing approaches to reducing the infection burden. Due to the frequency of the infections, even slight reduction in occurrence or amelioration of symptoms would be relevant on the population level, if adverse effects of the approach would be minimal. Probiotics have in recent years been evaluated as prominent nutritional
strategy to reduce respiratory tract infections (Hao et al. 2015; King et al. 2014). However, results are still partially conflicting, it is unclear which probiotic strain is the most effective, and also mechanisms of probiotics in viral respiratory infections are not fully understood.

The aim of this work was to clarify whether respiratory tract infections could be reduced in otherwise healthy children and adults by regular consumption of a widely researched probiotic strain, *Lactobacillus rhamnosus* GG (ATCC 53103). Furthermore, the work aimed to gain understanding about mechanistic aspects, specifically on the probiotic colonization in the pharynx, on probiotics effect on nasopharyngeal viral findings during an infection, and on the role of the probiotic viability.
2 REVIEW OF THE LITERATURE

2.1 RESPIRATORY TRACT INFECTIONS

2.1.1 DEFINITIONS AND SYMPTOM PROFILES

The common cold represents by far the majority of acute respiratory tract infection (RTI) cases. This generally mild illness is often linked to and shares similar symptoms with the other prevalent RTIs affecting the upper respiratory tract (maxillary sinusitis, acute otitis media, pharyngitis and laryngitis) and partially also with those affecting the lower respiratory tract (Table 1). The infections can be caused by multitude of viruses and also bacteria, and the exact aetiology can have an impact on the clinical manifestation of the disease (Heikkinen and Järvinen 2003). Usually most cases pass in two weeks, lower respiratory tract symptoms tending to last somewhat longer (Thompson et al. 2013). In the northern hemisphere, common seasonal pattern for most RTIs is to increase during the autumn towards peak incidence in the winter, and then decrease again in the spring.

Table 1. Typical symptom profiles of common respiratory tract infections (Korppi and Järvinen 2011; Ruuskanen and Heikkinen 2011).

<table>
<thead>
<tr>
<th></th>
<th>Fever</th>
<th>Runny nose</th>
<th>Stuffy nose</th>
<th>Sore throat</th>
<th>Cough</th>
<th>Wheezing</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper RTIs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common cold</td>
<td>x*</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinusitis^</td>
<td>x*</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOM^</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
<td>Ear ache, lowered hearing</td>
<td></td>
</tr>
<tr>
<td>Pharyngitis</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laryngitis^</td>
<td>x*</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
<td>Voice disturbance, difficulty breathing in</td>
</tr>
<tr>
<td><strong>Lower RTIs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchitis</td>
<td>x*</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

Abbreviations: RTI, respiratory tract infection; AOM, acute otitis media

* More commonly in children

^ Occurs most commonly with the common cold; common upper RTI symptoms are likely to be present

The common cold

The common cold is the term most often used by lay people when referring to a disease consisting of symptoms such as nasal discharge and stuffiness, sneezing, cough and sore throat (Heikkinen and Järvinen 2003). This term is widely used also in the medical literature, with acute nasopharyngitis and
rhinitis as the corresponding terms in the International Classification of Diseases (WHO 2016). The common cold is defined as an acute or mild infection of the upper respiratory tract, commonly self-limiting and most likely with viral origin (Allan and Arroll 2014; Heikkinen and Järvinen 2003). Most common initial and rapidly passing symptom is sore throat, usually followed by runny and stuffy nose (peaking at 2-3 days from the start of the symptoms) and accompanied by cough that is typically at its worst on the fourth symptomatic day (Gwaltney et al. 2003). Rhinorrhoea usually lasts throughout the clinical manifestation of the disease, nasal secretions starting off as watery and turning thicker towards the end (Igarashi et al. 1993). Fever lasting 2-5 days is a common symptom of the common cold in children (Putto et al. 1986). Usually most symptoms clear off by the end of the first week of the illness. A systematic review recently concluded that by day 15 the common cold was resolved in 90% of the cases (Thompson et al. 2013).

**Acute sinusitis**

Sinusitis is an infection in one or more of the sinuses, which develops in approximately 5-10% of viral URIs (DeMuri and Wald 2013). Due to the condition most commonly occurring with viral rhinitis, it is also referred to as rhinosinusitis. In sinusitis, the membrane-lined air spaces around the nose, sinuses, become infected. Most often this is due to the common cold virus spreading to the sinuses and causing mucosal oedema. Sinusitis can also be a bacterial complication, but differentiation between viral and bacterial aetiology is not possible based on symptoms (Ah-See and Evans 2007). Of the sinuses connected to the nasal passages (maxillary, frontal, ethmoidal and sphenoidal), maxillary sinuses are often affected by an infection. There are no reliable clinical criteria available to differentiate acute sinusitis from the common cold (Allan and Arroll 2014). Bacterial sinusitis can be suspected if symptoms of common cold show no sign of decreasing at 10 days since onset (DeMuri and Wald 2013). Typical symptoms of acute sinusitis are runny and stuffy nose, reduced sense of smell, facial pain and facial pressure, sometimes accompanied with symptoms such as toothache in the upper teeth, malaise or pain on stooping (Ah-See and Evans 2007). Fever is often associated with sinusitis in children, but not commonly in adults (Eccles 2011). Sinusitis is considered acute when symptoms last for less than four weeks (Ah-See and Evans 2007).

**Acute otitis media**

Acute otitis media (AOM) is an infection in the middle ear, affecting mainly children. AOM always originates from a symptomatic viral infection of the upper respiratory tract (Nokso-Koivisto et al. 2015), and occurring in over 60% of the cases in young children (Chonmaitree et al. 2008). In AOM, common cold symptoms are accompanied by more AOM-specific symptoms such as earache and lowered hearing (on average by 25 decibels). Earache
has traditionally been the hallmark symptom of AOM, but it is not necessarily present in all cases and neither is all earache caused by AOM (Blomgren and Pitkäranta 2005). Usually the complication occurs during the first week of the common cold episode (Kalu et al. 2011), and diagnoses of AOM are most commonly made on the third day from the cold symptom onset (Chonmaitree et al. 2008).

**Acute pharyngitis and tonsillitis**

Acute pharyngitis is an infection of the pharyngeal mucosa. When the primary site of the infection is tonsils, typically covered in light exudates, the condition is called acute tonsillitis. The most typical symptom of the infection is sore throat, causing difficulty in swallowing (Ebell et al. 2000). Cases with viral aetiology, associated with the common cold, are usually mild, with symptoms lasting only for a few days. In bacterial cases, accounting for 20-50% of the cases (Ebell et al. 2000), pain in the throat usually has a more abrupt onset and it is accompanied by fever, but differentiation of the aetiology based on symptoms alone is unreliable (Ebell et al. 2000; Wessels 2011).

**Acute laryngitis**

Acute laryngitis is an inflammation of the larynx, and it can be classified either as upper or lower respiratory tract infection. Typically laryngitis is preceded by common cold symptoms (Wood et al. 2014). The main symptoms of laryngitis include dry and barking cough, difficulty in breathing in, wheezing and voice disturbances. Upper part of the trachea is the tightest region in the upper respiratory tract of children, and thus swelling of the mucosa causes the difficulty in breathing. In adults swelling occurs in the vocal chords, which leads to voice disturbances. However, in the majority of the cases symptoms are mild.

**Acute lower respiratory tract infections**

In adults, bronchitis and pneumonia are the most common infections affecting the lower respiratory tract, and the distinction between the two conditions is sometimes vague. In bronchitis the main airways of the lungs are infected, whereas in pneumonia pulmonary alveoli or interstitial tissue are affected. Both conditions have similar symptoms and cough as the main symptom, resulting from excess mucus production. In children, infections affecting the lower respiratory tract are acute bronchitis, bronchiolitis, wheezing bronchitis, and pneumonia. Acute bronchitis is defined as cough associated with a viral infection, and it is the most common reason for cough in children (Tapiainen et al. 2016b). Bronchiolitis occurs in infants usually as apnoea and crepitations, whereas wheezing bronchitis occurs in toddlers (Tapiainen et al. 2016a).
2.1.2 EPIDEMIOLOGY

For investigating the incidence of RTIs as a whole, register or hospital based studies are not sufficient in revealing the epidemiology of the full clinical spectrum (Anders et al. 2015) as many RTI cases are treated at home without medical consultation or prescribed medications (Gruber 1995). Therefore, the most relevant data on the incidence of RTIs comes from longitudinal community-based cohort studies (Anders et al. 2015; Selvaraj et al. 2014). Even with the cohort data, it should be highlighted how varying definitions make the available evidence difficult to summarize and synthesize (Roth et al. 2015), and also the methods of surveillance have an impact on the result (Shapiro 1998).

Major data sets on the incidence of RTIs in high-income countries and community settings come from half a century ago. For example three large community-based studies were conducted in the US between 1940s and 1970s (Tecumseh Study, the Seattle Virus Watch, and the New York Virus Watch), with varying methodology. These studies recorded 4.5-6.7 acute RTIs in children under one year of age, 4.9-8.0 in 1-4 -year-old children, 3.0-6.2 in 5-9 -year-olds and 1.9-4.8 in 10-19 -year-olds (all frequencies per person-years) (Shapiro 1998). In a more recent longitudinal study, cohorts of households with children were followed for two full respiratory infection seasons (October to May), and were requested to report any cases of acute respiratory illnesses defined by two or more respiratory symptoms (Monto et al. 2014). The mean number of reported episodes in children was 1.1-1.4, 0.7-0.8 and 0.5 in children under the age of five, 5-11 -year-olds and 12-17 -year-olds, per season, respectively. In adults, the case numbers per season were 0.6-0.8 and 0.4 cases in 18-49 -year-olds and over 50-year-olds, respectively. A cohort study following 294 children (enrolled at the age of 0.5-3 years) for a year documented 5.1 episodes of RTI in the upper respiratory tract per child-year with surveillance relying on parents’ initiative to contact the study centres when symptoms occurred (Chonmaitree et al. 2008). Another study following 3463 children from the age of three months to four years, showed RTI incidence of 3.8 per person year with a combination of passive and active surveillance (Lönnrot et al. 2015). A long prospective birth cohort following 760 German children until age 12 years by means of a study diary filled out by parents found a mean annual number of 3.4, 2.3 and 1.1 respiratory infections in 0-2, 3-5 and 6-12 -year-olds, respectively (Grüber et al. 2008). Slightly lower numbers were reported in a follow-up study in Finland in the Finnish Otitis Media Cohort Study, where 329 healthy children were followed from two months to two years of age and parents were instructed to take the child to a study clinic in the case of symptoms of RTI in the upper respiratory tract for physician diagnosis (Nokso-Koivisto et al. 2002). During the study period, the median number of URI episodes was three for children cared for at home and six for children attending day care.
Taken together, different methodologies applied in the surveillance and categorization of RTIs make conclusions difficult to make, but it seems that today the incidence of acute RTIs in developed countries is between 2-5 episodes per year in pre-school aged children, decreasing on average close to one annual episode in school-aged children and to less than one yearly episode in adults. In the German cohort following children from birth to up to 12 years of age, also the occurrence of specific diagnoses was detailed (Grüber et al. 2008). Rhinitis occurred 2.3, 1.3 and 0.8 times, bronchitis 0.37, 0.32 and 0.07 times and AOM 0.31, 0.31 and 0.08 times annually in 0-2, 3-5 and 6-12 year-olds, respectively. All other recorded RTI diagnoses (pharyngitis, tonsillitis, sinusitis, pneumonia, laryngitis or unspecified) occurred on average less than 0.2 times annually. Also higher occurrences of AOM complications have been documented for instance by Chonmaitree et al. (2008), who reported a mean number of 1.7 AOM episodes per child-year in 6-72 -month-olds. Despite the extremely low rate of diagnosed sinusitis by Grüber et al. (2008), changes in x-ray referring to viral sinusitis have been found in 39% of the adult common cold patients when measured on the seventh symptomatic day (Puhakka et al. 1998). Different sources have estimated that sinusitis is diagnosed in every tenth adult patient with RTI (Louie et al. 2005), and in young children, bacterial sinusitis develops in 8% of the patients (Marom et al. 2014).

2.1.3 AETIOLOGY

Viruses are by far the most common causative agents of RTIs, with 200 different types of viruses known to cause infections. Frequently detected viral causes in RTIs are rhinovirus (RV), respiratory syncytial virus (RSV), influenza virus (IFV), parainfluenza virus (PIV), human metapneumovirus (MPV), adenovirus (AdV), coronavirus (CoV), human bocavirus (HBoV) and enterovirus (EV) (Nichols et al. 2008; Nokso-Koivisto et al. 2006). Of these, IFV, RSV, PIV and MPV are the most common viral pathogens in infections affecting the lower respiratory tract (Shi et al. 2015). RV is the most common respiratory virus in all age groups (Ruuskanen et al. 2013). The role of RV as the dominant causative agent in acute RTIs has been known since the 1960s, but knowledge of its importance further strengthened in the 1990s when polymerase chain reaction (PCR) techniques became widely available for virus detection and numbers of detected RV cases increased significantly (Ruuskanen et al. 2013). In otherwise healthy subjects, RV is associated with several clinical manifestations of RTIs: most significantly the common cold, but also AOM, sinusitis, pneumonia and bronchiolitis (Nichols et al. 2008; Ruuskanen et al. 2013). Subclinical infections are also common. Almost four out of five children have experienced at least one RV infection by the age of two (Blomqvist et al. 2002). RV infections peak in the fall and spring, but the
is some evidence that the RV cases occurring in the winter are more severe (Lee et al. 2012). No RTI symptoms can be directly associated to a specific virus so distinctly that a clinical diagnosis could be made based on it, but there are differences in the symptoms caused by different viruses (Nichols et al. 2008).

Many viruses can cause the common cold, but RV is the dominant pathogen. In adults, RV was detected in half of the subjects during a common cold episode, with the highest rate of 92% detected during a fall outbreak (Mäkelä et al. 1998). In children, RV has been found in 71% of the uncomplicated common cold cases, followed by HBoV with 14%, AdV with 12% and EV with 10% detection rates (Ruohola et al. 2009). HboV1 is only a decade ago discovered, persistent virus, that often appears as co-infection with other viruses, but recently it was confirmed that it also has an independent role in respiratory illness aetiology (Meriluoto et al. 2012). HboV2-4 have been discovered even more recently, and so far HboV2-3 have been associated with gastroenteritis (Berry et al. 2015).

AOM is essentially a bacterial complication of the common cold, but nowadays viruses are known to play an important role in the aetiology as in 5–10% of the cases no colonizing pathogenic bacteria can be detected (Nokso-Koivisto et al. 2015). A variety of viruses have a significant role in the aetiology of AOM, including RSV, AdV, CoV, EV, IFV, PIV and RV (Chonmaitree et al. 2008). In sinusitis, RSV, IFV and picornaviruses (EV, RV) have been detected (Louie et al. 2005). Bacterial aetiology is more rare than viral cause in sinusitis. Most common bacterial pathogens in both sinusitis and AOM are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. In pharyngitis, viruses have been detected in one third of the cases, bacteria in one fourth and both in one fifth of the cases (Esposito et al. 2004). Most common viral pathogens were RSV and AdV and most frequently detected bacterial pathogens *Mycoplasma pneumoniae*, *S. pyogenes* and *Chlamydia pneumoniae*. For tonsillitis, it has been estimated that virus may be the sole pathogen in one-third of all the cases (Nokso-Koivisto et al. 2006), most commonly RV, CoV or PIV, and that the most common bacterial pathogen involved is group A beta-haemolytic streptococcus (Bird et al. 2014). Laryngitis is primarily a viral infection, with PIV, RSV, HBoV, RV, EV and IFV as common pathogens, PIV being the one most often detected (Rihkanen et al. 2008).

Infections in the lower respiratory tract can be caused by all known respiratory viruses (Tapiainen et al. 2016a). In acute bronchitis, bacterial pathogens, most commonly *S. pneumonia*, have been detected in one fourth and viral pathogens, most commonly IFV, in one fifth of the cases in adults (Macfarlane et al. 2001). In children laryngitis, bronchitis, bronchiolitis and wheezing bronchitis are primarily viral infections, with RSV being the most
common virus detected in wheezing infants as well as in bronchiolitis, PIV in laryngitis and RV in wheezing children over one year of age (Tapiainen et al. 2016a). Bacterial pathogen, either alone or with a virus, has been detected in 15% of pneumonia cases in children (Jain et al. 2015a) and in 14% in adults (Jain et al. 2015b), the majority of cases being caused by viruses. RV and RSV were the most common viral findings in children, and RV and IFV in adults.

2.1.4 PATHOGENESIS

There are several different routes for respiratory viruses to spread: by direct contact with contagious secretions, by air in big or small particle aerosols, or by a combination of these routes (Heikkinen and Järvinen 2003). The most effective route of transmission differs between the viruses. RV can stay viable for several hours outside the body on contaminated surfaces (Winther et al. 2011). It can spread effectively through infected nasal discharge getting on the hands of a person via direct contact with an infected person or via infected surfaces, and then from the hands of the recipient to the eyes or nose. IFV spreads well through small particle aerosols that are able to linger in the air for an extended time (Richard and Fouchier 2016), which is also a possible transmission route for RV (Dick et al. 1987). RSV was previously though to transmit mainly by large droplets or by self-inoculation (Hall and Douglas 1981), but more recent evidence suggests that small airborne particles can also serve as one transmission route for RSV (Lindsley et al. 2010).

The details on the pathogenesis of respiratory viruses are best documented for the most common pathogen, RV. It is not currently known if RV can penetrate an intact mucus covering nasal epithelial cells or if RV moves to the nasopharynx by mucociliary clearance (Winther 2011). Nonetheless, nasopharyngeal secretion is where the RV shedding is most frequently detected. Most RVs enter the human cells using intercellular adhesion receptor molecule 1 (ICAM-1) receptors, which are located mainly in the nasopharynx. Data is still conflicting on the importance of different replication sites in the nasopharynx, and whether the replication environments could have a role in symptom development (Kennedy et al. 2012). However, due to the temperature sensitivity of RV, its replication is mainly restricted to the upper airways (Kennedy et al. 2012). RV infection does not destroy the epithelial barrier, like IFV and RSV infections do (Winther et al. 1990), but it does disrupt barrier function, which increases the permeability and translocation of pathogens (Kennedy et al. 2012).

The host response, including both innate and adaptive immune systems, has a critical role in symptom development (Kirchberger et al. 2007). With the first encounter with the virus, engagement of the recognizing receptors
induces the first cytokine expression, that can restrict virus replication and modulate cytotoxic and natural killer (NK) cell activity (Kennedy et al. 2012). Replication of the virus releases inflammatory mediators, such as proinflammatory cytokines. These mediators attract inflammatory leukocytes, granulocytes, dendritic cells (DCs) and monocytes to the site of the infection, resulting in a continuum of inflammatory reactions that are responsible for the symptoms (Kirchberger et al. 2007). Findings showing that positive viral culture is a stronger indicator of a symptomatic disease than a molecular assay detection indicate that high viral load could have a role in symptom severity, as tissue culture is less sensitive than molecular methods and detects only live virus and likely only larger quantities (Chonmaitree et al. 2008). It was indeed recently shown that RV load positively correlated with symptom scores, when measured on second and fifth day after RV infection (Tapiovaara et al. 2016a). After the infection, serotype-specific antibodies develop in the majority of the infected to form an effective antiviral mechanism against future encounters with the virus (Kirchberger et al. 2007). However, as a whole immune response triggered by infection and recovery from the infection are still incompletely understood areas in research (Kirchberger et al. 2007).

The time from an infection to symptom onset depends on the infecting virus, varying from half a day to six days (Lessler et al. 2009). In a systematic review pooling the available data for common respiratory viruses, it was found that median incubation time was 0.6 days for IFV B, 1.4 days for IFV A, 1.9 days for RV, 2.6 days for PIV, 3.2 for CoV, 4.4 days for RSV and 5.6 days for ADV (Lessler et al. 2009). The symptoms of infection also vary depending on the causative agent. Associations have been suggested between fever and IFV, MPV, CoV and PIV (Uitti et al. 2015). Rhinitis, nasal congestion and cough have demonstrated positive association with *M. catarrhalis*, and cough with RSV and PIVs in the same study. The fact that practically all respiratory viruses cause nasal symptoms is a likely reason for specific respiratory viruses not standing out in the multivariable models.

Virus infection can also facilitate later invasion bacterial pathogens (Hendaus et al. 2015). For example physical damage to the mucosa of the nasopharynx caused by IFV infection can weaken the local defence against bacteria, leading to more bacteria being able to translocate through the respiratory cell epithelial barrier. Bacterial adherence is also facilitated by viral infection upregulating the receptors utilized by bacteria. In AOM, dysfunction of the eustachian tube, passage connecting nasopharynx to the middle ear, can allow pathogens to access the middle ear to reproduce and cause local inflammation (Nokso-Koivisto et al. 2015). Bacterial complications of viral infections affecting the upper respiratory tract as a whole are in any case rare. In subjects with the common cold, rise in antibodies to bacterial pathogens occurred in only 3.5% of the cases, and in
most cases coinfection with a virus was also detected (Mäkelä et al. 1998). In hospitalized patients viral and bacterial coinfection compared to viral infection alone did not substantially affect the severity of the illness (Damasio et al. 2015).

2.1.5 DIAGNOSTICS

Clinical diagnostics

The diagnostic criteria of respiratory infections are not strict, which can leave physicians uncertain of the diagnosis (Blomgren and Pitkäranta 2005). Classification of acute RTIs according to World Health organizations (WHO) International Classification of Diseases (ICD-10) (WHO 2016) has been argued to be the area leaving most freedom for physicians on how to code a condition (Beckman 2014). Major challenge in diagnostics and clinical management of RTIs remains in the differentiation of uncomplicated viral infections from those with bacterial aetiology and thus indication for antimicrobial treatment (Zumla et al. 2014).

Diagnosis of the common cold can be simply done based on the clinical manifestation of the disease, and adult patients can even self-diagnose the common cold reliably (Heikkinen and Järvinen 2003). Examination of the viral aetiology is rarely needed as the virological diagnosis has no significant implication for the course of the disease or the treatment (Tregoning and Schwarze 2010). However, in the case of IFV for which antiviral medication is available, early identification of the aetiology is needed, as the possible treatment needs to be initiated during the first symptomatic days for it to be effective (Tapiainen et al. 2016a). Infections caused by the IFV cannot be differentiated based on the symptoms alone, and therefore virology needs to be tested for diagnosis. Diagnosis of sinusitis is most often done based on clinical signs and symptoms, the most typical symptom pattern being lack of improvement in the cold symptoms in 10 days (DeMuri and Wald 2013). Diagnosis of AOM relies on the inspection of the eardrum, and AOM can be accurately diagnosed based on signs and symptoms combined with tympanometry and pneumatic otoscopy (Blomgren and Pitkäranta 2005).

