Probiotics in the prevention of clinical manifestations of common infectious diseases in children and in the elderly

Katja Hatakka

Department of Medicine
Division of Infectious Diseases
Helsinki University Central Hospital (HUCH)

Institute of Dentistry
University of Helsinki and
Department of Oral and Maxillofacial Surgery
HUCH

Valio
Research and Development
Helsinki, Finland

Academic Dissertation

To be presented by kind permission of the Medical Faculty of the University of Helsinki for public examination in Lecture Hall 2, Biomedicum Helsinki, Haartmaninkatu 8, on June 1st, 2007, at 12 noon.

Helsinki 2007
To Tomi,
and our children Enni and Olli
### CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF ORIGINAL PUBLICATIONS</td>
<td>9</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>2 REVIEW OF THE LITERATURE</td>
<td>15</td>
</tr>
<tr>
<td>2.1 Common infectious diseases in children and in the elderly</td>
<td>15</td>
</tr>
<tr>
<td>2.1.1 Acute respiratory tract infections</td>
<td>15</td>
</tr>
<tr>
<td>2.1.1.1 Etiology and pathogenesis</td>
<td>17</td>
</tr>
<tr>
<td>2.1.1.2 Clinical manifestation</td>
<td>18</td>
</tr>
<tr>
<td>2.1.1.3 Treatment and prevention</td>
<td>19</td>
</tr>
<tr>
<td>2.1.2 Acute otitis media</td>
<td>20</td>
</tr>
<tr>
<td>2.1.2.1 Etiology and pathogenesis</td>
<td>21</td>
</tr>
<tr>
<td>2.1.2.2 Clinical manifestation</td>
<td>22</td>
</tr>
<tr>
<td>2.1.2.3 Treatment and prevention</td>
<td>22</td>
</tr>
<tr>
<td>2.1.3 Diarrhoea</td>
<td>24</td>
</tr>
<tr>
<td>2.1.3.1 Etiology and pathogenesis</td>
<td>24</td>
</tr>
<tr>
<td>2.1.3.2 Clinical manifestation</td>
<td>25</td>
</tr>
<tr>
<td>2.1.3.3 Treatment and prevention</td>
<td>26</td>
</tr>
<tr>
<td>2.1.4 Oral Candida infections</td>
<td>27</td>
</tr>
<tr>
<td>2.1.4.1 Etiology and pathogenesis</td>
<td>27</td>
</tr>
<tr>
<td>2.1.4.2 Clinical manifestation</td>
<td>28</td>
</tr>
<tr>
<td>2.1.4.3 Treatment and prevention</td>
<td>29</td>
</tr>
<tr>
<td>2.2 Probiotics in the prevention of infectious diseases</td>
<td>30</td>
</tr>
<tr>
<td>2.2.1 Probiotics and their health effects</td>
<td>30</td>
</tr>
<tr>
<td>2.2.2 Normal microbiota against pathogens</td>
<td>33</td>
</tr>
<tr>
<td>2.2.3 Probiotic mechanisms</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3.1 Antagonism to pathogens</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3.2 Immunomodulatory effects</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.3 Mucosal defence mechanisms</td>
<td>43</td>
</tr>
<tr>
<td>2.2.4 Clinical effects of probiotics against infectious diseases</td>
<td>45</td>
</tr>
<tr>
<td>2.2.4.1 Respiratory infections</td>
<td>45</td>
</tr>
<tr>
<td>2.2.4.2 Infectious diarrhoea</td>
<td>47</td>
</tr>
<tr>
<td>2.2.4.3 Candida infections</td>
<td>51</td>
</tr>
<tr>
<td>2.2.5 Safety of probiotics</td>
<td>52</td>
</tr>
<tr>
<td>3 AIMS OF THE STUDY</td>
<td>54</td>
</tr>
</tbody>
</table>
4 MATERIALS AND METHODS ................................................................. 55

4.1 Subjects and study designs (I-IV) ....................................................... 55
4.1.1 Respiratory and gastrointestinal infections in healthy children (I) .......... 56
4.1.2 Respiratory infections and acute otitis media in otitis-prone children (II). 57
4.1.3 Respiratory and gastrointestinal infections in the institutionalised elderly (III). 57
4.1.4 Oral Candida in the independent elderly (IV) ...................................... 58

4.2 Administration and doses of probiotics (I-IV) ....................................... 60

4.3 Diagnostics of infectious diseases ......................................................... 61
4.3.1 Acute respiratory tract infections (I-III) ........................................... 61
4.3.2 Acute otitis media (I-II) ................................................................. 61
4.3.3 Diarrhoea and gastrointestinal infections (I, III) ............................... 62

4.4 Bacteriological and serological methods ............................................... 62
4.4.1 Detection of nasopharyngeal rhino- and enteroviruses (II) ....................... 62
4.4.2 Cultivation of nasopharyngeal bacterial pathogens (II) ......................... 62
4.4.3 Salivary pneumococcal IgA antibodies (II) ........................................ 63
4.4.4 Cultivation of oral Candida (IV) ..................................................... 63
4.4.5 Lactobacillus GG in faeces (I) ....................................................... 63

4.5 Statistical methods .............................................................................. 64

4.6 Ethics ..................................................................................................... 65

5 RESULTS ............................................................................................... 66

5.1 Baseline characteristics (I-IV) ............................................................... 66

5.2 The effect of probiotics on acute respiratory infections (ARI) (I-III) ......... 69
5.2.1 Occurrence of ARI (including unpublished results) ............................... 69
5.2.2 Prevalence of rhinovirus and enterovirus (II, unpublished results) .......... 72
5.2.3 Interaction of virus positivity and probiotic treatment on the occurrence of ARI (II, unpublished results) ............................................ 72
5.2.4 ARI with complications (I-III) ....................................................... 73

5.3 The effect of probiotics on acute otitis media (AOM) (I-II) ...................... 74
5.3.1 Occurrence of AOM episodes (I-II) .................................................. 74
5.3.2 Carriage of otitis pathogens (II). ................................................ ..... 76
5.3.3 Interaction of pathogen carriage and probiotic treatment on the occurrence of AOM (II, unpublished results) ............................. 76
5.3.4 Pneumococci-specific antibodies (II, unpublished results) ................... 78

5.4 The effect of probiotics on diarrhoea and gastrointestinal infections (I, III) . 79

5.5 The effect of probiotics on oral yeast (IV) .............................................. 80
5.5.1 Prevalence of high Candida carriage ................................................ 80
5.5.2 Salivary secretion and oral mucosal lesions ......................................... 82
ABBREVIATIONS

AAD  Antibiotic-associated diarrhoea
ANCOVA Analysis of covariance
AOM  Acute otitis media
ARI  Acute respiratory tract infection
Bb-12  *Bifidobacterium lactis* 12
Bb99  *Bifidobacterium breve* 99
CDAD  *Clostrium difficile* associated diarrhoea
Cfu  Colony-forming unit
CI  Confidence interval
COPD  Chronic obstructive pulmonary disease
DC  Dendritic cell
GI  Gastrointestinal
GALT  Gut-associated mucosal tissue
HR  Hazard ratio
IBS  Irritable bowel disease
ICAM  Intercellular adhesion molecule
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
IQR  Inter-quartile range
ITT  Intention-to-treat
LGG  *Lactobacillus rhamnosus* GG
LC705  *Lactobacillus rhamnosus* LC705
LRI  Lower respiratory tract infection
MALT  Mucosa-associated lymphoid tissue
Md  Median
NK cell  Natural killer cell
OR  Odds ratio
PBMC  Peripheral blood mononuclear cell
PFGE  Pulsed-field gel electrophoresis
PCR  Polymerase chain reaction
PJS  *Propionibacterium freudenreichii* subspecies *shermanii* JS
PspA  Pneumococcal surface protein A
PhtD  Pneumococcal histidine triad
SD  Standard deviation
sIgA  Secretory immunoglobulin A
ssp.  Subspecies
TGF-β  Transforming growth factor-β
Th  T helper
TNF-α  Tumour necrosis factor α
URI  Upper respiratory tract infection
vs.  Versus
wk  Week
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:


The original articles are reprinted with the kind permission of the copyright holders.
ABSTRACT

Infectious diseases put an enormous burden on both children and the elderly in the forms of respiratory, gastrointestinal and oral infections. There is evidence suggesting that specific probiotics may be antagonistic to pathogens and may enhance the immune system, but the clinical evidence is still too sparse to make general conclusions on the disease-preventive effects of probiotics. This thesis, consisting of four independent, double-blind, placebo-controlled clinical trials, investigated whether Lactobacillus GG (LGG) or a specific probiotic combination containing LGG would reduce the risk of common infections or the prevalence of pathogens in healthy and infection-prone children and in independent and institutionalised elderly people.

In healthy day-care children, the 7-month consumption of probiotic milk containing Lactobacillus GG appeared to postpone the first acute respiratory infection (ARI) by one week (p=0.03, adjusted p=0.16), and to reduce complicated infections (39% vs. 47%, p<0.05, adjusted p=0.13), as well as the need for antibiotic treatment (44% vs. 54%, p=0.03, adjusted p=0.08) and day-care absences (4.9 vs. 5.8 days, p=0.03, adjusted p=0.09) compared to the placebo milk. In infection-prone children, the 6-month consumption of a combination of four probiotic bacteria (LGG, L. rhamnosus LC705, Propionibacterium freudenreichii JS, Bifidobacterium breve 99) taken in capsules appeared to reduce recurrent ARIs (72% vs. 82%, p<0.05; adjusted p=0.06), and the effect was particularly noticeable in a subgroup of children with allergic diseases (12% vs. 33%, p=0.03), although no effect on the presence of nasopharyngeal rhinovirus or enterovirus was seen. The 5-month consumption of the same probiotic combination did not show any beneficial effects on the respiratory infections in frail, institutionalised elderly subjects.

In healthy children receiving Lactobacillus GG, the reduction in complications resulted in a marginal reduction in the occurrence of acute otitis media (AOM) (31% vs. 39%, p=0.08; adjusted p=0.19), and the postponement of the first AOM episode by 12 days (p=0.04; adjusted p=0.09). However, in otitis-prone children, a probiotic combination did not reduce the occurrence of AOM or the total prevalence of common AOM pathogens (Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis), except in the case of children with allergic diseases, in whom probiotics reduced recurrent AOM episodes (0% vs. 14%, p=0.03). In addition, interaction between probiotics and bacterial carriage was seen: probiotics reduced AOM in children who did not carry any bacterial pathogens (63% vs. 83%), but the effect was the reverse in children carrying bacteria in the nasopharynx (74% vs 62%) (p<0.05).

Long-term probiotic treatment, either LGG given in milk to healthy children for 7 months or a combination of probiotics given in capsules to institutionalised elderly subjects for 5 months, did not reduce the occurrence of acute diarrhoea. However, when the probiotic combination (LGG, L. rhamnosus LC705, Propionibacterium JS) was given in cheese to independent elderly subjects for 4 months, the oral carriage of high Candida counts was reduced in the probiotic group vs. the placebo group (21% vs. 34%, p=0.01, adjusted p=0.004). The risk of hyposalivation was also reduced in the probiotic group (p=0.05).

In conclusion, probiotics appear to slightly alleviate the severity of respiratory infections by postponing their appearance, by reducing complications and the need for antimicrobial
treatments. In addition, they appear to prevent recurrent infections in certain subgroups of children, such as in infection-prone children with allergic diseases. Alleviating ARI by probiotics may lead to a marginal reduction in the occurrence of AOM in healthy children but not in infection-prone children with disturbed nasopharyngeal microbiota. On the basis of these results it could be supposed that *Lactobacillus* GG or a specific combination containing LGG are effective against viral but not against bacterial otitis, and the mechanism is probably mediated through the stimulation of the immune system. A specific probiotic combination does not reduce respiratory infections in frail elderly subjects. Acute diarrhoea, either in children or in the elderly, is not prevented by the continuous, long-term consumption of probiotics, but the consumption of a specific probiotic combination in a food matrix is beneficial to the oral health of the elderly, through the reduction of the carriage of *Candida*. 

**ABSTRACT**
1 INTRODUCTION

The intestinal normal microbiota is a metabolically active organ, which plays a significant role in the maturation and function of the immune system in the gut. Microbial colonisation begins at birth, is affected by the mode of delivery and the infant's diet, and by the age of two years gradually alters to resemble that of an adult. The mature intestinal microbiota provides a physical and immunological barrier against microbial and other harmful exposures from the environment (Salminen et al., 2006). The microbiota is continuously interacting with the environment, including other bacteria, the gut epithelium, and the mucosal and endocrine systems. By means of these interactions the microbiota resists the colonisation of pathogens, participates in the elimination of foreign antigens, and regulates the immune responses. In a diseased state, the healthy host-microbe interaction is disturbed, leading to increased intestinal permeability, inflammatory immune responses, and infection.

Probiotic therapy is based on the concept of maintaining a healthy, balanced microbiota. The term 'probiotic', meaning 'for life', initially comes from the Greek. Probiotics, in the form of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in fermented milk, have been ingested by humans for thousands of years. The original concept of probiotics originated over a hundred years ago, when the Russian Nobel Prize winner Elie Metchnikoff hypothesised that the longevity of Bulgarian peasants was the result of their consumption of fermented milk products. Since then, attention has increasingly been directed towards the possibilities of probiotic therapy for improving human health. Accumulating evidence suggests that administration of probiotics, either as mono- or mixed cultures, can be used to optimise the gut microbiota and to prevent and treat different diseases.

Infectious diseases, including oral, respiratory and gastrointestinal infections, are a major health problem among children and the elderly. They put a great health burden on patients and on their families, and an enormous economic burden on society in terms of consultations with doctors, direct medical costs, and the indirect costs of missed days from work or absences from school and day care (Bell et al., 1989; Nurmi et al., 1991; Fendrick et al., 2003). In children, acute respiratory infections, including their most prevalent complication, otitis media, account for 80% of all infectious diseases diagnosed in general practice (Rautakorpi et al., 2001). Viral gastroenteritis is the second most common infection (Rosenfeldt et al., 2005). Day-care attendance is known to be a major risk factor for infections in children (Louhiala et al., 1995).
In the elderly, respiratory infections account for approximately half (Rautakorpi et al., 2001), and gastroenteritis for one third, of all infections (Strausbaugh et al., 2003), while candidosis is the most common oral infection. As many as 75% of the elderly are known to harbour Candida in their oral cavity (Närhi et al., 1993).