Fever as an early symptom of infection in children is challenging, as it can be caused by the common cold but it can also be an indication of a severe bacterial infection (Heikkinen and Järvinen 2003). Sore throat is a typical symptom of both the common cold and streptococcal pharyngitis. Presence of runny or stuffy nose usually differentiates the two conditions, but for instance in Finland laboratory testing for group A streptococcal infection is recommended (Käypä Hoito working group 2013), whereas for example in the UK clinical diagnosis is considered sufficient (Bird et al. 2014).
Laryngitis, bronchitis and bronchiolitis in children are diagnosed based on clinical signs and symptoms as bacteria are an uncommon cause in all the conditions (Tapiainen et al. 2016a). Pneumonia can also be diagnosed indirectly based on clinical findings (Tapiainen et al. 2016b).

**Laboratory diagnostics**

Respiratory pathogen identification can be done from many different samples, such as nasopharyngeal aspirates, washes and swabs (alone or together with oropharyngeal swabs) as well as sputum, tracheal aspirates and bronchoalveolar lavages, and the type of sample can significantly affect the sensitivity and specificity of the pathogen detection (Passioti et al. 2014). Even though current available options for determining the pathogen in clinical practise are limited, due to e.g. challenges in availability and turnaround time (Zaas et al. 2014), major development has taken place in laboratory diagnostics of pathogens causing respiratory infections during the past decades. Bacterial diagnostic tests are more challenging, and culture remains the gold standard of bacterial identification, despite it taking several days (Zaas et al. 2014). Of the common respiratory bacteria, antigen testing is available for *S. pneumoniae* and group A *Streptococcus*. In terms of viral pathogens, point-of-care rapid antigen tests are available for the detection of IFV and RSV. Point-of-care test able to detect antigens from altogether eight respiratory viruses (IFV A and B, RSV, ADV, MPV, PIV1-3) as well as *Streptococcus pneumonia* has also been developed (Sanbonmatsu-Gámez et al. 2015). The molecular detection of respiratory pathogens is more feasible for viruses than for bacteria (Zaas et al. 2014). In the laboratory diagnosis of viral infection, PCR has for large part replaced the preceding methods, such as virus isolation in tissue culture and immunological methods detecting antibodies and antigens (e.g. enzyme linked immunoabsorbent assay (ELISA), direct and indirect immunofluorescence (IF) assays). PCR techniques are sensitive and relatively quick, with time from sample collection to results taking 2-24 hours. Development of multiplex PCR tests, able to detect several pathogens in one run with short throughput time, has further eased the viral detection (Zumla et al. 2014). Challenge from the clinical perspective remains in the fact that co-infections of both virus and bacteria can occur, and thus the detection of a virus does not rule out a concomitant bacterial infection. Furthermore, even multiplex PCR assays can miss virus detection, e.g. in the case of a mutated or novel virus, due to the assay being limited to the specific viruses included on the panel. Also losses in sensitivity have been reported when multiplex assays have been compared to singleplex assays in respiratory virus detection (Parker et al. 2015). Analysis of the host response via gene activation and suppression in blood samples for distinguishing bacterial infections from those caused by viruses is currently an actively researched area and potential diagnostic tool for the future (Herberg et al. 2016; Mahajan et al. 2016).
2.1.6 TREATMENT

Due to the multitude of pathogens causing RTIs, the development of a universal effective treatment is unlikely (Heikkinen and Järvinen 2003). Attempts to develop drugs targeting all phases of the life cycle of specific respiratory viruses have been numerous, but few have been successful (Passioti et al. 2014). To date, of all the respiratory viruses causing RTIs, antiviral drugs have been successfully developed only against IFV and RSV (De Clercq and Li 2016). Neuraminidase inhibitors can shorten the symptomatic period of IFV A and B infections on average by one day, symptoms may occur as less severe, and clinically meaningful reductions in complications measured by antibiotic treatments and hospitalizations have been achieved (Nguyen-Van-Tam et al. 2015). Challenge is that the treatment needs to start within two days from symptom onset, and data is also still very limited in regard to the treatment of children. Due to the rather modest effects, it has been suggested that the neuraminidase inhibitors should only be used in patients with a high risk of complications or who are markedly unwell. Ribavirin has shown to be active against both RSV and IFV, and is currently the only approved antiviral treatment in RSV-infections (Passioti et al. 2014). However, it is not in routine clinical use as only minimal clinical benefit has been demonstrated in reducing the duration of severe complications (Drysdale et al. 2016). Rapid development is currently ongoing in RSV drug development, and human challenge studies have shown promising results at least for two novel drug candidates, GS-5806 (DeVincenzo et al. 2014) and ALS-008176 (DeVincenzo et al. 2015).

Even though antibiotics are effective in the treatment of bacterial infections, challenges in their use in RTIs arise from the distinction of bacterial infections from those caused by viruses and many RTIs passing also without antibiotic treatment, and on the other hand from the clear need to avoid unnecessary antibiotic treatments due to the increasing antimicrobial resistance (Roca et al. 2015) and risk for adverse effects (Kenealy and Arroll 2013). Even though antibiotics are not effective against viruses, it was long thought that secondary bacterial infections could be prevented by prescribing antibiotics in viral RTIs, but there is no evidence to support this and it is thus not recommended (Kenealy and Arroll 2013). Sinusitis is commonly treated with antibiotics, even though 80% of sinusitis cases pass in two weeks without antibiotic treatment (Ahovuo-Saloranta et al. 2014). In the treatment of mild AOM, the expectant observational approach is often justified in terms of antibiotic treatment as most cases pass without treatment (Venekamp et al. 2015). Antibiotics show only modest benefit in the treatment of the sore throat, but when pharyngitis or tonsillitis have group A Streptococcus confirmed as the pathogen, the benefit of antibiotic treatment increases (van Driel et al. 2013). Antibiotics have no or very limited benefits in the treatment of laryngitis (Reveiz and Cardona 2015), bronchitis (Smith et al.
2014a) or bronchiolitis (Farley et al. 2014), which both have primarily viral aetiology. Pneumonia is treated with antibiotics (Prina et al. 2015).

**Symptom-alleviating pharmaceuticals**

Due to the lack of effective treatment options available for viral RTIs, the focus is in symptom alleviation. Adequate analgesia should be the priority in the treatment of all RTIs (Venekamp et al. 2015). Non-steroidal anti-inflammatory drugs, such as ibuprofen, can reduce pain associated with the common cold, such as head or ear ache, as well as sneezing, but are not effective in reducing the total symptom load (Kim et al. 2013). Acetaminophen (paracetamol) provides mild analgesia, and has shown to be more effective than placebo but less effective than ibuprofen in reducing fever in children (Allan and Arroll 2014). Also several other symptom-relieving medications and their combinations are commonly used in RTIs, but with varying data on their effects. Antihistamine-decongestant-analgesic combinations may provide some alleviation for the common cold symptoms (De Sutter et al. 2012). Antihistamine with decongestant was the most effective of the evaluated combinations, especially in regard to severity of sneezing, but it also increased the adverse events such as nasal dryness and insomnia. Nasal obstruction was relieved slightly with the combinations containing a decongestant. Rhinorrhea and cough were reduced slightly with antihistamine-decongestant-analgesic. All effects on single symptoms were less than one point on 4-5 point severity scales. Both antihistamine and decongestants alone have reached statistical significance in reducing some nasal symptoms, but relevance of minor decreases (e.g. 0.3 or less in 4-5 point scales for antihistamine) have been questioned from clinical perspective (Allan and Arroll 2014). Available data on decongestants or antihistamines in children do not support their use in acute sinusitis (Shaikh and Wald 2014) or in AOM (Coleman and Moore 2008). For intranasal corticosteroids only limited data is available, but currently there is no indication that they would be effective in alleviating the symptoms of the common cold (Hayward et al. 2012). Intranasal ipatropium bromide, an anticholinergic drug, has shown no effect on nasal congestion, but can alleviate runny nose (AlBalawi et al. 2013). Adverse events, such as nasal dryness, are commonly associated with the use of the drug intranasally. There is no good-quality evidence on the benefits of prescription-free medicines for acute cough (Smith et al. 2014b). Vapor rub, including camphor, menthol and eucalyptus oil, applied to the neck and chest has in one study showed modest improvement in sleep and cold symptoms (Paul et al. 2010).

**Nutritional approaches**

Several nutritional approaches have been researched on their potential in relieving RTI symptoms. In a meta-analysis of seven studies addressing
therapeutic vitamin C consumption, started only after the onset of symptoms (1-8 g per day once or for several days), vitamin C supplementation was concluded ineffective. A meta-analysis of 14 trials (13 in adults and one in children) showed that oral zinc supplementation started one or two days from symptom onset had no effect on symptom severity, but it shortened cold duration (Das and Singh 2014). The effect was more evident with high doses (at least 75 mg daily). However, adverse events, such as nausea, have been significantly higher in the zinc groups, which hinder the potential of zinc usability. Out of the six studies conducted on Echinacea preparations in shortening the durations of the common cold symptoms, only two have showed positive results (Karsch-Völk et al. 2014). A single daily dose of honey at bedtime showed weak positive effect on acute cough in children in a two-trial meta-analysis, but high risk of bias in the trials did not allow drawing of firm conclusions (Oduwole et al. 2012). Also the use of Chinese medicinal herbs for the treatment of sore throat has been evaluated, and a weak positive impact has been discovered, but with methodological criticism raised regarding the evaluated trials (Huang et al. 2012).

Other interventions

Five clinical trials conducted on adults and children have compared the use of nasal irrigation to routine care or use of nose sprays (King et al. 2015). Trials have methodological issues, but evidence points in the direction that nasal saline irrigation could reduce nasal secretion and obstruction in acute infections of the upper respiratory tract, even though more well-conducted trials are needed to confirm this. It has also been suggested that by breathing of humidified air, the steam could help congested mucus to drain better, and thus relieve symptoms. Recent review summarizing the findings from six trials concluded that the benefits of steam inhalation in the treatment of the common cold were inconsistent (Singh and Singh 2013). One study has indicated a lack of beneficial effects of mist therapy in bronchiolitis in children (Umoren et al. 2011). Mist has been concluded to be ineffective in reducing the symptoms of laryngitis in children (Tapiainen et al. 2016a).

2.1.7 GENERAL RISK FACTORS AND PREVENTION

Young age is a clear risk factor in RTIs, as the incidence of illness is highest in children under the age of two. Full breastfeeding for at least six months compared to only four months appears to reduce pneumonia and recurrent AOM (Chantry et al. 2006). Day-care attendance increases the frequency of common cold (Ball et al. 2002; Nokso-Koivisto et al. 2002). However, the attendance in large day care might serve as a protective factor against RTIs in school age, with difference evening out by teenage (Ball et al. 2002). Parental smoking significantly increases the risk of infections affecting the lower
respiratory tract in children (Jones et al. 2011). In adults, smoking (Bilello 2005), stress (Stover 2010) and short sleep (Prather et al. 2015) might increase susceptibility to the common cold, whereas interestingly positive emotional style has been shown to increase resistance to illness (Cohen et al. 2006). The evidence on the role of exercise is inconclusive (Grande et al. 2015). It has also been suggested that individual airway microbiota (Hasegawa and Camargo 2015) and genetic factors (Tregoning and Schwarze 2010) might have a role in respiratory infection susceptibility.

Many physical interventions, most importantly hand washing, especially around young children, and the use of surgical masks, have been shown to effectively reduce the spread of respiratory viruses (Jefferson et al. 2011). It has also been shown that ethanol hand rub is not as effective as hand washing with soap and water against RV (Savolainen-Kopra et al. 2012). One trial has shown that gargling with water decreases the number of RTI episodes significantly (Satomura et al. 2005).

**Immunization**

For respiratory viruses, globally available vaccine exists only against IFV. In 2-6-year-old children benefit of vaccination is clear, with six children needing to be vaccinated to prevent one influenza case (Jefferson et al. 2012). In older children 28 need to be vaccinated to prevent one case, and there is lack of evidence on the effectiveness in under 2-year-olds. In healthy adults influenza vaccination has been concluded to have limited benefits (Jefferson et al. 2014). The pooling of data in meta-analyses from several influenza vaccination trials can, however, be criticized. Details of individual trials in both children and adults show that when the antigen match between the circulating strains and those in the vaccine has been good, has the vaccine effectiveness also been high (Heikkinen et al. 2013). Furthermore, influenza vaccination has significantly reduced also the incidence of AOM in small children (Ozgur et al. 2006). In terms of bacterial pathogens, the introduction of pneumococcal vaccines has reduced not just pneumonia, but also AOM cases (Yildirim et al. 2015), for instance AOM decreasing by 6-8% in vaccinated children compared to controls (Nokso-Koivisto et al. 2006). There is on-going research to develop vaccines against several other respiratory viruses (e.g. RSV, RV, and PIV), but attempts have not yet been successful.

**Nutritional interventions**

A meta-analysis summarizing 11 placebo-controlled clinical trials on vitamin D found a protective affect against respiratory infections (range for number needed to treat 9-33), with daily doses showing a better response compared to large doses at long intervals (Bergman et al. 2013). A meta-analysis including 29 trials concluded that regular vitamin C supplementation does
not prevent colds in the general population, but it might be able to do so in heavily exercising adults (Hemilä and Chalker 2013). However, the same meta-analysis concluded that, based on 32 studies, regular vitamin C consumption (dose at least 0.2 g per day) can shorten the duration of common cold on average by 8% in adults and by 14% in children, and also decrease symptom severity (Hemilä and Chalker 2013). Prophylactic trials on Echinacea products have shown consistent albeit non-significant preventive trends in the 12 conducted trials, thus indicating some effect but with debatable clinical significance (Karsch-Völk et al. 2014). A meta-analysis on the prophylactic use of xylitol in the prevention of AOM in children pooled data from four trials with adequate methodological quality, and concluded that xylitol may reduce the occurrence of AOM by 25% (Azarpazhooh et al. 2011).

Research data on other potential components is more limited or conflicting. When supplementation of zinc was started in children at the onset of cold season, pooled data from two trials showed a cold incidence of 38% in the zinc group and 62% in the control group (Das and Singh 2014). Evidence is mixed regarding the benefit of zinc supplementation in the prevention of AOM (Gulani and Sachdev 2012). Effect of specific prebiotic supplementations on RTI outcomes has been addressed in two trials. Galacto-oligosaccharide-polydextrose-supplementation in preterm infants during the first two months of life was recently shown to lower the number of RTIs and specifically RV infections, compared to the control group during the one-year follow-up, and the benefits outweighed those achieved with probiotic Lactobacillus rhamnosus GG supplementation (Luoto et al. 2014). Earlier 6-month trial on term infants receiving an infant formula supplemented with a galacto-oligosaccharide-fructo-oligosaccharide combination, a tendency towards fewer infections in the upper respiratory tract in the prebiotic formula group compared to the control formula group was reported (Arslanoglu et al. 2007). Existing data for garlic in the common cold was also recently evaluated, with only one trial meeting the quality criteria (Lissiman et al. 2014). The one study suggested that garlic might be able to lower the occurrence of the common cold, but more research is needed to confirm this initial finding. Ginseng has also been studied for its ability to reduce the incidence of colds, but data is still limited to draw conclusions on its efficacy (Seida et al. 2011). There is indication that when taken preventatively, ginseng might shorten the duration of a cold, but the methodological quality of the studies has been questioned (Allan and Arroll 2014) and thus confirmatory trials are required. One clinical trial has studied the effect of bovine lactoferrin and whey protein immunoglobulin (Ig) -rich fraction on cold incidence (Vitetta et al. 2013). In this study, the treatment group experienced significantly less cold symptoms and episodes of cold compared to the control group, but no statistically significant difference was seen on the duration or severity of colds.
2.1.8 SOCIAL AND ECONOMIC IMPACT

In the latest Global Burden of Disease study from 2013, RTIs accounted for 4.8% of the total health lost when measured by disability-adjusted life years (Murray et al. 2015). Of this share 96% is attributed to infections in the lower respiratory tract, which are the third leading cause of disability-adjusted life years globally. Due to the disease burden and high incidence especially in children, the challenges associated with the infections are significant. Work or school absenteeism is the most evident social impact. In addition to this, due to the often mild nature of the illness, people may attempt to continue their lives normally by going to work or school. It has been suggested that RTIs cause reduced alertness and thus lower performance in some tasks (Smith 2013), which can have implications that are beyond the reach of the absenteeism measurements.

Even if RTIs are largely benign, due to the high incidence the economic consequences are significant, although very limited data is available on the financial burden. In the UK, it has been roughly estimated back in 2004 that the primary care costs of upper RTIs would be £31 million (Urquhart and McKenzie 2004). This estimation was a result of calculation taking into consideration the average number (value of 6-8 per year used), the frequency of taking the child to a physician when child is unwell (11%), a consultation cost estimation (£16) and the preschool population of 2.94 million in the UK at the time. In Finland, the economic consequences of illness in day care centres was estimated in 1990 (Nurmi et al.). In this evaluation infectious diseases in total caused 90% of illnesses and costs. The link between day care attendance, compared to other forms of care, and tendency to use more health care services has also been made (Silverstein et al. 2003). In Finland in 1999 it was estimated that each episode of AOM costs $228 in children attending day care (Niemelä et al. 1999).

2.2 PROBIOTICS

2.2.1 DEFINITION AND PROPERTIES

Food and agriculture organization (FAO) and WHO expert consultation group has defined probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO 2006). A recent consensus statement from the International Scientific Association for Probiotics and Prebiotics reinforced the definition as sufficiently accommodating also for anticipated future applications (Hill et al. 2014). Microbes from many genera have been identified as probiotics, but
Lactobacillus and Bifidobacterium have to date been the most commonly studied and used in food products and supplements. Probiotic strains should be able to survive and proliferate in the acidic conditions of the upper gastrointestinal (GI) tract created by bile and gastric juices (FAO and WHO 2006). It has further been specified that probiotics should demonstrate adhesive properties to human tissues, stimulate the immune system, protect the host from pathogens and beneficially affect host’s microbiota (Giraffa et al. 2010). Finally, probiotics should naturally be safe to consume, have no pathogenic or virulence properties and lack transferable antibiotic resistance (FAO and WHO 2006).

All probiotic strains are unique in their potential health benefits, and even strains belonging to the same species are known to confer different effects. Lactobacillus rhamnosus GG (GG) is one of the most researched probiotic strains worldwide, and it is available for consumers all over the world in numerous food products and supplements. GG was isolated from a faecal sample of a healthy human in the early 1980s. Its discoverers tested a number of Lactobacillus strains isolated from faecal samples for resistance to acid and bile, adherence to intestinal epithelial cells, production of antimicrobial substances and for their growth rate (Doron et al. 2005). Of the tested strains, GG possessed all the critical properties and demonstrated the highest growth rate. Since then, a multitude of studies have addressed the probiotic properties and health effects of GG. To date, most evidence on the health benefits of GG exist for atopic dermatitis (Foolad and Armstrong 2014), acute diarrhoea (Guandalini 2011) and antibiotic-associated diarrhoea (AAD) (Szajewska and Kołodziej 2015).

2.2.2 GENERAL MECHANISMS OF ACTION

It is believed that probiotic strains do not become permanent residents of the hosts’ intestinal microbiota (Corthésy et al. 2007). For the probiotic to be able to interact with the host and affect both the non-immune defence, e.g. the competitive exclusion of pathogens, and the immune system, adhesion to mucosal surfaces is of importance (Bermudez-Brito et al. 2012; Collado et al. 2007). Relatively recently, in genomic analysis of GG, fimbria-like surface structures, pili, were found to be essential for the adhesion of the probiotic to human intestinal mucus (Kankainen et al. 2009). Clinical trials on GG have demonstrated high levels of adhesion and persistence by analysing the recovery of the strain in GI mucosa. Adherence to intestinal mucosa has been demonstrated in colonic biopsies for GG after 12 days of consumption (Alander et al. 1997), and the temporary colonization was shown to remain for over a week after GG administration (Alander et al. 1999). In a study on a combination of Lactobacillus johnsonii La1 and Bifidobacterium longum BB536, administered twice daily for three days at two different doses, the
Lactobacillus strain was recovered from the colonic mucosa samples in 60% of the subjects with the higher dose and in 27% of the subjects with the lower dose, whereas the bifidobacterium strain could not be recovered from the mucosa (Gianotti et al. 2010). For nasopharyngeal adhesion, a trial on 40 children given GG for three weeks before adenotomy, GG was recovered from the adenoid tissue in all children receiving GG, but also in 76% of the children in the control group (Swanljung et al. 2015). Also one pilot study on six subjects demonstrated that oral consumption of L. plantarum DSM 9843 led to the recovery of the strain from a tonsil surface four hours after intake in all subjects, but after eight hours in only one of the subjects (Stjernquist-Desatnik et al. 2000). Furthermore, a non-randomized and non-controlled 10 day intervention trial on S. salivarius K12 showed that the strain could be recovered in only one out of 19 nasopharyngeal swab (NPS) samples, and in three out of seven adenoid samples (Power et al. 2008). GG has also been recovered from vaginal epithelium in one tenth of the subjects (Colodner et al. 2003), from the oral cavity in two thirds of the participants (Yli-Knuuttila et al. 2006) and from the middle ear effusion samples in one fifth of the subjects (Tapiovaara et al. 2014) after oral consumption of the probiotic.

Even though understanding of how probiotics generate their beneficial effects is not yet complete, it is known that effects are triggered through a combination of several different probiotic-host interactions. Figure 1 provides an overall framework of probiotic actions, but it is worth noting that strain-to-strain variations can be significant and that mediation of each health effect is likely to be a multifactorial process with mechanism profile differing between conditions. It is likely that some mechanisms of action are shared among all strains in specific genera, some among those belonging to the same species, while some mechanisms are strain-specific (Hill et al. 2014). For GG, several microbe-associated molecular patterns (MAMPs) have been identified, such as lipoteichoic acid molecules, secreted proteins, exopolysaccharides and specific DNA motifs. Using MAMPs, GG can communicate with the host via specific pattern recognition receptors (PRRs) located in epithelial and immune cells (Segers and Lebeer 2014; Wells 2011).