Infectious diseases are widely treated with antimicrobials; for example, respiratory infections account for 80% of all antibiotic prescriptions in primary care (Rautakorpi et al., 1999). Although the prescription of antibiotics is lowest in northern Europe compared to southern and eastern parts (Goossens et al., 2005), the enormous consumption of antibiotics is evidently leading to emerging antibiotic resistance, a major public health problem worldwide. The failure of antibiotics to change the host receptivity of pathogen recurrences, and the dearth of any effective means of preventing infectious diseases, have inspired scientists to find new alternative ways of reducing the risk of common infections, so the increased interest in probiotic bacteria is understandable. Nowadays, abundant experimental data support the hypothesis that probiotics affect the host beneficially by balancing the indigenous microbiota (Madden et al., 2005), by hampering the growth of pathogenic microorganisms, and by boosting innate and acquired immunity (Maldonado Galdeano et al., 2007). Probiotics may thus have the potential to reduce the risk of infectious diseases. However, evidence of the clinical relevance of these effects is still scarce and needs to be proven in well controlled, clinical trials.

The purpose of the present work was to evaluate the effect of specific probiotic bacteria in preventing common infections that occur in oral mucosa and in the respiratory and gastrointestinal tracts. To clarify these effects, four large-scale clinical studies, two with children and two with elderly subjects, were carried out.
2 REASON OF THE LITERATURE

2.1 Common infectious diseases in children and in the elderly

2.1.1 Acute respiratory tract infections

Acute respiratory infections (ARI) are the most common health problems among children and the elderly, though the incidence is high in all age groups. The majority of respiratory infections affect the upper respiratory tract, and are mild, self-limiting viral upper respiratory infections (URIs), also known as the common cold (for review, see Heikkinen and Järvinen, 2003). The frequency of respiratory infections shows clear seasonality; the occurrence increases rapidly in the autumn, reaching peak incidence during winter, and decreases in the spring (Butz et al., 1990; Rautakorpi et al., 2006).

In children, the prevalence of ARI is highest at the age of 2 years (Wald et al., 1991b). Children under 6 months of age experience less ARI than older children (Monto and Ullman, 1974), possibly due to antibodies originating from the mother. Based on population-based cohort studies, 1-2-year-old children experience an average of 3-8 ARI episodes per year, while children of over 3, an average of 4-6 episodes (Wald et al., 1991b). In addition to age, day-care attendance is known to be a major risk factor for ARI. Large numbers of children in close physical contact and the poor personal hygiene of infants and toddlers favour the transmission of infectious diseases. According to several population-based cross-sectional and cohort studies, the risk of respiratory infections and acute otitis media (AOM) is known to be 1.2-2.5 times higher (Bell et al., 1989; Wald et al., 1991a; Collet et al., 1994; Louhiala et al., 1995; Hernandez et al., 1999; Nafstad et al., 1999), and the risk of pneumonia as much as 9.7 times higher (Louhiala et al., 1995) in children attending day-care centres than in children looked after at home or in small groups. Thus, day-care children experience an average of 6.3 respiratory infection episodes compared to 3.9 episodes in children in home care (Wald et al., 1988). The risk is particularly noticeable during the first 6 months of enrolment in day care and in children under two years of age (Kamper-Jørgensen et al., 2006).
Approximately one quarter of children suffer from recurrent or prolonged infections (Nokso-Koivisto et al., 2002b), usually defined as having 4-12 ARI episodes/year. In Finnish children, the incidence of ARI episodes was 4.5 per person-year in children suffering from recurrent infections compared to 1.7 episodes in children without recurrences (Nokso-Koivisto et al., 2002b). Socioeconomic and environmental risk factors, such as day-care attendance (Wald et al., 1991a), number of siblings, parental smoking in the household (Nokso-Koivisto et al., 2002b), and short duration of breastfeeding (Douglas et al., 1994), have been associated with recurrent infections. The observation that boys experience recurrent infections more often than girls (Nokso-Koivisto et al., 2002b), suggests that genetic factors might account for a predisposition to infection. Deficiencies in the innate immune system, such as in complement activation via mannanbinding lectin, or via the classical pathway through complement factor C4, have also been associated with increased risk of recurrent infections in children and chronic sinusitis in adults (Cedzynski et al., 2004; Seppänen et al., 2006). However, immune deficiencies rarely explain susceptibility to infections at a population level.

Elderly subjects living in long-term care facilities are also at increased risk of infection because of the physiological changes that occur with aging (such as less flexible lungs and diminished clearance mechanisms for pulmonary secretions), because of underlying chronic diseases and the institutional environment where infections are easily transmitted (for review, see Garibaldi, 1999). Respiratory infections, including both upper and lower respiratory tract infections, are known to account for 36-70% of all infectious diseases in long-term-care patients (Beck-Sague et al., 1994; Orr et al., 1996; Engelhart et al., 2005). According to intervention trials, the incidence rate of URI in either the non-institutionalised elderly or elderly patients living in old people's homes, varies from 0.8-1.2 infections per person-year (Nicholson et al., 1997; Graat et al., 2002; Meydani et al., 2004). Although the incidence rate of respiratory infections does not appear particularly alarming, the impact is greater because infections in the elderly are associated with increased mortality (Beck-Sague et al., 1994; Thompson et al., 2006). There is also growing evidence to show that common respiratory infections cause the exacerbation of asthma and chronic obstructive pulmonary diseases (COPD) (Proud and Chow, 2006).

Viral upper respiratory infections are sometimes accompanied by a bacterial complication, causing otitis media (see Chapter 2.1.2) and sinusitis in the upper respiratory tract, or bronchitis and pneumonia in the lower respiratory tract. Sinusitis is known to occur in 0.5-2% of URI episodes (Heikkinen and Järvinen, 2003). However, in young children sinusitis is usually an extension of the common cold due to the spreading of infection to the paranasal sinuses, causing mucosal oedema and the accumulation of mucus without a bacterial co-infection (Kristo et al., 2003). Bronchiolitis and pneumonia are the most common manifestations of lower respiratory infections (LRIs) in infants. In the home-living elderly, as many as 65% of URIs are complicated by LRIs (Nicholson et al., 1997). LRIs represent about 60% of respiratory infections in the nursing-home elderly, acute bronchitis accounting for 84%, and pneumonia for 16% of the LRIs (Engelhart et al., 2005). Pneumonia is often a true bacterial complication, occurring with the prevalence of 0.3-5.8% (Garibaldi, 1999). In the Finnish population, the incidence of community-acquired pneumonia is known to be 12/1000 inhabitants per year, 36/1000 among children under 5, and 15-34/1000 among subjects over 60 years of age (Jokinen et al., 1993). Residents in long-term-care facilities are at particularly high risk of developing pneumonia.
2.1.1.1 Etiology and pathogenesis

Acute upper respiratory infections are mainly attributed to viruses. Viruses have been associated with two thirds of the common cold episodes (Mäkelä et al., 1998), and it is estimated that over 200 different types of virus cause URI (for review, see Eccles, 2005). The proportions of different viruses vary depending on the age of the subject, the season, and the methods of viral sampling and detection. Rhinoviruses are the largest group of viruses that cause URI, including at least 100 different serotypes (for review, see Nokso-Koivisto et al., 2006). Rhinoviruses have been consistently found to be the most predominant cause in all age groups, accounting for about 30-63% of common colds (Nicholson et al., 1997; Mäkelä et al, 1998; Vesa et al., 2001; Nokso-Koivisto et al., 2002b). For the most part rhinoviruses attack during the early autumn and in the spring (Mäkelä et al., 1998; Vesa et al., 2001). Although they are generally thought to cause mild common colds, in children they are frequently associated with LRIs. Human coronaviruses are not common causes of URI in children, but account for about 10% of URI episodes later in life (Nicholson et al., 1997), followed by influenza, accounting for 4-10% of colds in children, and in community-living elderly people (Nicholson et al., 1997; Mäkelä et al., 1998; Vesa et al., 2001; Nokso-Koivisto et al., 2002b; Peltola et al., 2005). Enteroviruses, frequently found in small children (Nokso-Koivisto et al., 2002a), used to be thought to cause mild disease, but recently they have also been associated with AOM (Nokso-Koivisto et al., 2006). Respiratory syncytial virus (RSV) has mainly been known as a primary cause of bronchiolitis in children (for review, see Hall, 1999), but is nowadays recognised as an important pathogen in adults as well (Hashem and Hall, 2003). In cohort studies RSV accounts for an annual incidence of 3-7% of URIs in healthy, community-living elderly subjects (Nicholson et al., 1997; Falsey et al., 2005). Other causative viruses, such as parainfluenza and adenoviruses, account for minor proportions of URIs (Nicholson et al., 1997; Heikkinen and Järvinen, 2003). Parainfluenza viruses are most often associated with laryngitis, but can cause a broad spectrum of respiratory diseases, ranging from mild upper respiratory infections to pneumonia (Nokso-Koivisto et al., 2006). Newly identified human metapneumoviruses and bocaviruses have been found fairly frequently in children, each accounting for 5-6% of infections (Arden et al., 2006).

The pathogenetic mechanisms of various respiratory viruses differ between the viruses themselves. The understanding of the pathogenetic events is mainly derived from rhinovirus infections. Rhinoviruses are transmitted mainly by small aerosol particles, and via direct or indirect contact with infected secretions. Virus titres peak 2-3 days after infection and may persist for as long as 3-6 weeks (Jartti et al., 2004). At the beginning of the infection, the rhinovirus invades the host by binding to the ICAM-1 receptor (intercellular adhesion receptor molecule 1), mainly located in the nasopharynx. After intracellular invasion and replication, the virus spreads intranasally to the pharynx. Replication evokes inflammatory and immune responses in the host, leading to vasodilatation, increased vascular permeability and cellular infiltration, through the release of inflammatory mediators. Elevated concentrations of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, in nasal lavage, result in a cascade of inflammatory reaction necessary to eradicate the virus (van Kempen et al., 1999).

Viral infections may pave the way for secondary bacterial infections (Peltola et al., 2006), leading to bacterial complications in the upper respiratory tract (such as otitis media and sinusitis), or in the lower respiratory tract (such as bronchitis and pneumonia). Viral infections
facilitate bacterial adherence and colonisation by upregulating the molecules that bacteria utilise as receptors (Hament et al., 1999; Peltola and McCullers, 2004). In addition, viruses cause physical damage to the respiratory epithelium and impair the local defence mechanisms, which leads to increased translocation of bacteria through the epithelial barrier of the respiratory cells (Hament et al., 1999; Peltola and McCullers, 2004). Mixed viral-bacterial infections seem to be relatively common in small children (Heikkinen et al., 2004), although bacterial co-infections in young adults have been found in only 4% of common cold events (Mäkelä et al., 1998). S. pneumoniae, H. influenzae or M. catarrhalis have been detected in 26% of young adults with sinusitis (Puhakka et al., 1998a), while viral infection has been detected in 82% of patients, with no significant increase in the levels of antibodies to bacteria or the elevation of CRP (Puhakka et al. 1998a). The pathogenesis of acute otitis media, the most common bacterial complication of upper respiratory infection in children, is reviewed in more detail in Chapter 2.1.2.1. Bronchiolitis in young children is mainly caused by viruses such as influenza, RSV, parainfluenza and metapneumovirus, and to a lesser extent by adeno-, rhino- and enteroviruses, while the prevalence of bacterial pathogens increases with advancing age (van Woensel et al., 2003; Klig, 2006). S. pneumoniae, H. influenzae, Moraxella catarrhalis, Chlamydia pneumoniae, Mycoplasma pneumoniae, and Staphylococcus aureus mixed with viruses, such as influenza and RSV, are important pathogens associated with pneumonia (for review, see Marrie, 2000).

### 2.1.1.2 Clinical manifestation

Acute respiratory infections are diagnosed on the basis of symptomatology. However, not all infections lead to clinical illness; symptomatic diseases are known to develop in 75% of infected persons (Gwaltney and Hayden, 1992). The clinical expression of ARI is variable, and is modulated depending on the infective virus and on the age, physiological and immunological state of the host. Upper respiratory tract infection involves inflammation of the respiratory mucosa from the nose to the lower respiratory tree, excluding the alveoli. URI causes localised symptoms such as cough, rhinitis, blocked nose, wheezing and sore throat, and systemic symptoms such as fever, malaise, headache and loss of appetite (for review, see Eccles, 2005).

Understanding of the mechanisms behind the infection symptoms is poor, but it has been suggested that clinical symptoms might not be caused by the direct cytopathic effect of the viruses on the epithelial cells, but are primarily due to the inflammatory response of the host (Eccles, 2005). The major cells protecting the host against the invasion of pathogens are macrophages, which produce cytokines, which lead to the recruitment of other immune cells and inflammation. The complex mixture of proinflammatory cytokines is responsible for systemic symptoms such as fever, while the inflammatory mediator, bradykinin, is believed to cause local symptoms. Symptoms arise after an incubation period that varies considerably between different viruses, ranging from 10-12 hours in rhinovirus infection, to 1-7 days in influenza infection (for review, see Heikkinen and Järvinen, 2003). The symptoms usually peak 2-3 days after infection and are resolved in 7-10 days, although in some patients symptoms can be present even for as long as 3 weeks (Eccles, 2005).

Rhinovirus infection typically starts with a sore throat, soon followed by watery nasal discharge, and later by nasal congestion and cough, which may persist for 3 weeks or even longer (Eccles 2005). In children, fever is often present during an acute respiratory infection (Putto et al.,
REVIEW OF THE LITERATURE

1986), but in adults fever occurs less frequently (Puhakka et al., 1998b). Fever of at least 39°C in children has been mainly associated with adenovirus and influenza A and B, but not so often with parainfluenza or RSV (Putto et al., 1986). The mean duration of fever varies from 3 to 5 days depending on the virus, and in one third of the children fever lasts five days or longer (Putto et al., 1986). However, high and prolonged fever does not differ between viral and bacterial infections. Influenza is often considered to be a separate disease from the common cold. However, the clinical manifestations of influenza range from an asymptomatic infection to severe illness, and therefore cannot be distinguished on the basis of the symptoms. Cough and fever occurring within 48 hours of symptom onset have been found to be the best predictors of influenza (Monto et al., 2000), but the clinical diagnosis of influenza is still extremely difficult. In a prospective outpatient study, fewer than 40% of all true cases were identified, and only one third of all clinically diagnosed cases could be verified by laboratory analysis (Peltola et al., 2005). Influenza, parainfluenza and RSV cause more symptoms in the lower respiratory tract than rhino- and enteroviruses (for review, see Klig, 2006). Although different viruses tend to have some variation in their typical clinical manifestation, it is not possible to identify the causative virus on the basis of symptoms. Lower respiratory infections are clinically characterised by a variety of symptoms, such as cough, fever, tachypnea, wheezing, chills and chest pain, but extrapulmonary symptoms such as nausea, vomiting and diarrhoea may also occur (for review, see Marrie, 2000). However, pneumonia, especially in the elderly, may also be latent and afebrile, or may manifest itself mainly as delirium with only a few respiratory symptoms.