Mechanisms of probiotics can be broadly categorized into three classes: exclusion or inhibition of pathogens directly or via modification of the commensal microbiota, enhancement of the epithelial barrier function and modulation of the host immune responses (Figure 1). These mechanisms have been demonstrated to a large extent using in vitro and animal models, thus more in vivo human data is needed to substantiate their role in the interaction with the host. Molecular profiling can offer insight of the complex host responses modulated via combinations of for instance different MAMP-PRR interactions and metabolites in vivo. For GG, host response studies have demonstrated effects on immune response and inflammation by gene expression analysis (Di Caro et al. 2005) and induction of the Th1 response.
by transcriptome analysis (van Baarlen et al. 2011) in the intestinal mucosa. Human mucosal transcriptome responses have, however, indicated that there is person-to-person variation in how probiotics affect the host, and it is suggested that genetic background, host microbiota as well as general lifestyle and diet could affect responsiveness to probiotics (van Baarlen et al. 2011). More in vivo research is needed to understand the determinants for responders and non-responders, although this can be a difficult task due to the observed inter-person variations in strategies for accomplishing mucosal homeostasis (Bron et al. 2012).

Figure 1. General mechanisms of action of probiotic bacteria (Bermudez-Brito et al. 2012; Lebeer et al. 2008; Segers and Lebeer 2014; Wells 2011).
2.2.3 PRODUCT MATRIX

When probiotic strain is added to a food product, further criteria are that the strain should not negatively affect the taste and it should survive the processing and storage conditions (Lee and Salminen 1995). Probiotics are most commonly consumed in milk products or as supplements, but also several other food products with probiotics exist on the market, such as juices and cereal products (Vijaya Kumar et al. 2015). The food product matrix can potentially protect probiotic strain in the GI environment. The protective properties of milk as a matrix are best documented, whereas the properties of non-dairy food matrixes with shorter history of use are less known (Vijaya Kumar et al. 2015).

A study on a four-strain probiotic combination (GG, L. rhamnosus Le705, P. freudenreichii subspecies (subsp.) shermanii JS, and B. animalis subsp. lactis Bb12) showed that within the studied matrixes (yoghurt, cheese, capsule), the matrix did not have an impact on the faecal recovery of lactobacilli, but it did affect the faecal quantity of propionibacteria and bifidobacteria, which were the lowest after the consumption of the probiotic cheese (Saxelin et al. 2010). A study on L. plantarum strain found that strain was recovered from faecal samples in higher amounts when consumed in fermented sausage compared to freeze-dried powder (Klingberg and Budde 2006). For B. animalis DN-173010, fermented milk and freeze-dried powder were equally good matrixes when measured by the faecal recovery of the strain (Rochet et al. 2008).

In terms of probiotic properties, an in vitro study on GG comparing different product matrixes and sources of the strain, no significant differences were found in the adhesion abilities, but pathogen exclusion by inhibition and competition varied significantly among the different isolates (Grześkowiak et al. 2011). For instance for pili, key adhesive structures in GG, industrial production procedures can be detrimental (Segers and Lebeer 2014). Industrial cultivations and production processes can for instance lead to the production of pilus-less GG if the genomic island carrying the pilus gene locus gets deleted during processing (Sybesma et al. 2013). These findings highlight that the product matrix and quality control of production are issues that should be taken into consideration also when human intervention studies are discussed and results interpreted.

In recent years, microencapsulation of probiotics has been suggested as a way to tackle the challenges of probiotic survival and stability both in the food product and in the GI tract (Islam et al. 2010). This approach would eliminate the possibility for local effects in the oral cavity and pharynx. Also growing interest has been targeted towards inactivated forms of the probiotic strains, which would tackle the same challenges. Furthermore, few trials
have administered probiotics in nasal spray instead of delivery via a food product or a supplement (Santagati et al. 2015; Skovbjerg et al. 2009). This approach has been well tolerated in subjects but health effects are yet to be studied.

2.2.4 VIABILITY

The often cited FAO&WHO definition (FAO and WHO 2006) includes a notion of the viability of the probiotic, and the ability of the strain to survive alive and grow in the harsh GI conditions is further emphasized in the critical properties of probiotics. Yet, there is uncertainty regarding how critical viability of the strain is for delivering the beneficial health effects. During the past decade, several in vitro and animal studies have compared the effects of inactivated and live probiotic cells on the immune system. It has been shown that in tumor necrosis factor (TNF) α induced Caco-2 cells, both live and heat-killed GG were effective in downregulating production of interleukin (IL)-8, but when there was no pre-existing inflammatory mediator stimulation live strain caused an increase in the IL-8 production, whereas heat-killed form caused a minimal response (Zhang et al. 2005). An equal effectiveness of unviable GG compared to the live strain was demonstrated for ultraviolet-inactivated GG using flagellin-induced IL-8 production in Caco-2 cells (Lopez et al. 2008). Another in vitro study on live and heat-inactivated L. acidophilus strains demonstrated that both forms were able to inhibit the attachment of pathogenic bacteria to Caco-2 cells in a similar manner (Ostad et al. 2009). Furthermore, in infant rats, heat-killed GG showed similar results to live GG by decreasing the lipopolysaccharide-induced proinflammatory mediators and increasing the anti-inflammatory mediators (Li et al. 2009). Due to the many recently recovered structural components of GG, for instance lipoteichoic acid and pili, it is possible that unviable GG, and possibly also other inactivated probiotic strains for which data is scarcer, could yield similar health effects compared to live strains. However, in terms of terminology it has been highlighted that nonviable forms do no fall under the probiotic construct (Hill et al. 2014).

Few studies have also attempted to test the clinical relevance of probiotic viability by comparing the effects of viable and unviable strains. An inactivated probiotic combination (GG, L. acidophilus La-5 and B. animalis subsp. lactis Bb12), included in the trial as a control group, was significantly less effective than a combination of the same strains in viable form in the prevention of AAD (Wenus et al. 2008). In a trial on atopic symptoms, no statistically significant differences were found for live or inactivated GG groups compared to the placebo group, but study was discontinued early when more GI symptoms appeared in the group receiving the inactivated GG
It was hypothesized by the authors that high numbers of bacteroides and clostridia might have responded to the administered probiotic antagonistically by secreting toxins that induce diarrhoea, and only live GG would have been able to restrain the antagonistic activity. Consumption of inactivated GG has also led to lower rotavirus immune responses compared to a live strain during rotavirus infections (Kaila et al. 1995). To conclude based on the clinical evidence, it appears that inactivated probiotics might not confer the same health benefits as the live strain. However, evidence is still limited and complicated by studies using different bacterial inactivation methods, for which details are not always reported.

2.2.5 DOSSING

Large variation exists in the dosing of probiotic strains in clinical trials, usual levels ranging from $10^8$ to $10^{13}$ colony-forming units (cfu) per day. The optimal dose is likely to depend on the strain and targeted health effect (Aureli et al. 2011). However, within specific strain or combination of strains, very few trials have attempted to reveal a dose-effect relationship to specific health effects (Bertazzoni et al. 2013). In AAD, a high dose ($10^{10}$ cfu/d) but not low dose ($10^8$ cfu/d) of a four-strain probiotic combination (L. acidophilus NCFM, L. paracasei Lpc-37, Bifidobacterium (B.) lactis Bi-07, B. lactis Bl-04) yielded a statistically significant reduction in AAD incidence compared to placebo, and significant linear trend indicated dose-depenency (Ouwehand et al. 2014). Dose-response was also seen in another trial on AAD, using a two-strain probiotic combination of L. acidophilus CL1285 and L. casei LBC80R (Gao et al. 2010). In this trial both studied doses were high ($10^{13}$ and $10^{14}$ cfu/d). Dose-dependency has also been demonstrated in the reduction of the whole gut transit time with the strain B. lactis HN019 at daily levels of $10^{12}$ and $10^{13}$ per day (Waller et al. 2011), and in the softening of stools using a combination of two strains (B. animalis ssp. lactis BB-12, L. paracasei CRL-431) at daily doses of $10^8$, $10^9$, $10^{10}$ or $10^{11}$ (Larsen et al. 2006). On the other hand, two high doses of GG ($10^{10}$ and $10^{12}$ cfu/d) decreased the frequency and duration of diarrhoea equally effectively (Basu et al. 2009). Thus, based on the available evidence, it can be the strain, the health effect in question, or both that are critical for the optimal therapeutic dose. Furthermore, synergistic or additional effect accomplished by combining two or more strains has been suggested, but evidence for this is conflicting, some studies suggesting even a negative outcome (Aureli et al. 2011). There is also lack of data available on whether probiotics would be more effective when consumed in intervals or continuously.

After the consumption of the probiotic, a standard method to demonstrate the consumption and evaluate the presence and the amount of the probiotic
in the GI tract is the analysis of faecal recovery. Most efficient and specific method to measure probiotic presence in faecal or mucosal samples is strain-specific PCR (Treven 2015). For GG, a quantitative strain-specific assay has been developed (Ahlroos and Tynkkynen 2009), which may detect also dead and dormant bacteria. Using this method, it has been demonstrated that after two week consumption of GG, almost one third of the subjects still carried the strain at the end of the three-week follow-up period (Saxelin et al. 2010). It has also been shown, using cultivation methods for GG detection, that GG can persist in colonic mucosa even after it has disappeared from the faecal sample (Alander et al. 1999). Long-term GG recovery has been addressed in a study on infants (Gueimonde et al. 2006). In this study, mothers received GG supplementation for four weeks before delivery, followed by infants receiving GG for the first six months of life directly or via breast-feeding mother. GG was recovered from the faecal samples of 58% and 78% of infants after the 6-month intervention and from 24% and 26% at 12 months in the GG group using colony identification and direct PCR, respectively. However, GG was also recovered in the faecal samples of children in the control group, in 28% and 43% at 6 months and 14% and 20% at 12 months by colony identification and direct PCR, respectively. In addition, one case study has been reported on faecal GG recovery rates in infants born to six women who had consumed GG during late pregnancy (Schultz et al. 2004). At 1 and 6 months of age, GG colonization was observed in all four delivered vaginally and in one of the two children delivered by cesarean section, even though children did not receive any GG supplementation after birth. Furthermore, GG was recovered in three children at 12 months, and in two children at 24 months.

2.2.6 SAFETY

European Food Safety Authority (EFSA) has in their qualified presumption of safety (QPS) assessments concluded that Lactobacillus, Bifidobacterium and Propionibacterium are safe for consumption (EFSA 2013). However, it was pointed out that some case reports on immunocompromised patients have emerged relating to the consumption of a strain belonging to Bifidobacterium and Lactobacillus genera, especially L. rhamnosus. These reports did not change the QPS recommendation of either of the genera, but it is recommended that clinical infections including lactobacilli species should be closely monitored. Since probiotics are most often administered as live strains, their possible infectivity or toxin production has indeed raised questions (Sanders et al. 2010). This is largely because probiotics’ safety has not been as systematically and rigorously evaluated as drugs’ but has rather relied on long and safe history of use.
To substantiate the safety of probiotics, retrospective evaluations have been conducted. A retrospective study looking at *Lactobacillus* bacteremia incidence in Finland demonstrated that significantly increased GG consumption following the introduction of GG to food products in Finland in 1990 did not lead to rising number of *Lactobacillus* bacteremia cases (Salminen et al. 2002). Further evaluation of the *Lactobacillus* bacteremia cases led to the conclusion that no risk groups of immunocompromized patients could be identified (Salminen et al. 2004a). GG has also been used in clinical trials in immunocompromised subjects such as low birth weight infants (Luoto et al. 2014; Manzoni et al. 2011), and HIV-infected patients (Salminen et al. 2004b), without serious adverse events. Reports of three individual cases of sepsis with GG-like bacteria have been published on patients with short gut syndrome have been reported (De Groote et al. 2005; Kunz et al. 2004). However, 12 years’ of experience on the routine use of GG in premature infants with low birth weight has been reported, with no cases of GG septicemia (Luoto et al. 2010). Recently, an analysis was published pooling together adverse event data from six GG trials with the total study population of 1909 healthy subjects concluded that GG is safe to consume with no rise in adverse events (Tapiovaara et al. 2016b). In patients with weakened intestinal barrier function, immune compromised state or central venous catheter use, probiotic consumption should in any case be carefully considered (Sanders et al. 2010).

### 2.3 PROBIOTICS IN RESPIRATORY TRACT INFECTIONS

#### 2.3.1 METHODS TO STUDY CLINICAL EFFECTS OF PROBIOTICS

When the aim is to assess the *in vivo* effect of a probiotic in RTIs, double-blinded randomized controlled clinical trials (RCTs) represent the most precise approach for determining if a causal relationship exists. Clinical trial conduction is guided by the principles of Good Clinical Practise (ICH 1996) as well as by the ethical principles laid down in the Declaration of Helsinki (World Medical Association 1964), which is regularly updated, most recently in 2013 (World Medical Association 2013). Prior to RCT conduction, protocol and all study materials are to be evaluated by a local ethical committee. At the recruitment phase, subjects need be well informed of all study procedures and written informed consent needs to be obtained from all participating subjects. Subjects also have the right to withdraw their consent at any stage of the trial. In RCTs, the active ingredient, in this case the probiotic, is to be compared to a group receiving no probiotic supplementation, serving as the control group. To allow the evaluation of the effects of the probiotic strain alone, an ideal study product is in every other sense similar to the placebo.
product, except for the active ingredient. Subjects are placed in the intervention groups according to a predetermined randomization list, and both subjects as well as all study personnel in contact with the subjects or the data should be unaware of who receives probiotic and who placebo, to comply with the double blinding. To achieve this, it is also of importance that active and control products are unidentifiable from each other. If control group receives no product or active and control products can be differed from one another, study will be an open trial instead of blinded and of lesser value in terms of drawing conclusions especially regarding subjectively evaluated effects. In the analysis phase, ideally all randomized subjects should be included in the analysis for it to fulfill the criteria of intention-to-treat analysis, and thus to ascertain that possible uneven dropout rate in different groups does not affect the results.

The approach used for determining the efficacy of a probiotic on the clinical outcomes of RTIs has been to study naturally occurring infections. Even though RTIs are common, due to the rather modest effects that have been demonstrated for most probiotics on the individual level, conducting RCTs requires large populations, which can be difficult to recruit, and long interventions, even when conducted during the highest RTI incidence, from fall to spring. Also possible pathogen verification of the infections can be challenging to arrange in large and long studies. Infection-inducing challenge studies provide an alternative to long intervention studies, and allow more controlled aetiology and follow-up of the course of the RTI. This alternative has not been utilized in probiotic trials.

When the ultimate aim is to reduce personal and socio-economic burden of RTIs, the experienced symptoms, the patient-reported outcomes, are a key endpoint in assessing the effects of probiotics. The Jackson scale is one of the most commonly used instruments to define and to evaluate colds and flu in adults, including eight symptoms rated as absent, mild, moderate or severe (Jackson et al. 1958). The instrument can be used for both self-assessment and with clinicians or researchers assistance. Wisconsin Upper Respiratory Symptom Survey (WURSS-21 and WURSS-44) is designed for the evaluation of the illness-specific quality-of-life outcomes in adult cold sufferers (Barrett et al. 2009; Barrett et al. 2002). For measuring colds in children, the Canadian Acute Respiratory Illness and Flu (CARIF) scale has been developed and validated (Jacobs et al. 2000). Many RCTs use non-validated questionnaires for daily symptom follow-up, and varying criteria is applied to for instance which number or combination of symptoms is considered an RTI case.

Endpoints on the clinical manifestation of RTIs do not, however, provide understanding on how the symptom effects are originated. For both pathogen antagonism and immunomodulation, adhesion to mucosal surfaces
is critical for the probiotic effects (Bermudez-Brito et al. 2012; Collado et al. 2007), and in respiratory illnesses probiotic ability to persist in the respiratory tract is of specific interest. Pathogen presence and load in the nasopharynx is a logical endpoint to be measured in clinical trials in order to understand if probiotics can reduce the pathogen load or if they are specifically effective against certain pathogens. Task force set by the International Life Sciences Institute has concluded that pathogen-specific immune responses (specific antibodies and T cell responses after infection) and vaccine-specific immune responses (seroconversion, seroprotection, specific antibodies and T cells) are useful tools in assessing the possible clinical relevance and involvement of the immune system in clinical trials on defence against pathogens (Albers et al. 2013). Furthermore, pathogen loads were considered clinically relevant endpoint, whereas the majority of \textit{ex vivo} assays (pathogen-specific B and T cell functions, phagocyte function and NK cell function) were considered relevant only in limited populations and merely indicative of immune function involvement. For instance for acute-phase response markers (e.g. C-reactive protein, TNF, IL-1 and IL-6), pro- and anti-inflammatory mediators, and ex-vivo produced cytokines it was concluded that their clinical relevance is unsubstantiated, but they can, however, provide mechanistic information and are most useful when combined with the follow-up of the symptoms of infection (Albers et al. 2013). Preclinical data can further support building of the understanding of probiotic mechanisms in RTIs. Also factors related to probiotic basic properties should not be overseen.

\section*{2.3.2 SYMPTOM IMPACT IN CLINICAL TRIALS}

The earliest study addressing probiotics' ability to reduce RTIs is from 2001 (Hatakka et al. 2001). Since then, numerous strains have been studied for this indication. Results on the effects of the strains with at least two double-blind RCTs addressing symptom endpoints in RTIs, either as primary or secondary endpoints, are reviewed in Tables 2a-i, together with criteria used for the endpoints. Only interventions with probiotic identified to the strain level and probiotic effect addressed separately rather than in combination with other active ingredients were considered in this context. Altogether six individual strains and three strain combinations filled these criteria, with 29 RCTs, all preventive, conducted on them in total.
**Table 2a. Effects of *Lactobacillus rhamnosus* GG on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
</table>
| Luoto et al. 2014 | 45 preterm infants                           | Powder mixed to 10 mL of breast milk or formula for 2 months from birth (1-2 x 10<sup>8</sup> cfu/d) | **Symptomatic RTI episodes:** at least one of the following symptoms: fever (>38°C), rhinitis, or cough (recorded by parents).  
**Viral RTIs:** virus confirmed from nasal swab.  
**Severity of viral RTIs:** median of parent-recorded severity scores for symptoms in one episode (scale of 1-3). | During first year of life:  
RTI episodes: 26 vs. 62.  
Incidence of RTIs: 1.2 vs. 2.5, RR 0.56, 95% CI 0.28-0.90 (P=0.022).  
**Rhinovirus-induced episodes:** RR 0.49, 95% CI 0.24-1.00 (P=0.051).  
**Severity of rhinovirus infections:** 2 vs 2.  
Duration of rhinovirus infections: 9.7 vs. 10.7 days. |
| Hojsak et al. 2010a | 681 children attending day care               | 100 mL of fermented milk for 3 months (10<sup>6</sup> cfu/d)                  | **Subjects with RTI/URI/LRI:** diagnoses made by physicians.                                   | **Subjects with RTI:** 43.2% vs. 67.6% (P<0.001)  
**Subjects with URI:** 41.7% vs. 66.9% (P<0.001).  
**Subjects with LRI:** 2.9% vs. 3.5% (P=0.759).  
**RTIs lasting >5 days:** 28.1% vs. 49.3% (P<0.001).  
**Duration of respiratory symptoms:** median 0 (0→21) vs. 4 (0→22) days (P<0.001). |
| Hatakka et al. 2001 | 571 children attending day care               | On average 260 mL of milk for 7 months (1.3-2.6 x 10<sup>6</sup> cfu/d)       | **Days with respiratory symptoms:** parent-reported symptoms of fever, runny nose, sorethroat, cough, chest wheezes, earache.  
**Subjects with RTIs:** physician-diagnosed.                                                        | **Days with respiratory symptoms:** 21 vs. 23 (P=0.28; age adjusted P=0.67)  
**Subjects with RTIs:** 39% vs. 47% (P<0.05; age-adjusted P=0.13). |
| Hojsak et al. 2010b | 742 hospitalized children                    | 100 mL of fermented milk product for the duration of hospitalization (10<sup>6</sup> cfu/d) | **Subjects with RTIs:** physician-diagnosed.                                                 | **Subjects with RTIs:** 2.1% vs. 5.5%  
**RRTIs lasting >5 days:** 2.1% vs. 5.2% |
| Kekkonen et al. 2007 | 141 adult marathon runners                   | 2 x 65 mL of milk-based fruit drink for 3 months (3.9 x 10<sup>6</sup> cfu/d) | **Number and duration of URI episodes:** 21 symptom on ≥2 consecutive days, based on subject-reported symptoms of URI (fever, rhinitis, sore throat, cough, wheezing, earache); with ≥3 days required to separate 2 episodes. | **Subjects with URI episodes:** 46% vs. 37% (P=0.52).  
**Number of URI episodes:** 0.7 vs. 0.5 (P=0.32).  
**Duration of URI episodes:** 7.9 vs. 6.3 days (I=0.69). |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; LRI, lower respiratory tract infection; cfu, colony-forming units; vs., versus; CI, confidence interval; NNT, number needed to treat.
Table 2b. Effects of *Lactobacillus casei* DN-114001 on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
</table>
| Merenstein et al. 2010 | 638 children attending day care or school | 200 mL of fermented milk drink for 3 months (2 x 10⁶ cfu/d)* | **Incidence rate of URI/LRI:** parent-reported symptoms categorized by researcher as infections, calculated per 100 person-days. | **Incidence rate of URI:** 2.7 vs. 3.3 (P=0.636).  
**Incidence rate of LRI:** 2.7 vs. 2.8 (P=0.829). |
| Prodeus et al. 2016  | 599 children attending day care   | 2 x 100 mL of fermented milk drink for 3 months (2 x 10⁶ cfu/d)* | **Number of URIs/LRIs:** reported by parents.  
**Severity of infections:** mild/moderate/severe, reported by parents. | No differences between the groups in the RTI outcomes (data not reported). |
| Toller et al. 2007   | 47 male military training participants | 3 x 100 mL of fermented milk drink for 1 month (dose not stated)* | **Subjects with RTI episodes:** based on subject-reported symptoms of RTI (recorded symptoms not specified) on two consecutive days. | **Subjects with RTI episode:** 46% vs. 57% (P=0.46).  
**Incidence of RTI episodes:** 0.8 vs. 0.6 (P=0.48).  
**Number of days with symptoms:** 5.5 vs. 6.1 (P=0.67).  
**Number of symptoms:** 0.7 vs. 1.3 (P=0.23). |
| Guillemand et al. 2010a | 1000 shift workers                 | 2 x 100 mL of fermented milk drink for 3 months (2 x 10⁶ cfu/d)* | **URIs/LRIs:** subject-reported symptoms and investigators clinical assessment.  
**Severity of infection:** mild/moderate/severe based on interference with daily activity evaluated by the investigator. | No differences between the groups in any RTI endpoints (data not reported). |
| Guillemand et al. 2010b | 1072 free-living elderly       | 2 x 100 mL of fermented milk drink for 3 months (2 x 10⁶ cfu/d)* | **URIs/LRIs:** subject-reported symptoms and investigators clinical assessment, ≥2 days between two events.  
**Severity of infection:** mild/moderate/severe based on interference with daily activity evaluated by the investigator. | **Duration of URI episodes:** 7.7 vs. 11.0 days (P=0.004).  
**Cumulative duration of URI episodes:** 8.5 vs. 11.6 days (P=0.004).  
No data not reported for other RTI endpoints. |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; LRI, lower respiratory tract infection; cfu, colony-forming units; vs., versus.  
* Control product unfermented product, thus the role of fermentation or started cultures cannot be fully separated from probiotic strains effect, and blinding could be compromised.
Table 2c. Effects of *Bifidobacterium animalis* subsp. *lactis* Bb-12 on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
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</table>
| Tai pale et al. 2011; Tai pale et al. 2016 | 69/64* infants/children          | Tablets given twice a day with a slow-release pacifier or a spoon from 1 month until 2 y of age (10⁶ cfu/d) | **Cumulative incidence of RTIs:** 2 symptoms on 1 day or 1 symptom ≥ 2 consecutive days (runny nose, nasal congestion, cough and shortness of breath) reported by parents.  
**Cumulative incidence of AOM:** all acute ear infections (parent-reported physician-diagnosed cases). | During first 8 months / 2 years of life:  
**Cumulative incidence of RTIs:** 65% vs. 94%, RR 0.69, 95% CI 0.53, 0.89 (P=0.014) / 87% vs. 100% RR 0.87, 95% CI 0.76, 1.00 (P=0.033).  
**Cumulative incidence of AOM:** 26% vs. 17%, RR 1.54, 95% CI 0.62, 3.87 (P=0.455) / 61% vs. 64%, RR 0.96 95% CI 0.66, 1.41. |
| Weizman et al. 2005 | 133 infants/children attending day care | On average 640 mL of cow's milk formula for 3 months (dose not stated) | **Days with respiratory symptoms:** parent-reported symptoms of runny nose, cough, shortness of breath and fever. | **Days with respiratory symptoms:** 0.68 vs. 0.60.  
**Respiratory Illness episodes:** 0.25 vs. 0.24. |
| Hojsak et al. 2015a | 210 children attending day care | Powder mixed to milk product, juice or water for 3 months (1 x 10⁹ cfu/d) | **Subjects with RTI/URI/LRI:** physician-diagnosed | **Subjects with RTI:** 56.7% vs. 57.5% (P=0.905).  
**Subjects with URIs/LRIs:** no difference (data not reported).  
**Duration of RTIs:** 3 vs. 3 days (P=0.41). |
| Hojsak et al. 2015b | 742 hospitalized children | Powder mixed to 20 mL of water during hospitalization (10⁹ cfu/d) | **Nosocomial RTIs/URIs/LRIs:** pediatrician-diagnosed, occurring within 48 h from hospital admission.  
**Duration of RTI-symptoms. Severity of RTIs:** physician-assessed. | **Incidence rate of RTIs:** 0.44 vs. 0.33, IRR=0.76, 95% CI 0.41, 1.36 (P=0.32).  
**Subjects with RTIs:** 3.3% vs. 3.6% (P=0.86).  
**Number of URIs/LRIs:** no difference (data not reported).  
**Severity of RTIs:** 1.75 vs. 1.0 (P=0.24). |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; LRI, lower respiratory tract infection; AOM, acute otitis media; cfu, colony-forming units; vs., versus; RR, risk ratio; CI, confidence interval; IRR, incidence rate ratio.