2.1.1.3 Treatment and prevention

Despite the fact that antibiotic treatment does not influence the clinical course of viral infection, uncomplicated respiratory infections are widely treated by antibiotics: In the USA, as many as 60% of patients with common colds receive antibiotic treatment unnecessarily (Mainous et al., 1996), the corresponding figure being 36% in Sweden (Andre et al., 2005) and 7-12% in Finland (Rautakorpi et al., 2006). Treatment of the common cold is mainly based on symptom-relieving medications, such as antihistamines for reducing rhinitis, decongestants and mucolytic cough medication, and non-steroidal anti-inflammatory drugs that reduce the fever. However, according to a recent review based on seven Cochrane reviews, these non-antibiotic treatments are mostly found to be ineffective (Arroll, 2005). Antiviral medication, such as the M2 ion channel-blocking drugs, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir, prevents approximately 60-80% of influenza cases, and shortens the duration of the fever by 1-2 days if the treatment is started within 48 hours of the onset of symptoms (Jefferson et al., 2006). Despite intensive research and drug development, no effective antiviral medication is so far available against other common respiratory viruses.

Preventive therapies against viral respiratory infections are also scarce. Preventive antimicrobial treatment has been shown to prevent the bacterial complications of URI in subjects carrying bacterial pathogens in the nasopharynx, but no benefits were seen in subjects without bacteria (Kaiser et al., 1996). However, antibiotic treatment did not improve the clinical course of acute sinusitis (van Buchem et al., 1997). Vaccine development against different viruses has been ongoing without any real success. The prevention of rhinoviruses would be the most beneficial, but because of its numerous different serotypes, the development of a vaccine seems unlikely. At present, the only vaccines commercially available are against influenza. According to a recent
systematic review, the effectiveness of the influenza vaccination was modest (23%) against influenza-like illnesses in subjects aged 65 years or more residing in long-term-care facilities, and was not effective in the community-living elderly (Jefferson et al., 2005a). However, vaccination seems to prevent pneumonia, hospital admission and death from influenza, pneumonia or cardiovascular diseases, as well as overall mortality. In children over two years of age, the effectiveness of live attenuated vaccines was 38%, and 28% for inactivated vaccines (Jefferson et al., 2005b). Vaccines were effective in reducing school absences, but had little effect on lower respiratory diseases, acute otitis media and hospital stay. Systematic reviews and a meta-analysis of the role of multivitamin and mineral supplementation in preventing infections in the elderly has showed a reduction of 18% in infections (El-Kadiki and Sutton, 2005), and vitamin C supplements show small benefits in reducing the duration and severity of infections (Douglas et al., 2004). Thus, the evidence for routine use of vitamin supplements is still weak and conflicting. Improved hygiene practice programmes have also shown modest results: Infections, mainly upper respiratory infections, have been reduced by 9% (Uhari and Möttönen, 1999), and absences due to infections, by 26% in day-care children (Pönkä et al., 2004), while the reduction of infections among long-term-care elderly subjects has been 34% (Makris et al., 2000).

2.1.2 Acute otitis media

Acute otitis media (AOM), inflammation of the middle ear, is the most common complication of a viral respiratory infection, and the main reason for consulting a doctor and for antibiotic prescription in children (for review, see Klein, 2001). 30-43% of upper respiratory infections are associated with AOM (Wald et al., 1991b; Koivunen et al., 1999; Vesa et al., 2001). AOM places a great burden on children and their families. Untreated AOM may lead to perforation of the tympanic membrane, suppurative complications and impairment of hearing, which may result in speech and language delays in small children (for review, see Rovers et al., 2004). Direct and indirect costs are also high: in Finland, the total annual cost associated with AOM reaches $US 138 M (Niemelä et al., 1999). The clinical picture of otitis media has changed over the past two decades: serious complications have decreased, while the incidence of recurrent infections has increased (Joki-Erkkilä et al., 2000). According to a cohort study in the Netherlands, the incidence rate of AOM and otitis media with effusion (OME) declined during the years 1995-2003 in children over two years of age, while the incidence rate in children under two increased by 46-66% (Plasschaert et al., 2006). Antibiotic prescriptions for AOM increased by 45% in all age groups.

Prospective cohort studies indicate that AOM peaks in children aged 6-11 months (Vesa et al., 2001), and approximately every fifth child is known to experience at least one AOM episode during the first year of its life (Aniansson et al., 1994). According to the Finnish Otitis Media (FinOM) cohort study, 62% of children experience at least one otitis media episode by the age of two, and 8% experience six or more recurrent episodes (Kilpi et al., 2001). When a child experiences at least three or more episodes of AOM within 6 months or at least four episodes within 12 months, s/he is considered prone to recurrent infections (Berman, 1997). Approximately 5-20% of the pediatric population suffers from recurrent otitis media episodes (Alho, 1997; Pichichero, 2000; Faden, 2001). According to a meta-analysis, day care outside
the home, siblings, parental smoking and the use of a pacifier were associated with increased risk of AOM, while breastfeeding for at least 3 months protected from AOM (Uhari et al., 1996). In addition, the occurrence of the first AOM episode during the first 6 months of life, age under 3 years, male gender, allergies and anatomic abnormalities of the eustachian tube in children with cleft palate or Down’s syndrome, are known to predispose to AOM (for review, see Klein, 2001). Studies have failed to demonstrate major immunological abnormalities in otitis-prone children, although differences, for instance in IgG2 antibody production between otitis-prone and healthy children, have been demonstrated (Faden, 2001). The prevalence of specific antibody deficiencies, such as an inadequate IgG response to polysaccharide antigens, is shown to be relatively common (15%) among children with recurrent infections (Boyle et al., 2006a).

2.1.2.1 Etiology and pathogenesis

Respiratory viruses play a crucial role in the development of acute otitis media. Viral respiratory infection precedes the development of AOM, which is frequently diagnosed on days 2-5 after the onset of acute upper respiratory symptoms (Koivunen et al., 1999). Viruses can be detected in 50-70% of AOM cases (Nokso-Koivisto et al., 2004; Ruohola et al., 2006), and in one fifth of the cases, virus has been the only pathogen found (Kleemola et al., 2006). Rhinoviruses, RSV and enteroviruses, in particular, have been found to be associated with a significant proportion of AOM cases (Vesa et al., 2001; Nokso-Koivisto et al., 2004; Kleemola et al., 2006; Nokso-Koivisto et al., 2006). The pathogenesis of AOM is multifactorial, involving the innate and adaptive immune system and genetic and environmental factors, as well as Eustachian tube function and pathogen load. Viral infections induce a release of inflammatory mediators from the nasopharyngeal cells, causing inflammation of the mucosa, and this provokes the dysfunction of the eustachian tube – known to be the most important factor in the pathogenesis of acute otitis media. Eustachian dysfunction causes a negative middle ear pressure, leading to the accumulation of mucus and pathogens from the nasopharynx to the middle ear and to impaired ventilation (for review, see Hendley, 2002). This enhances bacterial replication in the fluid of the middle ear, leading to bacterial otitis media. Two third of AOM cases are coinfections with both bacteria and viruses (Ruohola et al., 2006).

*Streptococcus pneumoniae* is the predominant bacterial pathogen associated with otitis media, followed by *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pyogenes* and *Staphylococcus aureus* (for review, see Faden, 2001). *S. pneumoniae* has been isolated as a causative agent in 22-26% of otitis events in Finnish children, *M. catarrhalis* in 17-23%, and *H. influenzae* in 13-23% of otitis cases (Kilpi et al., 2001; Kleemola et al., 2006). About 17% of the episodes remain etiologically obscure (Kilpi et al., 2001). On the basis of an intervention trial, the prevalence of *S. pneumoniae* carriage among Finnish day-care children varies between 27 and 46%, *M. catarrhalis* between 7 and 25%, and *H. influenzae* between 11 and 16% (Kontiokari et al., 2005). Age is strongly associated with pathogen carriage: according to a FinOM cohort study, the prevalence of *S. pneumonia* in children aged 2 months to 2 years gradually increases from 9% to 43% (Syrjänen et al., 2001). Approximately 50% of children carry *S. pneumoniae* at the age of 2 years, and the prevalence declines to 21% by the age of 7 (for review, see Harper, 1999). Colonisation stimulates the production of mucosal as well as serum antibodies against the pathogens. A specific IgA mucosal antibody limits
the frequency and duration of colonisation, while serum IgG mainly protects against the development of AOM but does not affect colonisation (Faden, 2001). Otitis-prone children probably fail to develop a broad-based immune response, and therefore experience repeated episodes of colonisation and infection. The nasopharyngeal carriage of bacterial pathogens is known to be higher in children with AOM than in children without AOM (Aniansson et al., 1994). The density of the bacteria in the nasopharynx and the adenoids is also higher in children with recurrent otitis than in non-otitis-prone children (Linder et al., 1997; Brook and Yocum, 1999; Brook and Gober, 2000). *H. influenzae* is known to be the major cause of recurrent AOM (Kilpi et al., 2001).

### 2.1.2.2 Clinical manifestation

Acute otitis media is defined as the presence of middle-ear effusion in conjunction with the rapid onset of one or more signs or symptoms of inflammation in the middle ear (for review, see Rovers et al., 2004). It is important to distinguish acute otitis media from otitis media with effusion, which is defined as fluid in the middle ear without local or systemic illness. Bacterial otitis media is characterised by a bulging eardrum that has purulent fluid behind it. Infectious symptoms usually appear in 48 hours, and are revealed by local or systemic findings such as earache, fever, rhinitis, cough, night restlessness, poor appetite, vomiting or diarrhoea. AOM is associated with common URI symptoms in 95% of cases, pain in 75%, and fever in 25% (Faden, 2001). However, symptoms of otitis media are considered non-specific. Earache has been most strongly associated with AOM, although as many as 40% of children with AOM might not have earache at all (Heikkinen and Ruuskanen, 1995; Kontiokari et al., 1998). Sore throat, night restlessness and fever have also been associated with AOM, especially in children of over two years of age, but they are of no value in distinguishing AOM from uncomplicated URI (Heikkinen and Ruuskanen, 1995; Kontiokari et al., 1998). As the infection begins to resolve, symptoms such as pain and fever lessen within 2 to 3 days, and most children become asymptomatic by 7 days (for review, see Faden et al., 1998), although URI symptoms may last an average of 11 days (Koivunen et al., 1999). However, effusion from the middle ear resolves relatively slowly: 40% of cases are resolved by 2 weeks, 60% by one month, but effusion may persist for as long as three months (Faden et al., 1998).

Myringotomy is considered the golden standard for diagnosing AOM. However, pneumatic otoscopy, which combines visual assessment of tympanic-membrane mobility and appearance, is widely used in general practice. Tympanometry, which is also used in clinical practise, is a simple and objective quantitative method of assessing tympanic mobility and function. The overdiagnosing of AOM is a common problem, especially among general practitioners, and there is only slight to moderate correlation between the diagnosis of paediatric residents and otolaryngologists (Steinbach et al., 2002). Although symptoms and signs associated with AOM have poor sensitivity and specificity, parents have been able to predict the presence of AOM to a sensitivity of 71% and a specificity of 80% (Kontiokari et al., 1998).

### 2.1.2.3 Treatment and prevention

Because the origin of acute otitis media may be viral or bacterial or a combination of the two, it is often unclear at the time of diagnosis whether antibiotic treatment will be effective or not.
According to a meta-analysis of randomised, placebo-controlled studies, AOM will resolve within a week without antibiotic therapy in more than 80% of children, and the benefit of antibiotics is only 13.7% over a placebo (Rosenfeld et al., 1994). Therefore the use of delayed antibiotic-prescribing strategy (waiting for 48 to 72 hours before prescribing antibiotics to see whether infection will resolve spontaneously) is appropriate (Spiro et al., 2006). On the basis of a recent meta-analysis, antibiotics seem to be beneficial in relieving pain or fever in children under two years of age, and in children with AOM and otorrhoea (Rovers et al., 2006). However, the increasing prevalence of antibiotic-resistant micro-organisms makes the management of otitis media complex and difficult (Goossens et al., 2005). During the last decades, multidrug resistance and macrolide resistance in S. pneumoniae has increased remarkably (Pihlajamäki et al., 2002), and has been clearly associated with the total use of β-lactam antibiotics (Wald et al., 2001; Nasrin et al., 2002), and macrolides (Bergman et al., 2006). According to the Finnish Study Group for Antimicrobial Resistance (the FiRe), the erythromycin resistance of pneumococci doubled during the years 1999-2005, being around 20% at the moment (www.ktl.fi/portal/english/projects/fire/finres/finres_antimicrobial_resistance_statistics). In general, 30-45% of H. influenzae and over 95% of M. catarrhalis are known to be β-lactamase producers (Brook, 1998). According to FiRe, the prevalence of ampicilloine-resistant H.influenza is around 20%, and of M. catarrhalis, around 90%.