* Analyzed number of subjects at 8 months / 2 years of age
Table 2d. Effects of *Lactobacillus casei* Shirota on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleeson et al. 2011</td>
<td>84 adult athletes</td>
<td>2 x 65 mL of fermented milk drink for 4 months (1.3 x 10^10 cfu/d)</td>
<td>URI-symptom weeks: weeks with subject-reported symptoms (sore throat, inflammation in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains, weakness, headache, loss of sleep). URI episodes: symptom score of ≥2 during the week and ≥1 week with symptom score of ≥2 in between two episodes. URI-symptom severity: subject-assessment for each symptom (1-3, light to severe).</td>
<td>URI-symptom weeks: 1.9 vs. 3.5 (P&lt;0.01). Subjects with URI-symptom weeks: 66% vs. 80% (P=0.021). Weeks with URI symptoms: 12% vs. 23% (P&lt;0.001). Number of URI episodes: 1.2 vs. 2.1 (P&lt;0.01). Severity of symptoms: 49 vs. 50 (P=0.928). Duration of symptoms: 7.9 vs. 7.6 days (P=0.801).</td>
</tr>
<tr>
<td>Shida et al. 2015</td>
<td>96 office workers</td>
<td>Fermented milk drink (amount not stated) for 3 months (1.0 x 10^10 cfu/d)</td>
<td>URI episodes: physician-evaluated, based on subject-recorded symptoms (fever, chill, headache, runny nose, stuffy nose, sneezing, cough, sore throat, sputum, malaise, muscular pain, joint pain), ≥2 symptom-free days separating 2 episodes. Incidence of influenza and common cold: URI classified as influenza based on virus test kit results; any other URI recorded as the common cold. Time to first URI: time-event analysis on the first episode. URI-symptom severity: subject-assessment for each symptom (none/light to severe).</td>
<td>Subjects with URI: 22.4% vs. 53.2% (P=0.002). Subjects with common cold: 18.4% vs. 44.7% (P=0.005). Subjects with influenza: 4.1% vs. 10.6% (P=0.201). Time to first URI: 0.47 vs. 0.78, hazard ratio 0.32 (95% CI 0.16–0.65). Number of URI episodes: 0.3 vs. 0.7 (P=0.64). Days with URI symptoms: 1.0 vs. 3.4 days (P=0.001). Duration per URI episode: 2.8 vs. 5.0 days (P=0.002). Mean severity score: 15.9 vs. 15.8 (P=0.966). Peak severity score: 17.4 vs. 18.2 (P=0.882).</td>
</tr>
<tr>
<td>Fujita et al. 2013</td>
<td>154 users of day care facilities for elderly</td>
<td>80 mL of fermented milk drink for 5 months (3.2 x 10^10 cfu/d)</td>
<td>Occurrence and duration of URIs: ≥1 caretaker-recorded day symptom (nasal/pharyngeal/bronchial symptom, headache, myalgia, conjunctivitis, fatigue or loss of appetite). Total symptom score: sum of the daily severity scores of nasal/pharyngeal/bronchial symptoms, headache, myalgia and conjunctivitis (0-6; none-severe), sneezing, fatigue, and loss of appetite (0/1: not present/present) and fever (6).</td>
<td>Occurrence of URI: 0.0066 vs. 0.0048 infection per observation day (P=0.89). Duration of infection per subject: 8.10 vs. 8.53 (P=0.83). Duration of infection per episode: 3.71 vs. 5.40 (P=0.037). Total symptom score: 0.0412 vs. 0.0372 per observation day (P=0.64).</td>
</tr>
<tr>
<td>van Puyenbroek et al. 2012</td>
<td>737 healthy elderly in nursing homes</td>
<td>2 x 65 mL of fermented milk for 6 months with influenza vaccination given after 3 weeks (≥1.3 x 10^10 cfu/d)</td>
<td>RTI symptoms: subject/care provider-recorded (runny nose, sore throat, fever, cough).</td>
<td>Days with RTI symptoms: 4.51 vs. 3.76 (P=0.342). Number of subjects with at least one day with symptoms: 46% vs. 42% (P=0.335).</td>
</tr>
</tbody>
</table>

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; cfu, colony-forming units; vs., versus.
Table 2e. Effects of *Lactobacillus fermentum* CECT5716 on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
</table>
| Maldonado et al. 2012 | 188 infants | On average 732 mL of follow-on infant formula from 6 months until 1 year of age (2 x 10^8 cfu/d). | **Incidence of RTI/URI/LRI/otitis:** pediatrician-diagnosed. | **Incidence of RTIs:** 1.093 vs. 1.473, IRR 0.74, 95% CI 0.580–0.957 (P=0.022).  
**Incidence of URIs:** 0.969 vs. 1.330, IRR 0.729, 95% CI 0.46, 1.38 (P=0.026).  
**Incidence of LRIs:** 0.124 vs. 0.143, IRR 0.87, 95% CI 0.40–1.90 (P=0.719).  
**Incidence of otitis:** 0.072 vs. 0.132, IRR 0.55, 95% CI 0.22–0.132 (P=0.177). |
| Gil-Campos et al. 2012 | 121 infants | Powdered infant formula from 1 month until 6 months of age (not stated). | **Incidence of RTIs:** pediatrician-diagnosed. | **Incidence of RTIs:** 0.689 vs. 0.716, IRR 0.977, 95% CI 0.623–1.530 (P=0.933). |
| Olivares et al. 2007 | 50 healthy adults | Capsule for 2 weeks before and after influenza vaccination (1 x 10^9 cfu/d) | **Influenza-like illness episodes:** fever with any systemic symptom (headache, myalgia, bone/joints pain, fatigue, anorexia, digestive disorder) and any respiratory sign (cough, nasal or pharyngeal symptoms) lasting for 23 days, based on subject-reported data. | During 5 months following vaccination:  
**Influenza-like illness episodes:** 25 vs. 40 (P=n.s.). |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; LRI, lower respiratory tract infection; cfu, colony-forming units; vs., versus; CI, confidence interval; IRR, incidence rate ratio.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatakka et al. 2007a</td>
<td>269 otitis-prone children</td>
<td>One gelatin capsule for 6 months (8–9 x 10⁹ cfu/strain/d)</td>
<td>URI episodes: ≥2 parent-reported symptoms (fever, earache, cough, rhinitis, sore throat, chest wheezing) during 1 day or 1 symptom during 2 days, with at least 7 asymptomatic days in between.</td>
<td>Number of URI episodes: 4.3 vs. 4.6 (P=n.s.).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Subjects with recurrent URI episodes (≥2/≥3/≥4/≥5/≥6): OR 0.50/0.67/0.56/0.87/0.59 (for ≥4 P=0.046; others P=n.s.).</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>Subjects with AOM: physician diagnosed.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Duration of AOM episodes: duration of respiratory symptoms after AOM diagnosis.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Subjects with recurrent AOM episodes (≥3): 18% vs. 17%, OR=1.04, 95% CI 0.87–2.52 (P=n.s.).</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Duration of AOM episodes: median 5.6 vs. 6.0 days (P=n.s.)</td>
<td></td>
</tr>
<tr>
<td>Hatakka et al. 2007b</td>
<td>265 elderly living in institutions</td>
<td>Two gelatin capsules for 5 months (6-12 x 10⁹ cfu/strain/d)</td>
<td>Number and duration of RTI episodes: based on nurse-recorded common cold symptoms (rhinitis, cough, sore throat and wheezing), with ≥7 asymptomatic days between 2 episodes.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Respiratory symptom days: number of days with at least one symptom.</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; AOM, acute otitis media; cfu, colony-forming units; vs., versus; CI, confidence interval; OR, odds ratio.
**Table 2g.** Effects of a combination of *Lactobacillus rhamnosus* GG, and *Bifidobacterium animalis* subsp. *lactis* Bb-12 on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rautava et al. 2009</td>
<td>72 infants requiring formula before the age of 2 months</td>
<td>Infant formula for 10-12 months (1 \times 10^{9}) cfu/strain/d</td>
<td><strong>Early RTI/AOM:</strong> age &lt;7 months, physician-diagnosed.  <strong>Recurrent RTI/AOM:</strong> physician-diagnosed, ≥3 before age of 1 year.</td>
<td><strong>Children with early RTI:</strong> 69% vs. 78%, RR 0.89, 95% CI 0.67, 1.18 (P=0.40).  <strong>Children with early AOM:</strong> 22% vs. 50%, RR 0.44, 95% CI 0.21, 0.90 (P=0.014).  <strong>Children with recurrent RTI:</strong> 28% vs. 55%, RR 0.51, 95% CI 0.27, 0.95 (P=0.022).  <strong>Children with recurrent AOM:</strong> 13% vs. 25%, RR 0.50, 95% CI 0.17, 1.45 (P=0.183).</td>
</tr>
</tbody>
</table>

| Smith et al. 2013 | 198 college students living on campus | 5 g of strawberry-flavoured powder for 3 months \(10^8\) cfu/strain/d | **Number and duration of URI episodes:** subject-reported feeling of sickness on ≥ 2 consecutive days, based on WURSS-21, with ≥7 asymptomatic days between ≥2 episodes.  **Severity of URI episodes:** subject-reported, based on WURSS-21. | **Number of URI episodes:** 84 vs. 83.  **Duration of URI episodes:** median 4.0 vs. 6.0 (P=0.001).  **Total severity score:** median 58 vs. 88 (P=0.0003). |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; AOM, acute otitis media; cfu, colony-forming units; vs., versus; CI, confidence interval; RR, risk ratio.

**Table 2h.** Effects of a combination of *Lactobacillus acidophilus* NCFM, and *Bifidobacterium animalis* subsp. *lactis* Bi-07 on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leyer et al. 2009</td>
<td>216 children attending day care</td>
<td>Powder added to 120 mL of milk for 6 months (1 \times 10^9) cfu/d including strains in 50:50 ratio</td>
<td><strong>Subjects with fever/cough/rhinorrhea:</strong> caretaker-reported.</td>
<td><strong>Subjects with fever:</strong> 16.1% vs. 63.3% (P=0.009).  <strong>Subjects with cough:</strong> 29.5% vs. 83.7% (P=0.005).  <strong>Subjects with rhinorrhea:</strong> 81.7% vs. 31.3% (P=0.03).  <strong>Subjects with any of the three symptoms:</strong> OR 0.55 (P=0.045).</td>
</tr>
</tbody>
</table>

| West et al. 2014 | 303 physically active adults | Powder dissolved to a beverage for 5 months \(1 \times 10^9\) cfu/d including strains in 50:50 ratio | **URI episodes:** For ≥3 days ≥2 symptoms (scratchy or sore throat, sneezing, stuffy or runny nose), with ≥2 between 2 episodes.  **Severity of episodes:** Subject-assessed as mild/moderate/severe based on impact on physical activity. | **Subjects with URI episode:** 35% vs. 45% (P=0.09).  **Rate of illness per month:** 0.026% vs. 0.035%.  **Time to first URI episode:** 3.4 vs. 2.5 months, hazard ratio 0.76 (P=0.14).  **Subjects with severe URI:** 16% vs. 20%. |

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; cfu, colony-forming units; vs., versus.
Table 2i. Effects of Lactobacillus fermentum VRI-003 (PCC) on symptoms of respiratory tract infections in randomized controlled double-blind clinical trials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Criteria for RTI endpoints</th>
<th>Results (probiotic vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>West et al. 2011</td>
<td>99 athletes</td>
<td>One capsule for 3 months (≥10^6 cfu/d)</td>
<td>Number and duration of URI episodes: ≥2 symptoms on ≥2 days with &gt;1 symptom-free day to separate 2 episodes, based on subject-reported symptoms (throat soreness, sneezing, a blocked or runny nose cough).</td>
<td>Males/females: Number, duration and severity of URI episodes: not reported. URI symptom load: ratio 0.66 (99% CI 0.23 to 1.78) / 1.78 (99% CI 0.96–3.37).</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>LRI episodes: as for URIs bit with chest congestion and wheezing, considered as symptoms.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Severity of symptoms: subject-assessed as mild/moderate/severe based on impact on training.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Symptom load: sum of symptom severity and number of days of the symptom per 100 days.</td>
<td></td>
</tr>
<tr>
<td>Cox et al. 2010</td>
<td>20 male elite distance runners (crossover design)</td>
<td>3 x 2 gelatin capsules with freeze-dried powder for 4 months (1.2 x 10^6 cfu/d)</td>
<td>RTI episodes: based on subject-reported symptoms of URI and LRI (sore throat, cough, runny nose, sneezing, chest congestion).</td>
<td>RTI episodes: 4 vs. 9 (P=0.24). Subjects with RTI episodes: 3 vs. 7 (P=0.27). Symptom days: 30 vs. 72 (P&lt;0.001). Severity of episodes: 1.0 vs. 1.7 (P=0.06).</td>
</tr>
</tbody>
</table>

Abbreviations: RTI, respiratory tract infection; URI, upper respiratory tract infection; LRI, lower respiratory tract infection; cfu, colony-forming units; vs., versus; CI, confidence interval.

Based on the trials on infants, both GG (Luoto et al. 2014) and B. animalis subsp. lactis Bb-12 (Taipale et al. 2011; Taipale et al. 2016), have shown promising effects in reducing RTIs but only in one trial. The combination of L. acidophilus NCFM and B. animalis subsp. lactis Bb-12 has shown some efficacy in one study (Rautava et al. 2009), and results for L. fermentum CECT5716 are conflicting (Gil-Campos et al. 2012; Maldonado et al. 2012).

In children, GG appears to be able to slightly reduce RTIs (Hojsak et al. 2010a; Hojsak et al. 2010b; Hatakka et al. 2001). Data from one trial supports the efficacy of the combination of L. acidophilus NCFM and B. animalis subsp. lactis Bi-07 (Leyer et al. 2009). The combination of GG, L. rhamnosus Lc705, B. breve 99, and P. freudenreichii supsp. shermanii JS has shown minor effects in one trial in children (Hatakka et al. 2007a). Results for L. casei DN-114001 are conflicting (Merenstein et al. 2010; Prodeus et al. 2016). B. animalis subsp. lactis Bb-12 has not been able to
reduce RTIs in children (Weizman et al. 2005; Hojsak et al. 2015a; Hojsak et al. 2015b).

In adult subjects, *L. casei* Shirota has shown most promise in reducing respiratory illness (Gleeson et al. 2011; Shida et al. 2015), *L. fermentum* CECT5716 (Olivares et al. 2007) and combination of GG and *B. animalis* subsp. *lactis* Bb-12 (Smith et al. 2013) have shown minor effects in single trials, whereas GG (Kekkonen et al. 2007), *L. casei* DN-114001 (Tiollier et al. 2007; Guillemand et al. 2010a), the combination of *L. acidophilus* NCFM and *B. animalis* subsp. *lactis* Bi-07 (West et al. 2014) or *L. fermentum* VRI-003 (Cox et al. 2010; West et al. 2011) have not been effective in adults.

Trials on the elderly have shown very minor to no effects with any probiotic. Both *L. casei* DN-114001 (Guillemand et al. 2010b) and the combination of GG, *L. rhamnosus* Lc705, *B. breve* 99, and *P. freudenreichii* supsp. *shermanii* JS (Hatakka) have shown minor positive effects in one trial. *L. casei* Shirota showed minor beneficial effect in one trial (Fujita et al. 2013) but was ineffective in the other (Van Puyenbroeck et al. 2012).

Out of the studies with only one RCT reported addressing RTIs, no effect was found for *L. casei* CRL431 (Agustina et al. 2012), *L. reuteri* DSM17938 (Agustina et al. 2012), *L. rhamnosus* HN001 (Cáceres et al. 2010), *L. rhamnosus* T cell-1 (Lin et al. 2009), *L. reuteri* SD2112 (Weizman et al. 2005), the combination of GG, *L. acidophilus* LA-5, and *B. animalis* subsp. *lactis* Bb-12 (Kloster Smerud et al. 2008) and the combination of GG and *B. animalis* subsp. *lactis* Bb-12 (Kalima et al. 2016). The following strains, with just one RCT conducted to date, gave at least some indication of potential in reducing respiratory symptoms: *L. reuteri* DSM 17938 (Gutierrez-Castrellon et al. 2014), *L. acidophilus* NCFM (Leyer et al. 2009), *L. delbrueckii* subsp. *bulgaricus* OLL0173R-1 (Makino et al. 2010), *B. animalis* subsp. *lactis* Bl-04 (West et al. 2014), *L. reuteri* protectis (Tubelius et al. 2005) *L. casei* 431 (Jespersen et al. 2015), and the combination of *L. gasseri* PA 16/8, *B. longum* SP 07/3, *B. Bifidum* MF 20/5 (de Vrese et al. 2006; de Vrese et al. 2005; Winkler et al. 2005), as well as the combination of *L. plantarum* HEAL-9 and *L. paracasei* 8700:2 (Berggren et al. 2011).

To conclude, data on the ability of specific probiotic strains to reduce RTIs is still limited as there is lack of trials confirming the findings of earlier trials with the same strain in similar populations and settings. It should also be noted that variety of RTI endpoints and criteria for these endpoints have been used in the studies, which makes it challenging to compare findings between trials. Furthermore, lack of studies comparing the effects of the most potential probiotics (strains for which efficacy is clinically demonstrated in earlier studies) is apparent. Comparative trials in RTIs have to date been conducted for comparing potential probiotic candidates to
strains with some data on clinical effects (Weizman et al. 2005; West et al. 2014). No dose-response studies assessing probiotic effects on RTIs have been conducted with any strain. Overall the research process of probiotics in RTIs, regarding clinical effect, effective dose, product matrix, mechanisms as well as safety has lacked systematicity that is present for instance in the phase process of pharmaceutical research.

2.3.3 RESPIRATORY PATHOGENS

Respiratory pathogen findings in clinical trials

Probiotic strains can possess properties able to inhibit the colonization of pathogenic micro-organisms (Bermudez-Brito et al. 2012). Probiotic effects on the upper respiratory tract or middle ear pathogen presence have been reported from six double-blind RCTs addressing the effects of two individual probiotic strains and one combinations of probiotic strains (Table 3). The results demonstrate that only GG and the combination of \( L. \text{rhamnosus} \ \text{GG} \), \( L. \text{rhamnosus} \ \text{Lc705} \), \( B. \text{breve} \ 99 \), \( P. \text{freudenreichii} \) subsp. \( \text{shermanii} \ \text{JS} \) have reached statistical significance between probiotic and control groups in reducing the presence of pathogens. RV-induced infections were fewer in the symptomatic infants who had received GG supplementation during the first two months of life compared to the control group (Luoto et al. 2014). The GG containing combination has reduced two of all the studied pathogens, \( M. \text{catarrhalis} \) and HboV, in asymptomatic otitis-prone children (Hatakka et al. 2007a; Lehtoranta et al. 2012). No effect has been demonstrated in respiratory pathogen reduction for any other probiotic strains or pathogen, but data is still limited for drawing of the final conclusions. In addition to the double-blind RCTs, an open prospective trial found that nasal colonisation of potentially pathogenic bacteria decreased slightly after the consumption of GG, \( B. \text{Bifidobacterium} \ \text{B420} \) (species not specified), \( L. \text{acidophilus} \ 145 \) and \( S. \text{thermophilus} \) (strain not specified) compared to placebo in healthy adults, but there were no significant differences between the probiotic and control groups (Glück and Gebbers, 2003).
Table 3. Upper respiratory tract and middle ear pathogens in double-blind randomized controlled clinical trials on probiotics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subjects &amp; intervention</th>
<th>Analyzed pathogens</th>
<th>Samples</th>
<th>Results, probiotic vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em></td>
<td>Preterm infants. Powder mixed to breast milk or formula for 2 months from birth (1-2 x 10⁸ cfu/d).</td>
<td>RV, EV, RSV (groups A and B), AdV, CoV, IFV (1-3), HBoV.</td>
<td>Nasal swabs taken from depth of 2-3 cm at home on days 1, 5, 10, and 15 of illness, and on study visits at the ages of 2, 4, 6 and 12 months analysed for viruses using nucleic acid testing.</td>
<td>During RTI / asymptomatic: <strong>Multiple viruses:</strong> 6 vs. 19 / 4 vs. 4. <strong>RV:</strong> 19 vs. 50 / 20 vs. 21. <strong>PIV3:</strong> 2 vs. 7 / 3 vs. 2. <strong>HBoV:</strong> 0 vs. 5 / 1 vs. 6. <strong>RSV A:</strong> 3 vs. 3 / 2 vs. 0. Other viruses detected max. 5 times. Symptomatic RV infection: <strong>RV load (day 1):</strong> median 5.9 vs. 5.6 log_{10} copies/sample. <strong>RV eradication:</strong> median 10-15 vs. &gt;15 days.</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>Children. 2 capsules mixed to dairy product for 3 weeks before adenotomy (2 x 10⁸ cfu/d).</td>
<td>RV, EV</td>
<td>Adenoid tissue collected during adenotonsilaval analysed for viruses using strain-specific real-time PCR.</td>
<td>RV: 4 (31%) vs. 3 (18%) (p=0.67). EV: 4 (31%) vs. 3 (18%) (p=0.67). <strong>Both RV and EV:</strong> 3 (60%) vs. 2 (40%) (p=0.63).</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>Children. 2 capsules mixed to dairy product for 3 weeks before tympanostomy (2 x 10⁸ cfu/d).</td>
<td>RV, EV, <em>H. influenzae</em>, <em>Neisseria</em> subsp., <em>S. aureus</em>, <em>S. pneumoniae</em>, <em>S. pyogenes</em>, <em>P. aeruginosa</em>.</td>
<td>Middle ear effusion samples, collected during tympanostomy, analysed for viruses using PCR for and bacterial pathogens using PCR and microarray assay.</td>
<td>RV: 10 (53%) vs. 2 (33%). EV: 1 (5%) vs. 0. <strong>Pathogenic bacterial DNA:</strong> 12 (63%) vs. 3 (50%) (p=0.65). <em>H. influenzae:</em> 10 (53%) vs. 2 (33%) (p=0.6). Only 3 samples with other bacteria than <em>H. influenzae</em>.</td>
</tr>
<tr>
<td><em>B. animalis</em></td>
<td>Children. Powder mixed to milk, juice or water for 3 months (1 x 10⁶ cfu/d).</td>
<td>Bacterial pathogens (details not provided).</td>
<td>Pharyngeal or nasal swabs collected and tested for bacteria when bacterial infection suspected (details not stated).</td>
<td>Pharyngeal swab: <em>S. Pyogenes:</em> 11 vs. 9. Nasal swab: <em>Pneumococcus:</em> 0 vs. 1 (p=0.283).</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>Otitis-prone children. 1 gelatin capsule for 6 months (8-9 x 10⁹ cfu/strain/d).</td>
<td><em>S. pneumoniae</em>, <em>H. influenzae</em>, <em>M. catarrhalis</em>, <em>S. Pyogenes</em>, HBoV (1-4).</td>
<td>Nasopharyngeal swabs samples collected from asymptomatic children through the nostril and analysed for bacterial pathogens with cultivation methods (end of the intervention), and for HBoV with RT-qPCR (mid and end of the intervention).</td>
<td><em>S. Pneumoniae:</em> 60 (47%) vs. 59 (47%) (p=n.s.). <em>H. Influenzae:</em> 29 (23%) vs. 22 (17%) (p=n.s.). <em>S. Pyogenes:</em> 0 vs. 1. <em>M. Catarrhalis:</em> 66 (52%) vs. 49 (39%) (p=0.028). HBoV (&gt;10,000 copies/ml; at either timepoint): 3 (6.4%) vs. 20 (19%), baseline-adjusted OR 0.29, CI 95% 0.07-0.94 (p&lt;0.039). No findings of HBoVt-4.</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>Military conscripts. 2 x 1 chewable tablet for 3 or 5-months (GG: 5 x 10⁸ cfu/d, BB-12, 2 x 10⁸ cfu/d).</td>
<td>IFV (A, B), RSV (A, B), PIV (1-4), AdV, MPV, CoV, EV, RV, HBoV.</td>
<td>Nasopharyngeal swab samples from asymptomatic subjects analysed for respiratory viruses by PCR-methods.</td>
<td>IFV A: 1 vs. 0 (p=0.46). IFV B: 2 vs. 0 (p=0.21). RSV: 1 vs. 0 (p=0.45). AdV: 0 vs. 2 (p=0.50). MPV: 0 vs. 3 (p=0.25). CoV: 3 vs. 9 (p=0.23). RV: 68 vs. 75 (0.51). EV: 9 vs. 11 (p=1.0).</td>
</tr>
</tbody>
</table>

Abbreviations: *L. Lactobacillus; B. Bifidobacterium; P. Propionibacterium; subsp., subspecies; H., Haemophilus; S., Staphylococcus/Streptococcus; M., Moraxella; P., Pseudomonas, IFV, influenza virus; RV, rhinovirus; EV, enterovirus; RSV, respiratory syncytial virus; AdV, adenovirus; MPV, metapneumovirus; PIV, parainfluenzavirus; HBoV, human bocavirus; CoV, coronavirus; PCR, polymerase-chain reaction; RTI, respiratory tract infection; n.s., not significant.
**Effect on pathogens in preclinical experiments**

Preclinical trials on lactobacilli and bifidobacteria strains have demonstrated that probiotics can have antagonistic activity towards e.g. *Escherichia coli*, *Salmonella*, *Helicobacter pylori*, *Listeria monocytogenes* and rotavirus. Probiotics can inhibit pathogen adherence by non-specifically covering receptor sites, as shown for instance for GG with *E. coli* and *Salmonella* subsp. (Lee and Puong 2002), or by competing for the same receptors. GG has also the ability to induce epithelial expression of mucin genes, which can inhibit adherence of *E. coli* to epithelial cells *in vitro* (Mack et al. 2003; Mack et al. 1999). Mucins on mucosal surfaces can potentially protect epithelial cells from pathogens either via supporting the physico-chemical barrier, or by binding to pathogenic bacteria or virus. For bacterial pathogens, combination of GG, *L. rhamnosus* Lc705, *P. freudenreichii* ssp. *shermanii* JS and *B. breve* 99 or *B. lactis* Bb12 was able to inhibit and to displace adhered pathogens by competitive exclusion (Collado et al. 2006), and same was shown for several individual commercial strains, such as GG, *L. rhamnosus* Lc705, *L. casei* Shirota, *L. acidophilus* NCFM, *B. lactis* Bb12, *B. breve* 99, and *P. freudenreichii* subsp. *shermanii* JS (Collado et al. 2007). The results highlighted that as strains differed in their ability to inhibit and exclude specific pathogens, case-by-case assessment would be beneficial when selecting probiotic to target specific pathogens. No similar comparative experiments have been reported for common RTI pathogens.

Probiotics may also produce antimicrobial substances, such as organic acids and antibacterial peptides, that can inhibit the growth of pathogens (Bermudez-Brito et al. 2012). It is also possible that probiotic strains release defensins, small peptides or proteins that are active against bacteria and viruses, from epithelial cells (Bermudez-Brito et al. 2012). Only a few *in vitro* trials have attempted to shed light to possible antagonistic activities of probiotics against respiratory viruses. Several *Bifidobacterium* and *Lactobacillus* strains have been shown to bind directly to a virus and thus inhibit its attachment to host cell in an eukaryotic cell culture mode (Botić et al. 2007). The same study demonstrated that tested probiotic strains secreted metabolic products that had direct antiviral effect. Studies on macrophages from animal cell lines have indicated that production of nitric oxide could also have a role in probiotics’ possible protective effects in RTIs (Ivec et al. 2007; Korhonen et al. 2001; Pipenbaher et al. 2009; Yeo et al. 2014). No data on such antagonistic effects is available for strains with clinical data in RTIs.
2.3.4 IMMUNOMODULATION

Immunomodulation in clinical trials

Few of the clinical probiotic trials on RTIs have attempted to shed light to the changes in immunological markers during the intervention. In healthy shift workers, in a study with no statistically significant effects in RTIs, two month consumption of L. casei DN-114001 did not affect hemogram parameters, C-reactive protein, NK cell count or activity, oxidative burst, or cytokine production analysed from blood samples (Guillemard et al. 2010a). However, when samples were taken during infections (both respiratory and GI infections combined), leukocytes, neutrophils, NK cell counts, and NK cell activity increased more, within the normal range, in the probiotic group compared to the control group. For leucocytes, the change was most emphasized during rhinopharyngitis, for NK cell count during all RTIs, rhinopharyngitis or sore throat, and for NK cell activity, during cases of sore throat. When the same strain was consumed by free-living elderly and a decrease in the duration of upper RTIs was observed, no difference was detected between the groups in the same biological and immunological parameters, but comparison of samples taken during an infection could not be done due to an insufficient number of infections (Guillemard et al. 2010b). A 3-month trial on L. casei Shirota on middle-aged office workers, in which probiotic reduced the infections of the upper respiratory tract, showed that after 1.5 months of probiotic consumption, NK cell activity was higher in the probiotic group compared to the control group, but difference was not observed at the end of the intervention (Shida et al. 2015). There were no differences in the salivary IgA or cortisol levels between the groups on either of the time points.

In heavily training adults consuming L. fermentum PCC® for three months was overall ineffective in reducing RTIs, but acute exercise-induced changes in anti-inflammatory (IL-1ra, IL-10), immuno-regulatory (IL-6) and pro-inflammatory (IL-8, granulocyte macrophage-colony stimulating factor, IFN-γ, TNF-α) cytokines were reduced in the probiotic group compared to the control group (West et al. 2011). However, no substantial differences were observed between the groups in the resting cytokine concentrations or in the mucosal immune measures of lactoferrin, lysozyme or IgA from pre- to post-supplementation, and no clear relationship between the reduced exercise-induced cytokine levels and symptoms could be identified. In another study on the same strain and in similar study population of athletes, where also symptom reduction was observed in the active group, probiotic consumption had no effect on salivary IgA or IgA1 concentrations compared to control group, and no statistically significant differences were observed in the measured cytokine responses (IL-4, IL-12, IFN-γ) even though it appeared that probiotic supplementation slightly attenuated the reduction in IFN-γ and IL-12 that was more apparent in the control group (Cox et al. 2010). In a
4-month study on *L. casei* Shirota on athletes, where RTI symptom and episode reductions were observed, saliva IgA concentrations were higher and monocyte counts slightly lower in the group receiving the probiotic compared to the control group after two and three months of supplementation (Gleeson et al. 2011). However, no differences were observed between the groups in the plasma concentrations of IgA and IgM, in other blood leukocyte counts than the monocytes, in lymphocyte subsets or in the stimulated whole-blood culture cytokine production. Based on this and the symptom findings, the authors concluded that probiotics ability to maintain saliva IgA levels could have a role in the reduction of the infection episodes of the upper respiratory tract observed in the trial. When hormonal as well as mucosal and cellular immune parameters were measured in commando training participants receiving *L. casei* DN-114001, no difference was observed in the RTI endpoints in the cell counts of leukocytes or lymphocytes, but a decrease in dehydroepiandrosterone sulfate, an immunostimulatory hormone, was observed in the probiotic group (Tiollier et al. 2007).

Few influenza vaccination trials have been performed in an attempt to reveal the possible capacity of probiotic consumption to modify humoral immune responses in human subjects. A clinical trial on GG demonstrated that the strain, given together with influenza vaccine, could enhance the immunogenicity of IFV H3N2 strain, but greater seroprotection was not observed for H1N1 and B strains (Davidson et al. 2011). Two clinical trials on *L. casei* DN-114 001 show that probiotic consumption increased seroconversion for IFV B virus, but statistically significant difference was not observed for H1N1 or H3N2 (Boge et al. 2009). *B. animalis* ssp. *lactis* Bb12 consumption led to an elevated vaccine-specific IgG antibodies in plasma and secretory IgA in saliva compared to control group in an influenza vaccine trial, but no difference was seen in plasma cytokines or innate immune parameters (Rizzardini et al. 2012). A four week consumption of *L. fermentum* CECT5716, and influenza vaccination given after two weeks of intervention, led to significantly higher antigen-specific IgA levels in the probiotic group at the end of the intervention, but no significant differences were detected between the groups in the antigen-spesific IgG or IgM (Olivares et al. 2007). In addition to changes in virus-neutralizing antibodies, increased TNF-α levels were observed in the probiotic group compared to the control group. In the elderly, the consumption of *L. casei* Shirota for three weeks before and five months following an influenza vaccination resulted in similar anti-influenza antibody titers in the probiotic and control groups when measured by the hemagglutination inhibition of A/H3N2 (Van Puyenbroeck et al. 2012). The humoral response to the vaccine, analysed by pre- and postvaccination geometric mean titres and rates of seroconversion and seroprotection, was also similar in both groups.
Immunomodulation in preclinical experiments

Results from numerous *in vitro* and animal experiments indicate that probiotics interact with mucosal immune cells and epithelial cells lining the mucosa, and via these cells modulate the mucosal immune system (Wells 2011). An important route for immunomodulation is via binding to pattern recognition receptors (PRR), such as Toll-like receptors, which can recognize microbe-associated molecular patterns (MAMPs), which then induce the production of innate effectors such as cytokines and chemokines. Balancing the production of the anti- and proinflammatory cytokines could be a key factor behind probiotics’ beneficial effects also in RTIs. Based on preclinical data, probiotics might also be able to affect epithelial signalling, T regulatory cells in the mucosa, the effector subsets of T cells, epithelial-associated DCs and macrophages, as well as humoral immunity, but little is still know about most of these interactions (Wells 2011). GG for example has demonstrated ability to modulate the cytokine expression patterns in human DCs and macrophages in several *in vitro* trials (Lehtoranta et al. 2012a; Miettinen et al. 2000; Veckman et al. 2004; Veckman et al. 2003).

In human macrophages, *L. rhamnosus* Lc705 induced IFN-dependent gene activation, correlating with the production of viral proteins and lower IFV A virus replication (Miettinen et al. 2012). A study on murine DCs demonstrated that *L. acidophilus* NCFM is able to induce the expression of viral defense genes (IFN-β, IL-12, IL-10) (Weiss et al. 2011). Several markers of cell-mediated immunity have been connected to infection endpoints in studies on animal models. Orally given *L. casei* Shirota has been shown to protect neonatal and infant mice against IFV infection, with increased pulmonary NK cell activity and IL-12 production in lymph node cells observed (Yasui et al. 2004). The same strain has been administered intranasally to mice in two studies, both of which demonstrated lowered IFV titers and increased mice survival, with increased IL-12, IFN-γ, TNF-α in mediastinal lymph node cells in the first study (Hori et al. 2001), and with increased NK activity of splenocytes and lung cells as well as induction of IFN-γ and TNF-α in nasal lymphocytes in the second study (Hori et al. 2002). Intranasally administered GG protected mice from IFV infection, with up-regulation of lung NK cell activity (Harata et al. 2010).
3 AIMS OF THE STUDY

The aim of this work was to study the clinical outcomes of *Lactobacillus rhamnosus* GG consumption in respiratory tract infections in healthy children and adults, as well as aspects that were hypothesized to have a role behind the clinical effects of the probiotic.

The specific aims were:
1. To investigate whether the intake of *L. rhamnosus* GG, as a single strain or as a part of a multispecies combination, would lead to the recovery of the probiotic in tonsil tissue in young adults (I)
2. To study the effect of long-term consumption of *L. rhamnosus* GG on respiratory tract infections in children attending day care (II)
3. To assess if a long-term consumption of *L. rhamnosus* GG can reduce the occurrence of common respiratory viruses in the nasopharynx in symptomatic children attending day care centers (III)
4. To assess the potential of human rhinovirus challenge model in probiotic trials and, using the model, to evaluate the effect of live and inactivated *L. rhamnosus* GG on prevention and symptom impact during rhinovirus infection in adults in pilot-scale (IV)
4 MATERIALS AND METHODS

4.1 SUBJECTS AND STUDY DESIGNS

Altogether three clinical trials were conducted, and one study was performed on a subgroup of one of the intervention studies. All three studies were double-blinded randomized, and placebo-controlled clinical trials. One of the studies had two parallel groups (control and live GG, study II) and two of the studies had three parallel groups (control, GG as single live strain and as the third group either inactivated GG in study IV or live GG together with three other live strains, *L. rhamnosus* Lc705, *B. breve* Bb-12 and *P. freudenreichii* supsp. *shermanii* JS in study I). Two of the studies, II and IV, were designed primarily for RTI follow-up. Study II followed naturally occurring infections in children attending day care during one infection season between September and May, and collected daily parent-reported respiratory symptom data as well as data from physician visits due to infections. Study IV utilized an experimental RV challenge model, in which subjects were inoculated mid-intervention with RV immunotype 39 (IND 12934) from an FDA-approved pool, administered in two inocula, 250 μl per nostril given 5-15 min apart. In this pilot study both subject and researcher recorded symptom data was collected, as well as several samples for the evaluation of the infection rates and endpoints related to it. Studies I and III were designed to address aspects that were hypothesized to have a role behind the symptom effects in RTIs, the recovery of GG in the upper respiratory tract in study I and the nasopharyngeal presence of respiratory viruses in study III. Study IV was also designed to assess the role of the viability of GG. Overview of study designs is presented in Figure 2 and of study populations in Table 4.
**Figure 2.** Overview of study designs of the randomized controlled trials. Abbreviations: GG, *Lactobacillus rhamnosus* GG; wk, week.

**Table 4.** Overview of study populations.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Subjects and setting</th>
<th>Number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Finnish tonsillectomy patients aged 18 to 30 years (mean 25)</td>
<td>Started the intervention</td>
</tr>
<tr>
<td>II</td>
<td>Finnish children aged 2 to 6 years (mean 4) attending day care</td>
<td>515</td>
</tr>
<tr>
<td>III</td>
<td>Subjects from study II who visited study physician due to symptoms of infection</td>
<td>n.a.</td>
</tr>
<tr>
<td>IV</td>
<td>American freeliving healthy adults aged 18 to 65 years (mean 23)</td>
<td>60</td>
</tr>
</tbody>
</table>

Abbreviations: GG, *Lactobacillus rhamnosus* GG; n.a., not applicable

In study II, the number of days with respiratory symptoms in a similar previous trial (Hatakka et al. 2001) was used to calculate the required sample
size, and 20% reduction in the symptom days was estimated to occur in the group receiving GG. The previous study was not directly used as the basis of a power calculation, as there were differences in the setup potentially affecting the outcome, such as the provision of milks also during absences from day care (compared to consumption only when in day care in the previous trial) and narrower age group. Thus, the total sample size of about 600 children (300 in each group) was estimated to be needed in order to detect a 20% difference between the study groups with a power of 85% and at a significance level of 0.05. Studies I and IV were pilot-scale trials, and in both studies it was estimated that sample size of 60 subjects (20 in each of the three groups) would give enough indication on if the issue is worth addressing in further larger trials with enough power for detecting a difference between the groups.

Subjects were recruited to the studies from patients on the waiting list for tonsillectomy (study I), via information delivered through day care centres participating in the study in Kainuu and Oulu regions (study II), and via advertisements in the local newspapers and e-mail distribution lists (study IV). Criteria for subject participation are presented in Table 5. In all trials, subjects were not allowed to participate in any other clinical trials during the present study, and they had to agree to follow the protocol. Recruitment of subjects for study I was done between October 2007 and November 2008. Study II was carried between September 2009 and May 2010, and study IV between August 2010 and November 2010.
Table 5. Inclusion and exclusion criteria in the trials.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Inclusion criteria</th>
<th>Excluding conditions or medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Age between 18 and 30 years, about to undergo tonsillectomy due to chronic or recurrent tonsillitis in the department of Otorhinolaryngology and Head and Neck Surgery at the Helsinki University Central Hospital.</td>
<td>Chronic gastrointestinal diseases, chronic sinusitis, allergy causing airway symptoms, alcohol or drug abuse, pregnancy, lactation, milk allergy, regular use of probiotic-containing products or antibiotics, or consumption of drugs associated with intestinal diseases, immunosuppressive drugs or inhaled asthma/allergy medications.</td>
</tr>
<tr>
<td>II, III</td>
<td>Age between 2 and 6 years, attending day care in the participating 60 day care centers in Kainuu and Oulu regions in Finland. All subjects who visited the study physician at least once were included in the study III population.</td>
<td>Milk allergy, lactose intolerance, congenital heart disease requiring regular medication, malignant diseases, diabetes, cytostatic treatment, use of biological rheumatic medication, continuous microbial medication, regular use of oral corticosteroids.</td>
</tr>
<tr>
<td>IV</td>
<td>Age between 18 to 65 years, healthy (stabilised chronic illnesses and regular medications accepted if not mentioned in the exclusion criteria), no previous participation in an experimental study with rhinovirus 39.</td>
<td>Significant allergic rhinitis, lower respiratory tract diseases, nasal abnormalities, pregnancy, lactation, history of alcohol abuse, drug abuse during the past 12 months, daily smoking within the past two years.</td>
</tr>
</tbody>
</table>

4.2 PROBIOTIC ADMINISTRATION AND DOSING

Subjects in all trials were randomly allocated to probiotic or control group according to a computer-generated randomization lists prepared by a statistician. In study I, randomization was stratified according to age and gender. Details of study products are presented in Table 6. In all trials, all groups consumed the same carrier product according to the same routine, the only difference between the groups being the probiotic supplementation of the active groups’ products according to the dosing specified in Table 6. In studies I and IV, products were only marked with study numbers and were otherwise blank. In study II, control and active products were packed in different coloured cartons, due to practical reasons, but consumed products had the same taste and appearance. In studies I and IV product doses were fixed, but in study II children consumed the study milks on three daily meals, without fixed amount. Prior to study it was estimated that children would consume on average 4.5 dl of milk per day (3 x 1.5 dl).
Table 6. Carrier products and probiotic supplementation in the active groups.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Product form</th>
<th>Consumption routine</th>
<th>Amount consumed daily</th>
<th>Carrier products for probiotic strains</th>
<th>Probiotic supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Capsules /yoghurt</td>
<td>Capsules mixed to a pot of yoghurt in the morning at home</td>
<td>2 capsules mixed to 150 g of yoghurt</td>
<td>GG alone 1. 2x10^10 cfu/capsule</td>
<td>1. GG alone 1. 2x10^10 cfu/capsule</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 capsules mixed to 150 g of yoghurt</td>
<td>GG with Lc705, PJS and Bb12 2. 1-5x10^9 cfu/capsule</td>
<td>2. GG 1x10^10, Lc705 1x10^10, PJS 6x10^9, Bb12 2x10^9</td>
</tr>
<tr>
<td>II, III</td>
<td>Milk</td>
<td>Served as a drink on three daily meals at the day care center and at home on days off</td>
<td>On average 4 dl</td>
<td>GG</td>
<td>GG 2x10^5 to 2x10^6 cfu/ml</td>
</tr>
<tr>
<td>IV</td>
<td>Fruit juice</td>
<td>Once a day in the morning at the study site</td>
<td>1 dl</td>
<td>1. live GG / 2. heat-inactivated GG</td>
<td>1x10^7 cfu/ml</td>
</tr>
</tbody>
</table>

Abbreviations: GG, Lactobacillus rhamnosus GG; Lc705, Lactobacillus rhamnosus Lc705; Bb12, Bifidobacterium breve Bb12; PJS, Propionibacterium freudenreichii supsp. shermanii JS; cfu, colony-forming units.