Because of difficulties in providing optimal treatment for otitis media, effective strategies for preventing the development of AOM would be of utmost importance. In that area, vaccine development against pneumococci has been extensive. Over 90 different serotypes of S. pneumoniae, based on the capsular polysaccharide antigens, are currently known. The most frequent serotypes in Finnish children are 6B, 6A, 11, 14, 19F, and 23F (Syriänen et al., 2001), and serotypes 6, 14, 19, and 23 have been shown to be responsible for a large proportion of both invasive and local pneumococcal diseases in children (Sniadack et al., 1995; Kilpi et al., 2001). The first 23-valent capsular polysaccharide vaccine has been constructed by using 23 of the most common pneumococcal polysaccharide capsule serotypes, but its efficacy has been limited in children under 2 years of age, because polysaccharides are not immunogenic in small children (Bernatoniene and Finn, 2005). Conjugating polysaccharides to protein conjugates makes the combination more immunogenic. A seven-valent pneumococcal conjugate vaccine, consisting of capsular polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, conjugated to a carrier protein, has been found to be highly effective against vaccine serotype invasive pneumococcal infections (Black et al., 2000; O’Brien et al., 2003; Whitney et al., 2006), and moderately against pneumonia (Black et al., 2002). The overall efficacy against AOM has only been around 6-7% (Eskola et al., 2001), but efficacy against pneumococcal episodes and episodes due to vaccine serotypes has been higher: 35% and 57% respectively (Eskola et al., 2001). The major secondary advantages of vaccination include the reduction of the nasopharyngeal carriage of vaccine strains and antibiotic-resistant serotypes in vaccine recipients (Musher, 2006). In the USA, after introducing conjugate vaccine into the immunisation schedule, the total amount of invasive pneumococcal diseases decreased to about half, while the rate of antibiotic-resistant invasive pneumococcal infections caused by vaccine serotypes decreased by 87% among children under two years of age, but also significantly among people 65 years of age or older (Kyaw et al., 2006). Thus the reduction in the carriage rate results in reduced spreading of infective strains to unvaccinated subjects, a phenomenon known as “the herd effect”.

23
REVIEW OF THE LITERATURE

2.1.3 Diarrhoea

Acute diarrhoea is a non-specific response of the intestine to several different factors and conditions. Infection is the most common cause of acute diarrhoea, but other conditions, such as drugs, functional bowel disorders or inflammatory bowel diseases may also cause diarrhoea. Acute infectious diarrhoea remains a major cause of morbidity and mortality worldwide. In industrialised countries, infectious diarrhoea is mainly a problem of morbidity and has a major financial impact on health care systems, but in terms of increased mortality, the burden is much heavier in developing countries. Therefore, the prevention of diarrhoeal illnesses would have considerable public-health implications (for review, see Cheng et al., 2005).

In children, acute gastroenteritis is the second most common infectious disease after the common cold. In prospective cohort studies of children aged 6 months to 7 years attending day-care centres, 46-51% of the subjects developed acute gastroenteritis during a 6-12-month follow-up (Hjelt et al., 1987b; Rosenfeldt et al., 2005), the annual incidence of diarrhoea being 0.9–1.1 episodes per child (Hjelt et al., 1987b; Louhiala et al., 1997). The incidence is age-dependent and highest among children under two years of age (Rosenfeldt et al., 2005). Infants have been reported to have 3.6 diarrhoea episodes per year, decreasing to 1.3 episodes in children older than 18 months of age (Staat et al., 1991). Day-care attendance is a major risk factor for diarrhoea in children, and the incidence of diarrhoea seems to be higher in boys than in girls (Staat et al., 1991), while breastfeeding is known to be a protective factor in infants (Gianino et al., 2002). Children in day-care centres experience an average of 0.4-4.2 diarrhoea episodes per year; the risk of acute diarrhoea being 1.2-3.5 times higher than in children taken care of at home (Thacker et al., 1992; Louhiala et al., 1997). The proportion of diarrhoeal episodes attributable to day-care centres is 49% in 1-year-olds and 37% in two-year-olds (Louhiala et al., 1997). The peak incidence of diarrhoea occurs during the winter, between January and April (Hjelt et al., 1987b; Rosenfeldt et al., 2005).

Nursing-home facilities provide an ideal environment for the spreading of gastrointestinal infections, since residents share sources of air, food and water in crowded institutional settings. Age-related achlorhydria and the frequent use of antimicrobial agents predispose residents to gastrointestinal infections. Visitors, staff and residents bring pathogens from the hospital and the community, and residents with dementia, incontinence or behavioural disturbances facilitate the person-to-person transmission of GI pathogens (Strausbaugh et al., 2003). Among institutionalised elderly patients, gastroenteritis is the second most common infection after respiratory infections, accounting for 20-35% of all infections (Li et al., 1996; Strausbaugh et al., 2003; Engelhart et al., 2005). In a German nursing-home survey, the incidence of gastroenteritis during a one-year follow-up, was 1.2 per 1000 resident days (Strausbaugh et al., 2003).

2.1.3.1 Etiology and pathogenesis

Although most cases of diarrhoea are the results of an infection, specific organisms can be identified in only a minority of patients. More than 20 organisms that cause diarrhoea have been detected (for review, see Glass et al., 2006). In children, rotavirus is the most frequent cause of acute gastroenteritis, resulting in annual winter epidemics of high attack rates (over 50%), and predominantly affecting young children (Hjelt et al., 1987b; Waters et al., 2000; Rosenfeldt
et al., 2005). It accounts for 24-40% of the diarrhoeal episodes in day-care centres, while bacterial pathogens are detected in only 6%, and parasites in 2% of the episodes (Hjelt et al., 1987b; Waters et al., 2000; Rosenfeldt et al., 2005). Rotavirus has been found to be more often the causative organism in children admitted to hospital with symptoms of acute diarrhoea (36%), than in the diarrhoeal episodes of children attending child-care centres (15%) (Waters et al., 2000). However, rotavirus infection does not necessarily lead to symptomatic infections: the incidence of nosocomial rotavirus infections in 420 Italian infants and toddlers was 28%, of which 17% were symptomatic and 11% remained asymptomatic (Gianino et al., 2002). Rotavirus is transmitted by the faecal-oral route with small infectious doses. During the incubation period of 18-36 hours, the virus enters the epithelial cells, where it elaborates toxin, causing diarrhoea. It continues to destroy the epithelium, leading to extensive damage and the shedding of huge masses of virus in the stools (Glass et al., 2006). Other viruses commonly found as causative agents in community-acquired diarrhoea among toddlers and children are sapporovirus (19% of outbreaks), astrovirus (7%), adenovirus (4%), torovirus (3%) and noroviruses (2%), (Mitchell et al., 1993; Waters et al., 2000; Rosenfeldt et al., 2005). Among bacteria, Campylobacter jejuni and Clostridium difficile have occasionally been found in children (Rosenfeldt et al., 2005).