4.3 DATA COLLECTION

All data collection, including study product consumption follow-up, health data and collection of biological samples, is summarized in Table 7.
Table 7. Collection of biological samples and information on health and study product consumption in the clinical trials.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Study product use</th>
<th>Health data</th>
<th>Biological samples</th>
</tr>
</thead>
</table>
| I            | Counting of leftover capsules returned by the subjects. | **Study diary:** filed out daily by subjects including data on infections or other illness and medications (during intervention), and bleeding or pain at site of the operation and presence of fever (during follow-up after tonsillectomy). | Feecal samples: at the end of the run-in and intervention periods.  
Tonsil samples: from tonsillectomy at the end of the intervention period.  
Blood samples: 4 h after tonsillectomy. |
| II, III | Consumption at home recorded in study diary by parents. Consumption at day care recorded by day care personnel on each meal. | **Study diary:** filed out daily by parents including data on respiratory symptoms (fever, runny nose, stuffy nose, sore throat, cough, increased mucus production, wheezing, ear ache and eye discharge), medications, health care visits, absences from day care.  
**Health care visit forms:** from visits due to symptoms of infection filled out by treating physicians. | Feecal samples: at the end of the run-in and intervention periods.  
Nasopharyngeal swab samples: throughout the trial on study physician visits due to symptoms of infection. |
| IV | Study product consumption recorded daily at the study site (consumed under surveillance of study staff). | **Study diary:** filed out daily in the evening by subjects, including question on cold symptoms (sneezing, runny nose, stuffy nose plus sore throat, cough, headache, malaise and chilliness), severity of each symptom from the prior 24 h (five-point scale, ranging from zero (none) to four (very severe)), and subjective impression of having a cold.  
**Study site symptom follow-up:** same symptom follow-up conducted by study nurse prior to virus inoculation and each morning on the five days following virus inoculation. | Feecal samples: at the end of the run-in and intervention periods.  
Blood samples: just before the inoculation and at the end of the intervention.  
Nasal lavage samples: just before the inoculation, and on five days following the inoculation. |

During all interventions, the most important source of health data was subject-recorded symptoms, or in the case of children parent-recorded, and the symptom data was collected by means of paper study diaries (Table 7). In study II, the study diary used an unvalidated questionnaire of respiratory symptom presence, without the classification of symptom severity, which was based on a questionnaire used in the previous trial with a similar set-up (Hatakka et al. 2001). The parent-recorded data was supplemented by physician-recorded data from physician visits due to symptoms of respiratory or GI infection. To follow the normal procedures of when a child gets an infection as closely as possible, families were instructed to visit the study physician only in the cases in which they would normally take the child to a doctor due to symptoms of respiratory or GI infection. Study physicians worked at private health centres and were familiarized with the study protocol including filling out a structured questionnaire with detailed information of the visit, and collection and storage of an NPS sample. If
subject was taken to a physician who was not involved in the study, parents were requested to ask the physician to write information of the visit to the study diary. In study IV, in addition to subjects filling in study diaries at home in the evenings throughout the study, data on the same endpoints was recorded by a study nurse at the study site in the morning prior to the virus inoculation and on five mornings following this. Follow-up of study product consumption was done via study diaries, day care centre personnel and study site personnel.

Faecal, tonsil, blood, NPS and nasal lavage samples were collected during the trials (Table 7). Faecal samples were collected at home in all trials, and subjects or their parents were advised to deliver samples immediately to a study centre. In study II, if immediate delivery was not possible, instructions were to store the samples in a fridge for up to two hours or in a freezer for up to two days. In study I, two tonsils collected during the tonsillectomy from each subject were divided into ten parts, and frozen in liquid N₂. Venous blood samples were drawn four hours after the operation in study I, and in study IV, all serum samples were collected in fasting conditions. In study III, NPS samples were collected by a flocked-tip nylon swab, which was inserted into the nasopharynx through the nose. After collection the swab was placed immediately into a vial containing 3 mL of universal transport medium. To collect the nasal lavage specimens in study IV, 5 mL of 0.9% sterile saline was installed into each nostril with subject sitting head tilted back. Head was bent forward when saline started running towards the oropharynx, to allow the collection of lavage fluid to a cup. All samples were stored at -70°C until analysis.

4.4 CRITERIA FOR SYMPTOM-BASED ENDPOINTS

The criteria and data sources for endpoints based on respiratory symptom data collected in studies II and IV are presented in Table 8. In study IV, Jackson scale was used to score symptom presence and severity. The used method of symptom scoring and of diagnosing illness is based on a modification (Gwaltney 1992; Gwaltney et al. 1980) of a previously described method (Jackson et al. 1958).
Table 8. Criteria and data sources for endpoints on clinical manifestations of respiratory tract infections.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Endpoint</th>
<th>Data source</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Days with RTI symptoms</td>
<td>Parent-reported symptoms in study diaries</td>
<td>Number of days with at least one parent-reported RTI symptom.</td>
</tr>
<tr>
<td></td>
<td>RTI symptom episodes</td>
<td>Parent-reported symptoms in study diaries</td>
<td>Number and length of episodes defined as at least one RTI symptom on two consecutive days, or at least two symptoms on one day with at least seven days without respiratory symptoms between two episodes.</td>
</tr>
<tr>
<td></td>
<td>Physician-diagnosed RTIs</td>
<td>Physician recordings from health care visits</td>
<td>Number of children with at least one study physician-diagnosed RTI.</td>
</tr>
<tr>
<td></td>
<td>Antibiotic treatments</td>
<td>Parent recordings in study diary and physician recordings from health care visits</td>
<td>Number of children with parent and/or physician reported antibiotic treatments.</td>
</tr>
<tr>
<td>IV</td>
<td>Daily cold symptom score</td>
<td>Subject-reported symptoms in study diary</td>
<td>Daily scores for severity of all seven cold symptoms (each evaluated separately on a scale of 1-4), with data from all days before/after the inoculation combined by calculating the area under the curve, divided by the total time.</td>
</tr>
<tr>
<td></td>
<td>Post-inoculation symptom score</td>
<td>Study personnel recordings of symptoms on study visits</td>
<td>Daily symptom scores from the five days following the inoculation for severity of all seven cold symptoms (each evaluated separately on a scale of 1-4) with before virus challenge scores subtracted for each symptom.</td>
</tr>
<tr>
<td></td>
<td>Diagnosis of a cold</td>
<td>Evaluations done at the study site on the day of the virus challenge and on five days following the inoculation</td>
<td>Total symptom score of at least six (score for each cold symptom present before virus challenge subtracted from the daily score for the symptom, and scores for individual symptoms added together) and either the presence of rhinorrhoea on at least three days or the subjective impression of having a cold.</td>
</tr>
</tbody>
</table>

Abbreviations: RTI, respiratory tract infection

4.5 BACTERIAL, VIROLOGICAL AND IMMUNOLOGICAL ANALYSES

Real-time quantitative PCR of GG in faecal samples (I, II, IV)

Strain-specific real-time quantitative PCR assay was used for faecal samples to quantify GG according to a published method (Ahlroos and Tynkkynen 2009). Samples were analysed from all subjects who provided a sample both before and at the end of the interventions. The frozen samples were thawed, suspended in the ratio 1:10 with 50mM-EDTA in blender bags and
homogenised with a stomacher blender (Seward, Worthing, UK) for two minutes. The suspension was then diluted in the ratio 1:10 using 50 mM-EDTA, reaching final faecal dilution of 1:100. Following this, 1 mL of the dilution was centrifuged at 14 000 g for two minutes. Cells were collected and resuspended in 480 mL of 50 mM-EDTA, and 100 mL of 50 mg/mL lysozyme (Amresco, Solon, USA) and 20 ml of 50 U/mL (0.08 kat/l) mutanolysine were added (Sigma). The mixture was incubated at 37°C for one hour and then centrifuged for two minutes at 14 000 g. The cell pellet was extracted according to the manufacturer’s instructions with Wizard® Genomic DNA Purification Kit (Promega, Madison, USA), and purified DNA suspended in 200 mL of Tris–EDTA buffer. Genomic DNA from pure culture GG (cells from 1 mL of the culture collected and the DNA isolated using the same method) was used as a standard in quantitative PCR. The strain-specific detection limit for GG on the log₁₀ scale was 5.18 log₁₀ genome copies/g in study I, and 5.5 log₁₀ genome copies/g in studies II and IV.

Real-time PCR of GG in tonsil samples (I)

Tonsil aliquots of approximately 0.5 g were immersed in 480 mL of 50 mM-EDTA followed by cell lysis, according to the assay described for faecal samples. The samples were repurified with the Wizard® Genomic DNA Purification Kit (Promega) before PCR due to impurities caused by the blood contained in the samples. The DNA dilutions were made to 100 mg/mL, of which 50 ng was used to detect GG in real-time PCR. Because the tonsil samples were different in terms of the amount of tonsil surface and thus differences in the potential adhesion area of GG in each sample, quantitative analyses were not possible to perform.

Viral nucleic acid extraction and PCR assays for respiratory viruses in NPS samples (III)

Fourteen respiratory viruses were analysed from the NPS samples: RV, EV, AdV, RSV, IFV A (H1N1)pdm09, IFV A(H3N2), IFV B, PIV1–3, and HBoV1–4. BioSprint 96 One For All Vet-kit (Qiagen, Hilden City, Germany) was used to purify viral nucleic acids from 100 mL of NPS samples with an automated KingFisher ml purification system (Thermo Fisher Scientific, Vantaa, Finland). Viral RNA and DNA samples were tested using real-time PCR methods for RV and EV (based on TaqMan chemistry were), multiplex and singleplex real-time quantitative PCR assays for HBoV1–4 (Allander et al. 2007; Kantola et al. 2010), real-time or reverse transcriptase PCRs for AdV, IFV A and B, RSV, and PIV1–3 (Akinloye et al. 2011), and RT-PCR for the specific detection of IFV A(H1N1) pdm09 (Rönkkö et al. 2011). For HboV, samples with over 10 000 copies/mL were considered to be of high viral load, and those positive samples were included in the analyses.
Rhinovirus shedding in nasal lavage specimens by isolation in cell cultures (IV)

An aliquot of nasal lavage fluid was inoculated into two tubes of human embryonic lung fibroblast cells (MRC-5 or WI38). Samples were incubated in roller drums at 33 °C for 14 days, and identification of rhinovirus was done based on the development of a cytopathic effect (Couch 1990).

Serum neutralizing antibody response (IV)

Neutralising antibody response was tested from the sera collected before the inoculation and at the end of the study by a standard method (Hamparia 1979). A four-fold increase in antibody titer between the paired sera was considered a serologic response to the challenge virus.

Rhinovirus infection (IV)

If subject had at least one positive rhinovirus culture or at least a four-fold rise in antibody to the challenge virus, the subject was considered infected.

4.6 SAFETY

Adverse events during the trials were followed, recorded and evaluated throughout the whole interventions for continuous monitoring of safety. Adverse events were followed up by means of study diaries in trials I and II. In study IV, subjects visited the study site and were questioned on possible adverse events by study personnel daily during the intervention. In studies II and IV detailed adverse event forms were filled out for all adverse events.

4.7 STATISTICAL ANALYSES

The statistical analyses in studies were performed using STATA (StataCorp, Texas, USA) or SPSS (SPSS Inc, Chicago, IL, USA) software. Data are presented as counts and percentages, medians and interquartile ranges (IQR), or means and standard deviations (SD) or 95% confidence intervals (CIs).

In study I, Fisher–Freeman–Halton test was used for the comparisons between the groups for dichotomous and ordinal level outcomes, and Kruskal–Wallis test for continuous variables, and Dwass–Steel–Critchlow–Fligner test for pairwise comparisons. Kappa statistic (κ) and Jaccard similarity index (Chamberlain’s positive agreement) were applied for the comparison of the tonsil and faecal recovery of GG. In this analysis, the level of agreement is considered poor with κ<0.20, fair with κ = 0.21 to 0.40, moderate with κ=0.41 to 0.60, substantial with κ=0.61 to 0.80, and very
good with $\kappa > 0.80$. The 95% CI for Kappa statistic were obtained using bias correcting bootstrapping. In Jaccard index, the proportion of positive agreement (both faecal and tonsil sample are positive) is divided by all positive findings in either of the samples. Jackknife equation was used to calculate the 95% CIs for Jaccard index.

In study II, chi square test was used for the comparison of dichotomous outcomes between groups and Poisson regression models were used for analysis on the number of episodes and symptom days per month, incidence rate ratios (IRRs) and P-values between the groups.

In study III, chi-square test was used for categorical variables and the independent samples t-test, permutation test, or Wilcoxon rank sum test was used for a continuous variable in the demographic characteristics between the study groups. Poisson regression model was used for analysis of the number of symptom days per month (incidence rates) and P-value between groups concerning this endpoint. Permutation type probit regressions were used for the testing of differences between the two study groups in viral findings, with standard error adjusted for the number of children (194 clusters). The 95% CI for the number of symptoms were obtained by bias-corrected bootstrapping (2000 replications).

In study IV, chi square test, bootstrap type analysis of variance and Kruskall-Wallis test were used for the between-group comparisons depending on the distribution of the outcome. Generalising estimating equation models with exchangeable correlation structure and appropriate contrasts were used for the analysis of the repeated measurements. Data from different time points was combined by calculating the area under the curve (AUC) divided by the total time in question. No adjustment was made for multiple testing, because this information can be obtained, e.g. after Bonferroni, by multiplying the actual P-value by the number of comparisons made.

4.8 ETHICS

All studies were conducted according to the guidelines laid down in the Declaration of Helsinki (World Medical Association 1964), the updates that have followed, latest being from 2013 (World Medical Association 2013) and following good clinical practise. All procedures involving human subjects were approved by the local ethics committees (Ethics Committee of Helsinki University Central Hospital in study I, Ethics Committee of Joint Authority of Kainuu Region in study II/III, and Human Investigation Committee at University of Virginia in study IV). Subjects for the studies were volunteers who themselves or their parents gave a written informed consent to participate in the study.
5 RESULTS

5.1 BASELINE CHARACTERISTICS (I-IV)

In study I, the mean age of subjects was 24.5 years (24.4, 25.5 and 23.9 years in GG, multispecies and control groups, respectively), and 68% of the subjects were female. 21% of the subjects smoked at least weekly, and half of the smokers were in the control group. Subjects did not consume any regular medications that would be of relevance regarding the study endpoints, nor had they any diagnosed diseases.

In study II, the mean age of subjects was four years, and 47% of the subjects were girls in both groups. The mean number of siblings was 1.3 in both groups. The median number of months in day care was 24 and 20 months in the GG and control groups, respectively. Median duration of exclusive breastfeeding was four months and partial breastfeeding seven months in the GG group. The corresponding durations were three and eight months in the control group. 10% of children in the GG group and 6% in the control group had been diagnosed by a physician with atopic eczema earlier in life, but were no longer suffering from symptoms, where as proportions for diagnosed and still symptomatic children were 8% and 18% in the GG and control groups, respectively. Numbers of other allergic diseases and asthma were low, and they were similarly distributed between the groups. There was more adenoidectomies performed to children in the GG group (14%) compared to those in the control group (10%). Health of children during the past year preceding the trial was similar in both groups.

Study III presented a subpopulation of study II, including children with at least one visit to a study physician with NPS sample collected. There were no significant differences in the baseline characteristics between the GG and control groups in this subpopulation. Compared to the children in study II, the children in study III were slightly younger with mean age of 3.7 (SD 1.3) and 3.8 (SD 1.4) in the GG and control groups, respectively, and had also spent less time in day care at the start of the intervention (median duration 18 months in both groups).

In study IV, the mean age of subjects was 22.5 years (24.3, 23.3 and 22.5 years in live GG, inactivated GG and control groups, respectively), and 13% of the subjects were female.
5.2 PROBIOTIC RECOVERY IN FAECAL AND TONSIL SAMPLES (I, II, IV)

Recovery of GG in faecal samples (I, II, IV, including unpublished results)

Summary of received faecal samples and recovery of GG is presented in Table 9. Both samples (before and at the end of the intervention) were received from 43% of the subjects in study II, to up to 97% of the subjects in study IV. At the start of the intervention, GG was found in 63% (study I, with a one week run-in period), in 34% (study II, with a run-in period of 2-3 weeks), and in 12% (study IV, with a run-in period of three weeks) of analysed faecal samples. The number of samples with GG above the detection limit increased from the start to the end of the intervention in all groups receiving GG. GG concentration also increased in the positive samples in all groups receiving GG except for one group (study I, group receiving GG as part of a multispecies combination). Correspondingly, in the control groups the GG concentration decreased, except for study I.

In an ideal case in terms of evaluating the effect of the probiotic intervention, GG would be below the detection limit before the intervention in all subjects’ fecal samples in all groups, and above the detection limit at the end of the intervention in all of the subjects in the GG groups and below the detection limit in the control group. The subjects who filled these criteria were termed as the “completed cases”, and they represented one third of the subjects in study I, 59% in study II and 86% in study IV. In study II, the baseline characteristics of this subgroup were similar for children in the GG and control groups, but the age of the children differentiated this subgroup from the whole study population, as the children in this subgroup were older than the rest of the children in this study (mean 4.3 [SD 1.3] vs. 3.9 [SD 1.3], P=0.031).
Table 9. Summary of analysed faecal samples and faecal recovery of *Lactobacillus rhamnosus* GG in these samples analysed by strain-specific real-time quantitative PCR method (detection limits 5.2, 5.5 and 5.5 log_{10} genome copies/g for studies I, II and IV, respectively).

<table>
<thead>
<tr>
<th>Study number</th>
<th>Both samples received and analyzed, n (% of total trial n)</th>
<th>Before the intervention</th>
<th>End of the intervention</th>
<th>&quot;Completed cases&quot;%, % of analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG group</td>
<td>Control group</td>
<td>GG group</td>
<td>Control group</td>
</tr>
<tr>
<td>I</td>
<td>18 (90)</td>
<td>19 (95)</td>
<td>72</td>
<td>47</td>
</tr>
<tr>
<td>*</td>
<td>17 (82)</td>
<td>7</td>
<td>9.1</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>119 (47)</td>
<td>98 (39)</td>
<td>27</td>
<td>42</td>
</tr>
<tr>
<td>IV</td>
<td>18 (95)</td>
<td>20 (100)</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>^</td>
<td>19 (95)</td>
<td>6</td>
<td>6.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: GG, *Lactobacillus rhamnosus* GG

1 Concentration in samples with GG above the detection limit
2 In the GG group, GG below the detection limit before the intervention and above the detection limit at the end of the intervention and in the control group, GG below the detection limit in both samples
3 Group receiving *L. rhamnosus* GG as a part of a multispecies combination
4 Group receiving inactivated *L. rhamnosus* GG

Recovery of GG in tonsil samples (I) and its association with faecal recovery of GG (III)

In the tonsil samples, GG was recovered from 40% of the samples in the GG group, 42% in the multispecies group and 30% in the control group (P-value between the groups 0.79). Out of the 51 subjects who provided both tonsil and faecal samples at the end of the intervention, 80% (41 subjects) demonstrated a positive faecal recovery of GG, and of these 41% also demonstrated a positive recovery of GG in the tonsil tissue. Out of the 10 subjects with negative recovery of GG in faeces, none had GG recovered from the tonsil sample. Out of the six subjects in the control group, from whom GG was recovered in the tonsil tissue, GG was also found in the faecal sample in all subjects with the end of the intervention sample available (one of these six subjects failed to provide the sample). Furthermore, four of the six subjects had GG recovered from the faecal sample already at the start of the intervention. The observed agreement between faecal and tonsil GG recovery was 53%, with Jaccard index of 0.41 (95% CI 0.26, 0.57), and the κ-statistic of 0.22 (95% CI 0.10, 0.40).

5.3 EFFECT OF PROBIOTIC ON SYMPTOMS OF RESPIRATORY TRACT INFECTIONS (II-IV)

Results on clinical manifestations of RTIs in studies II and IV are presented in Table 10. No statistically significant differences were observed in either of the studies on the complete study populations.
Table 10. Symptom impact of *Lactobacillus rhamnosus* GG in respiratory tract infections in studies II (n=501) and IV (n=59).

<table>
<thead>
<tr>
<th>Study number</th>
<th>Endpoint</th>
<th>Control</th>
<th>Live GG</th>
<th>Inactivated GG</th>
<th>Between the group</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Days with RTI symptoms(^1) per month, mean (95% CI)</td>
<td>5.17 (5.05–5.29)</td>
<td>5.03 (4.92–5.15)</td>
<td>n.a.</td>
<td>IRR 0.97, 95% CI 0.64–1.00, P=0.098</td>
</tr>
<tr>
<td></td>
<td>RTI symptom episodes(^2) per month, mean (95% CI)</td>
<td>0.55 (0.52–0.59)</td>
<td>0.59 (0.55–0.63)</td>
<td>n.a.</td>
<td>IRR 1.06, 95% CI 0.96–1.16, P=0.24</td>
</tr>
<tr>
<td></td>
<td>Length of RTI symptom episodes(^2), median (IQR)</td>
<td>8 (5, 12)</td>
<td>8 (5, 12)</td>
<td>n.a.</td>
<td>P=0.25</td>
</tr>
<tr>
<td></td>
<td>Children with physician-diagnosed RTIs, n (%)</td>
<td>122 (49)</td>
<td>121 (48)</td>
<td>n.a.</td>
<td>P=0.89</td>
</tr>
<tr>
<td></td>
<td>Children with antibiotic treatments(^3), n (%)</td>
<td>86 (34)</td>
<td>89 (35)</td>
<td>n.a.</td>
<td>P=0.80</td>
</tr>
<tr>
<td>IV</td>
<td>Cold symptom score on the 3-wk pre-inoculation period(^4), mean AUC (95% CI)</td>
<td>0.47 (0.22–0.71)</td>
<td>0.16 (0.06–0.26)</td>
<td>0.24 (0.05–0.43)</td>
<td>P=0.064</td>
</tr>
<tr>
<td></td>
<td>Cold symptom score(^5) on the 3-wk post-inoculation period, mean AUC (95% CI)</td>
<td>2.09 (1.07–3.12)</td>
<td>1.25 (0.81–1.70)</td>
<td>1.49 (0.96–2.03)</td>
<td>P=0.33</td>
</tr>
<tr>
<td></td>
<td>5-day post-inoculation symptom score(^5), mean AUC (95% CI)</td>
<td>4.11 (2.77–5.44)</td>
<td>2.88 (1.51–4.25)</td>
<td>3.36 (2.02–4.69)</td>
<td>P=0.45</td>
</tr>
<tr>
<td></td>
<td>Diagnosis of a cold(^6), n (%)</td>
<td>13 (65)</td>
<td>12 (62)</td>
<td>13 (65)</td>
<td>P=0.99</td>
</tr>
</tbody>
</table>

Abbreviations: GG, *L. rhamnosus* GG; RTI, respiratory tract infection; CI, confidence interval; IRR, incidence rate ratio; AUC, area under the curve; n.a., not applicable.

\(^1\) Days with at least one symptom of RTI based on parent-recorded symptoms in study diaries

\(^2\) ≥1 symptom on 2 consecutive days, or ≥2 symptoms on 1 day with at least 7 days without symptoms between two episodes

\(^3\) Number of parent and/or physician reported antibiotic treatments

\(^4\) Sum of daily scores for severity (score of 1–4) of all 7 respiratory symptoms from the 3-week period before/after the inoculation

\(^5\) From the 5 days following the inoculation, the sum of daily scores for severity (score of 1–4) of all 7 respiratory symptoms with before virus challenge scores subtracted for each symptom

\(^6\) Total symptom score of at least 6 (score for each cold symptom present before virus challenge subtracted from the daily score for the symptom, and scores for individual symptoms added together) and either the presence of rhinorrhea on at least 3 days or the subjective impression of having a cold.

In study II, in the subgroup of completed cases in terms of recovery of GG in faecal samples (n=128), less days with at least one respiratory symptom was observed in the GG group compared to the control group (4.71/month [95% CI 4.52–4.90] vs. 5.67/month, [95% CI 5.40–5.94], IRR 0.83; [95% CI 0.78–0.88], P<0.001). In study III, in the subgroup of 194 children who visited the study physician at least once, there was significantly fewer days with respiratory symptoms in the GG group (6.48, 95% CI 6.28–6.68) compared to the control group (7.19, 95% CI 6.98–7.41) (P<0.001). There was no statistically significant difference in the duration of respiratory symptom
episodes related to a study physician visits (16 days [95% CI: 13–19] in the GG group vs. 18 days [95% CI 14–23] in the control group) or in the number of respiratory symptoms recorded by a study physician on a visit with NPS sample collection (3.6 [95% CI 3.3–3.8] in the GG group vs. 3.7 [95% CI 3.5–4.0] between the GG and control groups.

In study IV, on the three-week period preceding the rhinovirus challenge, there was a trend towards statistically significant difference between the groups in the total symptom score (P=0.064). All other illness endpoints were systematically lowest in the live GG group, but no statistically significant differences were detected. As this study was designed as a pilot-study, it was calculated that with a power of 80% and at a significance level of 0.05, 103 subjects would be needed to detect a difference in the symptom scores during the five days following the virus challenge. Symptom endpoints following the inoculation were also analysed for the subgroup of infected subjects (n=50). In this subgroup, the mean AUC values of the 5-day post-inoculation symptom scores were 4.51 in the control group (95% CI 3.12-5.91), 3.81 in the live GG group (95% CI 2.23-5.40) and 3.61 in the inactivated GG group (95% CI 2.21-5.01). During the 3-week post-inoculation period, the mean AUC value in the infected subjects was 2.28 (95% CI 1.21-3.35) in the control group, 1.63 (95% CI 1.21-2.06) in the live GG group and 1.63 (95% CI 1.07-2.20) in the inactivated GG group. There was no statistically significant difference in either of the analysed symptom scores in the infected subjects subgroup (P=0.65 for both comparisons).

5.4 EFFECT OF PROBIOTIC ON VIROLOGICAL AND IMMUNOLOGICAL FINDINGS (III, IV)

Nasopharyngeal findings of respiratory viruses in symptomatic children (III)

In study III, at least one NPS sample (mean 1, range 1–6) was collected from 194 symptomatic children, who then formed the subgroup for the virological analyses. The total number of collected NPS samples was 315 (159 in the GG group and 156 in the control group), and 202 (64%) of these samples were positive to at least one virus. Two or three viruses were found in 14% of the studied samples. No statistically significant differences were found in the number of children who gave at least one positive sample nor in the number of virus positive samples between the two groups. Procentual distributions between the groups in positive findings per virus, from most often detected virus to the least frequently found, are presented in Figure 3. There was more PIV1 detected in the GG group compared to the control group (P=0.035), but also non-significant trends towards less EV (P=0.083) and AdV (P=0.095) findings in the GG group compared to the control group.
Figure 3. Distributions between the groups in viral findings presented per virus in study III (the number of total findings of the virus in brackets). Abbreviations: RV, rhinovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; EV, enterovirus; IFV A, influenza A virus; ADV, adenovirus; HBoV, human bocavirus.

Association of viral findings with respiratory symptoms (III)
The mean duration of respiratory symptom episodes related to a study physician visit was similar in the GG group (16 days, 95% CI 13-19) compared to the control group (18 days, 95% CI 14-23) in the subgroup of 189 children from whom a study diary was received from the timing of the study physician visit (P=0.42). On the study visits where NPS samples were collected, the mean number of recorded respiratory symptoms was 3.6 (95% CI 3.3–3.8) in the GG group, and 3.7 (95% CI 3.5–4.0) in the control group. When numbers of respiratory symptoms associated with positive viral finding were analysed, no statistically significant differences were found between the study groups.

Viral cultures, antibody responses and infection rates (IV, including unpublished data)
In study IV, positive viral culture was found in 48 (81%) subjects (13/19 [68%], 18/20 [90%] and 17/20 [85%] in the live GG, inactivated GG and control groups respectively, P=0.20). Two of these positive viral cultures were identified as wild viruses (one subject in the inactivated GG group and one in the control group). An antibody response was observed in 31 (52%) subjects (9/19 [47%], 10/20 [50%] and 12/20 [60%] in the live GG, inactivated GG and control groups, respectively; P=0.70). Infection, defined as at least one positive culture on five days following inoculation and/or at
least four-fold rise in the antibody response to the challenge virus, occurred in 50 out of 59 (85%) subjects (14/19 [74%] in live GG group, and 18/20 [90%] in both inactivated GG and control groups, P=0.36). Of the infected subjects, 19 had rhinovirus shedding without sero-conversion and two subjects did not have rhinovirus in the nasal washes but sero-conversion occurred. It was calculated that, if the trend of lower infection rate in the live GG group holds true in a larger-scale trial, 102 subjects per group would be needed in order to detect a difference between the live and control group, with a power of 80% and at a significance level of 0.05.

5.5 COMPLIANCE (I-IV)

In study I, the capsules of 53 subjects were available for counting as four subjects failed to return the leftover capsules. Of these subjects, 79% consumed at least 95% of the advised amount of capsules during the intervention. In study II, the average daily milk consumption was 4 dl in both groups, based on the recordings by the day care personnel and parents’ reporting in the study diaries. In study IV, 99.8% of the daily study product doses were consumed, based on the recordings of the study personnel from the direct surveillance of the product consumption at the study site. Furthermore, the recovery of GG in faecal samples indicated good compliance as the number of subjects with GG recovered increased in all groups receiving GG in all trials when the baseline and the end of trial faecal samples were compared.

5.6 SAFETY (I-IV)

In study I, symptoms during the intervention and also after the intervention and operation were followed as a measure of safety. Data from 70% of the subjects were available for the analysis of the symptoms as the rest of the subjects failed to return the study diary. Respiratory symptoms occurred in 25% of the subjects (3/13, 3/12 and 4/15 in the GG, multispecies and control groups, respectively) and 10% of the subjects experienced GI symptoms (0/13, 1/12 and 3/15 in the GG, multispecies and placebo groups, respectively) during the intervention at least on one day, without differences between the groups (P=0.99 for respiratory symptoms and P=0.33 for GI symptoms). During the post-tonsillectomy follow-up period, there was no difference between the groups in the number of subjects experiencing post-operative bleeding or pain, nor in the number of days with bleeding or pain between the groups (P-values 0.32, 0.40, 0.99 and 0.62, respectively; data not shown). There was, however, statistically significant difference between the groups in the total number of subjects having a temperature of >37.5°C post-operatively (2/13, 4/12 and 0/15 in the GG, multispecies and control
groups, respectively; P=0.036). Furthermore, all post-tonsillectomy blood cultures were negative for bacterial growth.

In study II, altogether 22 adverse events were reported during the intervention, of which 15 were related to GI problems such as nausea or abdominal pain, and seven to skin problems such as rash. Of all the adverse events, eight occurred in the GG group (five GI related and three other) and 14 in the control group (10 GI related and four other). None of the reported adverse events were serious. In study IV, 105 adverse events were reported during the study, and 84% of these were cold symptoms occurring prior to rhinovirus challenge. None of the reported adverse events were assessed as having a probable relationship to the study product consumption and 16 were considered to have a possible relationship to the study product consumption. Of these 16 adverse events, nine occurred in the control group and seven in the live GG group. No serious adverse events were reported during any of the trials.
6 DISCUSSION

The series of studies presented in this work consists of three double-blind randomized controlled trials, all aimed at investigating the symptom impact or mechanistic aspects of the widely researched probiotic, *L. rhamnosus* GG, in respiratory tract infections. The clinical manifestation of infections, nasopharyngeal presence of respiratory viruses, and colonization of GG in the upper respiratory tract were addressed as primary endpoints, and also probiotic viability and effects as a single strain versus as part of a multispecies combination were explored in specific studies. Two of the RCTs were pilot-scale studies, and thus aimed at collecting preliminary data. One of these applied a novel clinical set-up in probiotic efficacy studies, experimental rhinovirus challenge model.

6.1 RECOVERY IN TONSIL TISSUE

The present study was the first RCT to address the pharyngeal recovery of a probiotic strain. At the time of the protocol development of study I, only one unrandomized and uncontrolled pilot study on six subjects had addressed the tonsil recovery of a *Lactobacillus* strain, and shown that *L. plantarum* DSM 9843 could be recovered from a tonsil surface four hours after oral intake in all subjects, and after eight hours in just one subject (Stjernquist-Desatnik et al. 2000). Furthermore, it had been previously demonstrated that GG possesses adhesive properties in intestinal gut mucosa (Alander et al. 1999; Alander et al. 1997). With this background, study I, a pilot-scale double-blind RCT was set-up to investigate whether an extended oral consumption of GG would lead to the recovery of the probiotic strain in the tonsil tissue, and whether the supplementation of GG as a single strain or together with three other probiotic strains would affect the adhesion. In this study GG was recovered in the tonsil tissue of less than half of the subjects who received GG as a single strain or as a part of the multispecies probiotic combination, but also in almost one third of the subjects in the placebo group. The recovery rates were similar for both single-strain and multispecies groups. Thus the present study did not give any indication that other probiotic strains would either boost nor hinder the adherence of GG in the tonsil tissue.

The finding that recovery of the probiotic in the upper respiratory tract did not occur in all the adult subjects after the oral consumption is in line with the findings from studies on other probiotic strains (Power et al. 2008; Stjernquist-Desatnik et al. 2000). It appears that there is individual variation in the tonsillar adherence, as in the present study the compliance of study
product consumption was good, confirmed by the analysis of faecal recovery of the strain and counting of leftover study products. On the contrary, in children three weeks GG consumption led to the recovery of the strain in all subjects’ adenoid tissue (Swanljung et al. 2015). Based on this it can be speculated that the adherence in the upper respiratory tract might be more effective in children, or that there are disparities in the adherence in different areas. Mechanisms of probiotic adherence in the upper respiratory tract mucosa have not been studied but it is likely that pili structures of GG have a crucial role in adherence similarly as in the gut (Lebeer et al. 2012). In the middle ear, GG has been recoverable in only fifth of the children (Tapiovaara et al. 2014). Mechanisms by which GG migrates from the nasopharynx to the middle ear are not known, but based on the recovery rates it appears that the invasion mechanism is not fully effective.

The high rates of GG recovery in the faecal and tonsil samples in the control group at the end of the intervention were unexpected, as only one subject with a positive faecal recovery and none with positive tonsillar recovery in the control group reported accidental one-time consumption of products containing GG. Furthermore, two thirds of the subjects in the control group with a positive tonsillar recovery of GG had GG recovered from the faecal sample already at the start of the intervention. This raises the speculation on the possibility of a persistent colonization of GG in the upper respiratory tract. GG has been documented to be recoverable from the faecal samples by PCR methods in almost one third of adults three weeks after consumption has ceased (Saxelin et al. 2010) and in one quarter of infants six months after the intervention (Gueimonde et al. 2006). Cases with GG colonization persisting up to 18 months (Gueimonde et al. 2006) and up to 24 months (Schultz et al. 2004) have also been reported. The finding of high nasopharyngeal recovery of GG in the control group is not unique. Even a higher recovery rate of GG was found in the control group in a study on adenoid tissue recovery in children, with similar study set-up to the present trial (Swanljung et al. 2015). In this study GG was recovered from three quarters of the control adenoid samples. It could be that the persistence of GG is stronger in children compared to adult subjects or persistence might differ in different parts of the upper respiratory tract mucosa.

Even though no studies have yet addressed the issue, it is plausible that nasopharyngeal probiotic colonization would have an effect on the microbiota residing in the upper respiratory tract. In terms of the intestinal microbiota, a follow-up study on the cohort from study II recently demonstrated that GG does influence the intestinal microbiota in children and also appears potent to prevent some the changes resulting from antibiotic use (Korpela et al. 2016). Understanding of the airway microbiota as a whole is still incomplete (Taylor et al. 2016). It has been concluded that the nasopharyngeal microbiota can have an impact on the risk of developing
RTIs (Biesbroek et al. 2014; Hasegawa and Camargo 2015). Furthermore, the severity of acute respiratory symptoms has been shown to be associated with the microbiota composition (Teo et al. 2015). It was recently presented that for instance RSV interaction with the nasopharyngeal microbiota modulated the host immune response and had an impact on the clinical disease severity (de Steenhuijsen Piters et al. 2016). The role of the airway microbiota, regulating local immune functions, and the gastrointestinal microbiota, affecting the respiratory immunity (Taylor et al. 2016) in RTIs and the possibility of probiotics to beneficially modify both is an interesting future research area.

**Methodological considerations**

Subjects recruited to study I were young adult patients about to undergo tonsillectomy due to chronic or recurrent tonsillitis. The subjects represented only a small age group of 18-30 year-olds, and results might differ in other age groups. Adhesion could be affected for instance by the tonsillar microbiota, which has been shown to be different in children and adults (Gaffney et al. 1991; Loganathan et al. 2006). Furthermore, the health status of the subjects in the present study, history of recurrent or chronic tonsillitis, is also known to impact the tonsillar microbiota (Jensen et al. 2013). Thus it cannot be outruled that the results could differ in subjects without any history of tonsillitis or in other age groups.

Due to the pilot nature of the study, it was short in duration, with intervention lasting only three weeks, and run-in period, without the consumption of probiotic products, for one week. This was considered adequate because events during the intervention were not an efficacy endpoint, and time from the start of the run-in to tonsil sample collection was four weeks, which was estimated as long enough for any probiotic colonization originating from the time preceding the trial to clear off. As high recovery rates were detected in the control group, a longer run-in period should be applied in future studies, but role of the unconscious GG consumption during the run-in and intervention periods cannot be ruled out as the source of GG colonization in the control group.

Regarding the detection method of GG in the tonsil samples, the original aim was to use strain specific GG quantification for tonsil samples, similarly as was done for faecal samples. However, when tonsil samples were collected, the tissue was divided into several pieces and surface and core were not identified. Therefore it was considered in the analysis phase that quantification would not give trustworthy results due to the possible differences in the amount of the tissue surface in the analysed pieces, and simple real-time PCR was carried out instead.
Taken together, GG can be recovered from tonsil tissue after oral consumption in some but not all adult subjects, and it thus appears that individual variation exists in the attachment to tonsil tissue. Colonization might also be persistent at least in some subjects. Combining GG with other strains does not seem to affect the adhesion rate. Due to the possible individual variation in the tonsillar colonization of GG, future studies combining the follow-up of respiratory tract infection endpoints to the analysis of GG recovery in the upper respiratory tract would be of interest.

6.2 SYMPTOMS OF RESPIRATORY TRACT INFECTIONS

Prior to the studies II and IV, the effects of GG in RTIs had only been addressed in one study on children (Hatakka et al. 2001) and one in adult marathon runners (Kekkonen et al. 2007). The present studies were conducted to clarify the findings of the previous trials, as well as to increase understanding behind the health effects, if these were to be observed. In the present studies, GG supplementation was not able to significantly affect the clinical manifestation of RTIs in healthy children attending day care (II), nor in healthy adults challenged to RV (IV). In children, there was no difference observed in the number of days with respiratory symptoms, nor in the number of episodes between the GG and control groups. However, when numbers of days with respiratory symptoms were explored in the “completed cases” subgroup of children based on the recovery of GG in faecal samples (II), or in the subgroup of children taken to a study physician during the intervention (III), children in the GG group had less symptomatic days. In adults, the study does not provide a definitive answer on the efficacy of GG in RV infections as it was designed as a pilot-scale trial and thus not powered to detect a statistically significant difference between the groups, but the symptom endpoints systematically favoured the group receiving live GG. Based on a post-trial power calculation, an RCT with over 100 subjects per group would be needed to detect a statistically significant difference in the symptoms scores between the live GG and the control group, assuming that the observed trends would hold true in the large-scale trial.

The results of the present study II are conflicting with the studies on GG that have demonstrated significant respiratory symptom reduction in children (Hojsak et al., 2010a; Hojsak et al., 2010b; Luoto et al., 2014), and more in line with the modest trends detected in one study (Hatakka et al. 2001). One possible explanation for conflicting results is the daily GG dose. The dose has been on average $10^8$ cfu per day in the interventions that have resulted in the more modest or no effects (Hatakka et al., 2001; study II) and $10^9$ cfu in the studies where clear benefits have been demonstrated (Hojsak et al., 2010a; Hojsak et al., 2010b). No dose-response studies have been conducted on
respiratory infections for any probiotic strain, and thus the effect of the dose can only be speculated. Dose-response of GG has been investigated in GI infections, but only using higher doses ($10^{10}$ and $10^{12}$ cfu/d), resulting in both doses being effective (Basu et al. 2009). For other strains with dose-response studies also with lower doses, it has been shown for instance that dose of $10^{10}$ cfu but not $10^8$ cfu per day of a four-strain probiotic combination was effective in reducing AAD (Ouwehand et al. 2014). Recent study on infants interestingly showed that daily GG supplementation of $10^9$ cfu/d only during the first two months of life was able to have an effect on RTI episodes during the first year of life (Luoto et al. 2014). Furthermore, a follow-up study on a subgroup of children from study II showed that GG appeared to prevent some bacterial infections for up to three years following the intervention, indicated by reduced antibiotic purchases (Korpela et al. 2016). In study II, in contrast to a previous trial on GG with similar trial setting (Hatakka et al. 2001), probiotic product was also given to subjects to be consumed at home on the days when a child was not attending day care, such as weekends, holidays and absences due to illness. It was hypothesized that the daily consumption would boost the health effect compared to consumption only in day care, but results of the present study do not support the hypothesis. These findings indicate that doses of at least $10^9$ cfu/d appear to be more effective, but more research is needed to identify the optimal dose and its long-term impacts in terms of health effects, feasibility and cost-effectiveness in RTIs.

The need to explore the “completed cases” subgroup separately in study II rose from the observed relatively high number of children with GG recovered from the faecal sample at the beginning of the intervention as well as numerous faecal GG detections in the control group at the end of the intervention. These findings were speculated to affect the results of the trial. It seems clear that the two to three week run-in period was not sufficiently long to clear off GG, and during the intervention children in the control group were either ingesting GG unconsciously via some other products, even though families had a list of products with GG to be avoided, or GG colonization persisted throughout the intervention in some children. In a 7-month GG intervention study, 9% and 15% of children in the control group had GG-like bacteria recovered from the faecal samples using cultivation methods at four and seven months, respectively (Hatakka et al. 2001). Furthermore, in children who had received GG during first six months of life, GG was still recovered in one quarter of the infants six months after the intervention, and few of these children still carried the strain 18 months after the intervention (Gueimonde et al. 2006). In this study, the subjects with GG recovered from faecal samples after the intervention had decreased frequency of atopic eczema at two years of age compared to the subjects without GG detected in the faeces (Gueimonde et al. 2006). In study II, the effects of GG supplementation were similarly more emphasized in the
“completed cases” subgroup. Not a lot is known about how the possible prolonged GG colonization affects the health outcomes in vivo. It has been shown that early life probiotic consumption can have long term impact on RTIs (e.g. Luoto et al. 2014), hypothesized to be incurred through timely immunomodulation during the “programming” of the immunologic phenotype, but it is not known if beneficial effects could be reflected as long-term probiotic colonization.

The children taken to a study physician at least once (III) had more days with respiratory symptoms compared to the total group of followed subjects (II), and the RTI episodes were longer, thus indicating that this subgroup represented children more effected by RTIs. In this subgroup of more symptomatic children, a significant reduction in the number of days with RTI symptoms was observed in children in the GG group compared to those in the control group, although there was no difference in the number of study physician consultations between the groups. This suggests that GG might incur some minor symptom reducing effects in children more prone to infections, even if effects are not significant enough to reduce more severe infections that require physician consultation. Previously a GG-including probiotic combination was unable to reduce AOM-episodes in otitis-prone children, but daily symptom data was not reported (Hatakka et al.). No other studies have addressed the effects of probiotics in RTI-prone subgroups, and thus future research on the issue is warranted, with follow-up and reporting of daily symptoms in addition to physician-diagnosed infections and infections episodes.

The results of the pilot-scale trial on adults (IV) implied that GG might be effective in reducing RTI symptoms also in healthy adults, but findings were not statistically significant and need to be assessed in an appropriately powered study. Previously, the effects of GG on respiratory symptoms in adults have only been addressed in marathon runners, where no effect was demonstrated (Kekkonen et al. 2007). Authors concluded that with the study being conducted during summertime when pathogen exposure is low, can the effect of GG, which is likely to be small, be impossible to see, even if it would exist. The post-trial power-calculation of study IV, which indicated that over 100 subjects per group would be needed to confirm if the observed trend between GG and control group would hold true in the large-scale trial, demonstrates that if GG has an effect in adults in reducing RTIs, the effect is likely to be small, requiring large study populations, even in the challenge model exposing all subjects to a virus. However, no difference between the groups was seen in the diagnoses of a cold, determined based on predefined symptom scoring and the subjective impression of having a cold. In terms of other probiotic strains, there is indication that some strains, most notably L. casei Shirota, could reduce RTIs significantly also in healthy adults (Shida et
al. 2015), but majority of probiotic studies on RTIs in adult populations have shown negative results.

In the present study (IV), respiratory illness endpoints were systematically on a lower level in the live GG group, and thus it suggests that inactivated GG is not as effective as the live strain. However, the pilot-scale of the study allows only hypothesis creation and no final conclusions can be drawn based on it. It is possible that the superiority of live GG would be mediated through the lower rhinovirus infection rate in the live GG group, as when results were analysed only for the infected subjects, live and inactivated GG were on similar levels in symptom scores, both groups still maintaining a lower level compared to the control group. Based on these findings it can be speculated that perhaps only live GG is able to prevent the infection altogether for instance by binding directly to the virus, whereas both live and inactivated forms are able to modulate the immune response and thus reduce the symptom impact of the viral infection. *In vitro* and animal models have previously suggested that similar immunomodulation patterns would occur with both live and inactivated strains (Li et al. 2009; Lopez et al. 2008; Zhang et al. 2005).

**Methodological considerations**

Subjects in studies II and IV did not have serious illnesses, and results thus apply to healthy population in the studied age groups. Subjects in study II were children aged two to six years old attending day care, thus covering the most significant age groups in day care, and making the study group representative of children attending day care in Finland in general. Despite randomization, there was slightly more atopic eczema in the placebo group compared to the probiotic group. A previous study has found that effect of GG is more pronounced in children with a history of allergic diseases (Hatakka, 2007b). Furthermore, there were slightly more adenoidectomies in the probiotic group compared to the placebo group. However, there were no differences in the infections rates between the groups during the past 12 months preceding the trial, and thus it can be presumed that groups were similar in terms of their proneness to RTIs. The median number of months children had attended day care before the trial was four months more in the GG group compared to the control group. However, results regarding the effect of the length of the day care attendance on RTIs are conflicting (Hatakka et al. 2010; Hurwitz et al. 1991), and thus it is difficult to conclude to which direction the difference could possibly have skewed the results. Subjects in study IV were healthy, mainly young adults. There is no indication that respiratory illness would be significantly different within adults of different ages (excluding the elderly), thus making the results relevant for healthy adult population in general.
In study II, the sample size estimated to be required to demonstrate a statistically significant difference between the groups could not be met. The intervention was set to start by latest at the beginning of October 2009 before the peak respiratory infection season. Owing to limited time available for the recruitment and unexpectedly challenging recruitment of subjects, the sample size goal could not be met despite extensive expansion of the study area and inclusion of altogether 60 day care centres. The number of randomized subjects was 87% and those with primary outcome data available 84% of the sample size that according to pre-study power calculation would have been needed to detect a statistically significant difference between the groups. Furthermore, the effect size was smaller than expected. Study IV was designed as a pilot-scale trial, and no power calculation was performed, and thus no statistically significant differences between the groups in the followed endpoints were expected. In study II, only subjects with at least one primary outcome data available, in practise meaning one study diary filled out by parents including the respiratory symptom data, were included in the analysis. The approach can be criticised for not fulfilling the intention-to-treat criteria. However, also excessive imputation of data should be avoided, and thus the exclusion of subjects from whom no primary outcome data was available from analyses was considered as the best option available for undistorted results.

The symptoms of respiratory tract infections were evaluated and reported by the parents of the children in study II and by both the subjects themselves as well as by the study nurse in study IV. In study II, the daily symptom questionnaire in the study diary was unvalidated. The CARIFS scale (Jacobs et al. 2000) would have been a validated option, but lack of awareness of the scale, due to rare use in clinical trials at the time, led to it not being considered as an option in the protocol design phase. In retrospect, it would have been a possible option, although linguistic validation to Finnish would have been required. It would also have been slightly more laborious for the families, which could have been a challenge due to the length of the trial. The chosen form of symptom monitoring was based on a study diary used in a previous trial with similar trial setting (Hatakka et al. 2001), and thus its usability had been tested in practise in a long intervention trial. Another drawback in the symptom follow-up was that severity of symptoms was not assessed, but merely if a symptom was present or not. In study IV, data on the common colds was collected using the Jackson scale (Jackson et al. 1958), which has been successfully used for decades in clinical trials investigating the common cold. From societal perspective, a drawback of the Jackson scoring is that it does not measure functional impairment or quality-of-life (Barrett et al. 2006). To overcome this the method could have been supplemented with the use of WURRS that specifically assesses illness-specific quality-of-life outcomes in adult cold sufferers (Barrett et al. 2009; Barrett et al. 2002). Overall challenge with symptom questionnaires and
their validation is that there is no gold standard for assessing RTIs (Barrett et al. 2006). However, strive towards the implementation of validated forms of RTI follow-up should be taken to increase the quality and comparability of the data.

One challenging aspect in RTI studies is defining infections. For subject-reported symptom data, the most common approach is to report either the occurrence of all respiratory symptoms or episodes, defined using varying criteria. In some studies physicians verify the infections, but this again can be done according to varying methods. Closer virus type follow-up has also shown that new episode can begin already during the previous one (Peltola et al. 2013), which can further complicate the interpretation of episode data and thus support symptom days as a preferable endpoint. In study II, symptom days, episodes and physician diagnoses were all reported to give a versatile picture of the illness rates in the study population. In study II, episode was defined by at least one respiratory symptom on two consecutive days or at least two symptoms on one day. Similar criteria have been applied also in other probiotic studies (Kekkonen et al., 2007; Tiollier et al., 2007; Taipale et al., 2011; Hatakka et al., 2007a). In addition, seven symptom-free days were required in between two episodes. Some studies have applied the same one week requirement (Hatakka et al., 2007a), but shorter periods, e.g. three asymptomatic days sufficing to separate two episodes have also been reported (Kekkonen et al. 2007), and many publications do not specify the length of this period at all, implying that possibly only one symptom-free day is considered enough to separate different episodes. The number of episodes detected in study II, approximately four during the whole 28-week follow-up during which most annual respiratory infections occur, is in line with the higher end of RTI incidence reported in previous trials for pre-school aged children (Chonmaitree et al. 2008; Lönnrot et al. 2015; Nokso-Koivisto et al. 2002; Shapiro 1998), but more than in some of the reports (Grüber et al. 2008; Monto et al. 2014). It is logical that the incidence is in the higher end of the scale as the subject were attending day care, which is known to increase the incidence of RTIs (Ball et al. 2002). These findings suggest that the episode criteria used in study II reflected the epidemiological findings.

To summarize, GG did not significantly reduce the clinical manifestations of RTIs. The results imply that in children, proneness to infections could increase and intestinal GG colonization originating from outside the trial minimize the possible small symptom impact of GG, but it was clearly shown that the used dose could not reduce the clinically defined infection cases. Use of a lower GG dose than what has been applied in previous studies on children with positive results is one possible factor behind the negative results. Preliminary pilot-scale results
suggest that live GG is a more promising option compared to inactivated strain in reducing RV infections in adults.

6.3 VIRAL PATHOGENS OF RESPIRATORY TRACT INFECTIONS

Study III was the largest probiotic trial based on the number of subjects to analyse viral aetiology including all the common causative viruses in RTIs, and study IV was the first viral challenge study addressing probiotic efficacy. No significant differences in viral findings were observed in the symptomatic children attending day care (III) or in the healthy adults challenged with RV (IV). In the child cohort, probiotic and control groups did not differ in the number of symptomatic children with at least one positive viral finding, the number of virus positive samples or the occurrence of most of the individual respiratory viruses analysed from the NPS samples (RV, RSV, IFV A (H1N1)pdm09, IFV A(H3N2), IFV B, PIV2–3, and HBoV1–4). PIV1 appeared to occur less frequently in the control group, and EV and AdV less frequently in the GG group. No difference was seen between the groups regarding the major pathogen, RV. Oppositely, a recent study on GG in preterm infants showed a significant reduction in RV-induced infections during the first year of life, but no effect was seen on the presence of other respiratory viruses (Luoto et al. 2014). In comparison to findings of study III, in the study by Luoto et al PIV1 was not recovered in any of the samples in symptomatic or asymptomatic infants, and no difference was observed between the groups in EV or AdV-findings. Similarly to the present study, in symptomatic military conscripts GG and B. animalis supsp. lactis Bb-12 combination did not reduce the detection rates of any of the analysed respiratory viruses (Lehtoranta et al. 2014). In asymptomatic children, GG has not had an effect on the presence of common respiratory viruses (Luoto et al. 2014; Swanljung et al. 2015; Tapiovaara et al. 2014), whereas probiotic combination containing GG has reduced HboV in otitis-prone children (Lehtoranta et al. 2012). In the present symptomatic cohort, no effect on HboV findings was seen.

In the adult population challenged with RV, the number of positive viral cultures and infection rate (defined by either positive viral cultures and/or rise in the antibody response) were lowest in the group consuming live GG, but the study was pilot-scale trial and difference was not statistically significant (IV). Inactivated GG had no effect on the before mentioned parameters. The recently demonstrated reduction in RV-induced infections in preterm infants makes this promising findings on live GG specifically interesting (Luoto et al. 2014). From the present adult study population (IV), also RV loads have been analysed, demonstrating the lowest RV loads in the live GG group (Tapiovaara et al. 2016a). Interestingly, no effect on RV load
was seen in the study on preterm infants (Luoto et al. 2014). Even though no effect was observed for GG in RV-reduction in the symptomatic children (III), further research to clarify ability to GG to reduce RV-induced infections or RV loads is warranted to clarify the conflicting results.

The nasopharyngeal microbiota can influence not just the pathogen colonization but also the disease process after pathogen colonization, such as symptom severity (de Steenhuijsen Piters et al. 2016; Teo et al. 2015). It has already earlier been demonstrated that upper respiratory tract microbiota has a role in the regulation of virulence that can occur also without differences in pathogen colonization (Roos et al. 2001). Thus it should be noted that lack of difference between the probiotic and control groups in the respiratory virus findings does not rule out clinical effects. In study III, symptom severity was not measured, but the fact that both groups consulted study physicians at equal frequency suggests lack of or at best minor differences in infection severities. Interestingly there was an overall lower symptom rate observed, but as no nasopharyngeal samples were taken outside the physician consultation, the present data is not able to clarify if differences in pathogen counts would have explained the symptom reduction in cases which did not lead to physician consultation. As this subgroup could be considered more prone to infections due to the higher symptom rate, it is of interest that in otitis-prone children, trend towards a lower number of HboV findings was detected in asymptomatic children consuming GG-including probiotic combination compared to the control group (Lehtoranta et al. 2012), even though the occurrence of AOM was not reduced in the same study population (Hatakka et al. 2007a). In that trial, differences in the daily symptoms were not reported by intervention groups, and thus findings cannot be fully compared to the present study. However, the majority of the results on GG’s effect on viral findings in asymptomatic children have been negative (Luoto et al. 2014; Swanljung et al. 2015; Tapiovaara et al. 2014).

Methodological considerations

Subjects in study III consisted of children who visited the study physician at least once, and this subgroup represented subjects suffering from more respiratory symptoms and longer episodes compared to the complete study population, thus representing children more affected by RTIs. The study was designed to follow the patterns of normal health seeking behaviour, and thus parents were instructed to take the child to a study physician only in the cases when they would normally seek consultation. It is therefore likely that many mild infections were treated at home, and are thus not represented in the analysed samples. It should, however, be noted that these factors affecting health seeking behaviours have most likely affected the two groups in a similar manner. It is also possible that some samples were missed if parents for instance due to practical reasons consulted a physician who was not involved in the study.
All study staff collecting specimens were instructed to take samples in a similar manner and to follow guidelines regarding sample storage and transportation. Analysis procedures used validated PCR-methods. Furthermore, the reliability of analysis results is further supported by the facts that findings of respiratory viruses in study III followed the typical seasonal pattern and in study IV infection rate achieved with the model in the control group was similar compared to the previous studies.

**Taken together, GG was not effective in reducing the overall presence of common respiratory viruses in the nasopharynx of symptomatic children. A pilot-scale RV challenge trial in adults suggested possibility for the effectiveness of live GG, but due to the small scale of the trial no firm conclusions can be drawn. Data in the area remains scarce and conflicting, GGs ability to reduce symptomatic RV infections appearing most promising. To clarify the data currently available, it would be of relevance to include viral detection also in future GG trials in RTIs, ideally also in the cases of mild infections and including the detection of viral loads.**

### 6.4 SAFETY

In study II, very few adverse events were reported and the majority of them occurred in the control group. In study IV, a higher number of adverse events were reported, likely due to the methodological differences, as in study IV subjects were asked daily by the study personnel of any adverse events and also cold symptoms occurring before the rhinovirus inoculation were reported as adverse events. However, no adverse events had a probable and only 16 has a possible relationship to the study product consumption, most of them occurring in the control group. Recently published analysis on the adverse events of six GG trials, one of which was study II, also demonstrated the safety of GG in healthy individuals (Tapiovaara et al. 2016b). In study I on tonsillectomy patients, more post-operative fever was observed in the GG groups, but no bacteraemia cases occurred in the study population. In a recent study, post-tonsillectomy bacteraemia was detected in 2.1% of patients (Shishegar and Ashraf 2014), and previously rates as high as 25% have been reported (Kaygusuz et al. 2001). In the earlier study, 70% of bacteraemia was caused by bacterium recovered on a tonsil surface and in deep tissue cultures (Kaygusuz et al. 2001). In the more recent study, the growth of the same potentially pathogenic bacteria were found in both blood and tonsillar tissue cultures, suggesting that bacteraemia could originate from the bacteria present in tonsillar tissue (Shishegar and Ashraf 2014). In study I, all post-tonsillectomy blood cultures were negative for bacterial growth, and further correlation of GG findings in tonsil tissue to blood cultures was not required.
To conclude, the results from this work support the safe use of GG in healthy subjects.

6.5 CONTEXT OF FINDINGS AND CONSIDERATIONS FOR FUTURE TRIALS

The sosio-economic burden of RTIs is high, due to their frequency of between two and five episodes in small children to approximately one in adults per year, and lack of effective preventive or treatment options available in the majority of the cases because of viral aetiology. For large part are symptoms of RTIs, such as runny and stuffy nose, sore throat and cough, merely a nuisance, but also a risk for a very severe illness is present, especially when the lower respiratory tract is affected. Physical interventions, especially hand washing, remain the central strategy for preventing the spread of the respiratory viruses, but such nutritional interventions that have a low risk for adverse effects and a low cost of implementation could have significant impact on the socioeconomic burden of RTIs on the population level, even if effects are modest on an individual level. Recent meta-analyses have concluded that probiotics could help to reduce RTI burden (Hao et al. 2015; King et al. 2014). Even economic modelling has been performed concluding a significant budget impact of probiotic consumption (Lenoir-Wijnkoop et al. 2015), but analysis of the individual studies reveals inadequate data regarding specific strains and target populations.

The results of this work on GG add to the conflicting data on the effects of the probiotic in RTI prevention and symptom alleviation. Instead of confirming the mainly positive previous findings of GG in children, study II showed no effect on the RTI outcomes on the total population of children. However, two explorative subgroup analyses showed almost one day less per month with respiratory symptoms, and thus further research is warranted, as GG remains the strain with most promising effects in children (Luoto et al. 2014; Hojsak et al. 2010a; Hojsak et al. 2010b; Hatakka et al. 2001) together with *B. animalis* subsp. *lactis* Bb12 that has recently demonstrated positive results in infants (Taipale et al. 2011; Taipale et al. 2016), but not in children (Hojsak et al. 2015a; Hojsak et al. 2015b). Study III showed no effect for GG on viral findings, even though the effect on rhinovirus findings was recently seen in infants (Luoto et al. 2014). Furthermore, study IV suggested, in a controlled setting, the possible ability of GG to have an effect against rhinovirus, even though the pilot-scale of the study did not allow drawing of any firm conclusions. Thus, also the aspect of pathogen counts should be clarified in new trials. Study I offered an interesting insight to the ability of GG to adhere to the pharyngeal mucosa in only part of the subjects; a factor
which role in responsiveness to probiotic supplementation in RTIs would be of interest to address in future trials.

Taking into account the distribution of the RTI burden and the results on GG, it would be of greatest relevance to see the probiotic research on RTIs focusing on infants and children in the future. It was recently demonstrated that only 2-month intervention in the very beginning of life could have effects on RTI outcomes during the first year of life (Luoto et al. 2014). These type of cost-effective and feasible supplementation strategies should be further explored and compared to continuous supplementation. Previous data on GG has shown that doses of at least $10^9$ cfu/day yield better results, and thus it is not advisable to use doses lower that this in future studies, and ideally a dose-response intervention should be carried out in the future. An often side-lined aspect in clinical trials is the manufacturing method of the study products, which can have an effect on the probiotic properties (Grześkowiak et al. 2011), and therefore rigorous quality control and reporting of production details in publications should be applied. The currently available data, to which study IV contributed, does not encourage the use of inactivated strain in future trials, even though data is still scarce.

Furthermore, since there are implications that probiotic consumption during infancy could affect the RTI outcomes (Luoto et al. 2014), if other population groups than infants are studied, data regarding probiotic use earlier in life is critical to collect. This type of data is naturally subject to recall bias, but as probiotics are freely available, there is no other means to control their use retrospectively. Even if infants are the target group and probiotic consumption preceding the study is not a challenge, a careful follow-up of probiotic intake other than the study product should be applied, especially if the trial is conducted in a country with high probiotic product availability such as Finland.

The adherence and persistence of GG recovery also deserve attention in future studies. As study I suggested, there might be individual variation in the probiotic adherence in the upper respiratory tract, and thus the effect of the adherence on the airway microbiota and the role in RTIs would be of interest to address. Also the assessment of faecal recovery is suggested, as it can possibly have an effect on the clinical outcomes, like study II preliminarily suggested. Furthermore, the run-in period of over three weeks without consumption of probiotic products is warranted if the aim is to clear off prior GG colonization. Study II demonstrated that approximately one third of the subjects still carried GG in faeces after the 2-3 week run-in period. Collection of both nasopharyngeal and faecal samples for GG quantification at several time points during the trial would be of interest to clarify the role of GG adherence, persistence of colonization and correlation to clinical RTI endpoints. Explorative subgroup analysis in study II indeed
suggested that clearing off the “uncompleted” cases based on faecal GG recovery from the study population, be it due to persistent colonization or incompliance, had an effect on the clinical outcomes.

Another challenging aspect in RTI trials, not just on probiotics but in general, is the wide variety of endpoints and criteria for these used in the studies, which makes the comparison of results challenging. First step to clarify this in the future would be the wider application validated questionnaires such as WURSS or CARIFS. The former is more targeted for measuring the illness specific quality of life, but the latter is specifically targeted for measuring colds in children. The next step would then be to harmonize the criteria for RTI endpoints. The present work also suggested that simply reporting days with symptoms is of interest in order to better understand the likely modest effects of GG in RTIs, and ideally also symptom severity assessment should be included.

To follow the results of the present work and other data published of GG in RTIs, and taking into consideration the challenges mentioned in this chapter, an ideal way forward in this research area would be to conduct a prospective randomized, controlled and double-blind birth cohort study with follow-up lasting throughout childhood to cover the years with the highest RTI burden. Preferably GG supplementation during only the first few months of life would be compared to continuous supplementation, although this would surely propose a challenge in the time scale suggested. In addition to the follow-up of the symptoms using a validated questionnaire, faecal and nasopharyngeal samples would be collected at regular intervals, to study persistence of GG colonization, its effect on the airway microbiota and its role in symptom endpoints. Ideally also infection aetiology would be assessed, in both mild and more severe cases. If overall results would be positive, economic evaluations of the interventions should be performed. Ideally societal perspective, which aims to look at the costs of an intervention to the society as a whole, should be applied in the evaluation. With appropriately calculated power, taking into consideration the relatively high drop-out rates of such study set-ups, this type of cohort would offer high-quality data to either substantiate or challenge the current understanding of potential modest benefits of GG consumption in reducing the symptom burden caused by RTIs, and if the approach would be cost-effective from a societal perspective.
7 CONCLUSIONS

The studies included in this work addressed the clinical outcomes of *Lactobacillus rhamnosus* GG consumption in respiratory tract infections in children and adults. Based on the results, the following conclusions can be drawn:

1. *L. rhamnosus* GG could be recovered from the tonsil tissue of young adults after three week oral consumption as a single strain or as part of a multispecies probiotic combination, but only in less than half of the subjects receiving the probiotic.

2. *L. rhamnosus* GG did not reduce respiratory tract infections in healthy children attending day care. This finding, partially conflicting with previous results from studies mainly on higher daily doses, needs to be clarified in further studies. Explorative analysis on specific subgroups, completed cases based on faecal GG recovery and subjects who visited a study physician due to an infection during the trial, suggested that GG could reduce the number of days with respiratory symptoms, warranting further research to clarify the role of GG colonization and susceptibility to infections.

3. *L. rhamnosus* GG did not reduce viral findings in the nasopharynx of symptomatic subjects, or reduce the number of symptoms associated with the viral findings.

4. The experimental rhinovirus model was demonstrated a functional controlled approach to studying probiotic efficacy in viral respiratory tract infections. The pilot-scale results favored the live *L. rhamnosus* GG over the inactivated form of the strain in reducing infections ans symptoms, but larger-scale trial would be needed to see if significant difference between the groups exists.
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