In institutionalised elderly subjects, the etiology of diarrhoea may sometimes be non-infectious, but epidemic diarrhoeal illnesses caused by a variety of bacterial and viral agents are also common (for review, see Garibaldi, 1999). Viruses cause the majority of outbreaks of GI infections in old people's homes, the Caliciviridae family, including noroviruses, playing a dominant role (Marshall et al., 2003; Strausbaugh et al., 2003). During recent years, a striking increase in norovirus outbreaks have been detected in Europe due to the emergence of a new predominant norovirus variant (genogroup II-4) (Lopman et al., 2004; Kroneman et al., 2006). Nine countries out of 11 reported an increase of 2-3 times in the number of outbreaks (Kroneman et al., 2006), most of them occurring in hospitals (42%), homes for the elderly (31%), and locations with children (13%) (Krisztalovics et al., 2006). Norovirus was detected in 42% of outbreaks in aged-care facilities, while rotavirus accounted for 13% and astrovirus for 2% of outbreaks (Marshall et al., 2003). Norovirus is highly infectious because of the small infectious dose. Transmission occurs by the faecal-oral route and via aerosol sprays, as well as through the ingestion of contaminated food and water. Norovirus is very stable and resistant to many disinfectants, and may shed for prolonged periods of up to one month (for review, see Estes et al., 2006). Salmonella and toxigenic Escherichia coli are the major bacterial agents that cause diarrhoea, as well as Clostridium difficile, which may be carried asymptomatically or may cause diarrhoea as a result of antibiotic therapy. Infectious nosocomial diarrhoea is mainly caused by C. difficile. The prevalence of C. difficile colonisation in long-term-care facilities in the absence of an outbreak has ranged between 4-20%, while up to 30% of residents have been found to harbour C. difficile during outbreaks (Simor et al., 2002). Other infectious agents that have been occasionally detected in diarrhoeal outbreaks in nursing homes, include Shigella, Campylobacter, Aeromonas, and Yersinia, and parasite agents such as Entamoeba histolytica, Giardia lamblia and Cryptosporidium parvum (Strausbaugh et al., 2003).

2.1.3.2 Clinical manifestation

Diarrhoea is defined as an alteration in the normal bowel movement characterised by an increase in the water content, volume or frequency of stools. Acute diarrhoea is usually defined as the passage of three or more soft or liquid stools/day lasting 14 days or less (for review, see
Guerrant et al., 2001). It is considered persistent when it lasts more than 14 days, and chronic when it lasts over 30 days. Infectious diarrhoea is often accompanied by symptoms of nausea, vomiting and abdominal pain (Guerrant et al., 2001). Other symptoms, such as headache and myalgia, may also be present. Diarrhoea is generally a self-limiting illness lasting 5-7 days. The median duration of diarrhoea among day-care children has been 33 hours, ranging between 5-169 hours (Rosenfeldt et al., 2005). Severe sequelae associated with acute diarrhoea are rare in developed countries, although dehydration may be a major problem in children, in the elderly, and in immunocompromised persons (for review, see Cheng et al., 2005).

Rotavirus infection is associated with initial fever and vomiting for 2-3 days, especially in children under two years of age (Hjelt et al., 1987a), followed by profuse diarrhoea (Cheng et al., 2005). A norovirus outbreak is characterised by an illness of short duration, usually lasting 1-3 days, following a short incubation (24-48 hours) (Cheng et al., 2005). The most frequent symptoms observed in geriatric patients during a norovirus outbreak were nausea (82%), abdominal pain (80%), diarrhoea (71%), asthenia (68%) and vomiting (63%) (Odelin et al., 2006). Norovirus infections in hospitalised patients are more severe than those seen in otherwise healthy people, and subjects over 65 years of age are at greater risk of prolonged illness (Estes et al., 2006). Clinical manifestations of C. difficile diarrhoea range from asymptomatic carriage to mild or moderate diarrhoea lasting less than 10 days in the majority of cases. However, one third of the patients may suffer from a prolonged duration of the disease and severe complications such as pseudomembranous colitis, toxic megacolon, dehydration, hypokalaemia, gastrointestinal bleeding or bowel perforation (Simor et al., 2002). Inflammatory infectious diarrhoea, most commonly caused by Salmonella, Shigella and Campylobacter, is characterised by the presence of fever, systemic illness and blood or pus in the stools (Cheng et al., 2005).

2.1.3.3 Treatment and prevention

The management of diarrhoea is mainly directed toward symptomatic relief, the reduction of the duration of the illness and the correction of fluid loss and electrolyte imbalance (for reviews, see Farthing, 2000; Cheng et al., 2005; Gadewar and Fasano, 2005). The development of an oral rehydration solution has provided a simple approach for maintaining hydration in children suffering from acute watery diarrhoea. Anti-diarrhoeal drugs are widely used by adults to treat the condition, but are contra-indicated in young children. Motility-altering medication may even worsen the colonisation or invasion of infectious organisms by paralysing intestinal motility. There is also a continuous search for agents that will reduce fluid and electrolyte losses by directly inhibiting the intestinal secretory mechanisms. One candidate, racecadotril, has recently been proposed as a safe and effective anti-secretory drug (Farthing, 2006). Routine use of antimicrobial agents for treating diarrhoea is not recommended. However, appropriate antimicrobial therapy can shorten the illness and reduce morbidity in bacterial and parasitic infections, and can be life-saving in invasive infections (Farthing, 2000; Cheng et al., 2005; Gadewar and Fasano, 2005).

Improving hygiene is the most effective way of preventing enteric illnesses. On the basis of a recent meta-analysis of 17 case-control and intervention trials, proper hand washing resulted in a 47% reduction in diarrhoea risk (Curtis and Cairncross, 2003). Vaccine development against
several pathogens including *Salmonella typhi*, *Cholera, Campylobacter, Shigella*, and rotavirus has also been under extensive study during the past decades (Gadewar and Fasano, 2005). Two live rotavirus vaccines (containing an attenuated human monostrain or a combination of five bovine-human reassortant strains) are currently licensed in 35 countries in Europe and America (Glass *et al*., 2006). In Finland, both rotavirus vaccines are commercially available but are not yet included in a general immunisation schedule. The safety and efficacy of both vaccines have been proved in large-scale clinical trials (Ruiz-Palacios *et al*., 2006; Vesikari *et al*., 2006). A monovalent, live, attenuated human rotavirus vaccine showed an efficacy of 85% against any rotavirus diarrhoea and 100% against severe disease, in a large, multicentre trial of 63,000 infants in Latin America and Finland (Ruiz-Palacios *et al*., 2006). In a randomised clinical trial performed in 11 countries among 68,000 infants, the efficacy of a pentavalent human-bovine reassortant rotavirus vaccine, containing human serotypes G1, G2, G3, G4 and P, was 74% against G1-G4 rotavirus gastroenteritis, and 98% against severe gastroenteritis (Vesikari *et al*., 2006). The vaccine also reduced the need for hospitalisation and emergency department visits. The vaccination was found to be safe, since the cases of intussusception, a potential complication of vaccination, occurred similarly in the active and in the placebo groups in both vaccine trials.

### 2.1.4 Oral *Candida* infections

Candidosis is the most common infection involving oral mucosal tissues in the elderly. As many as 75% of elderly people in Finland harbour oral yeast (Närhi *et al*., 1993). The prevalence of oral candidosis, based on clinical signs and symptoms or high yeast counts, ranges between 37 and 51% in geriatric patients (Wilkieson *et al*., 1991; Budtz-Jørgensen *et al*., 1996; Paillaud *et al*., 2004). The level of *Candida* in non-institutionalised elderly people is known to be lower than in the institutionalised elderly (Honda, 2001). The elderly are vulnerable to *Candida* infection provoked by chronic diseases, medication, poor oral hygiene, reduced salivary flow or the impairment of the immune system (for review, see Shay *et al*., 1997). Studies have consistently demonstrated a higher frequency of *Candida* carriage among subjects using full or partial dentures and among those suffering from hyposalivation, both common factors in the elderly (Wilkieson *et al*., 1991; Närhi *et al*., 1993; Närhi *et al*., 1999; Ikebe *et al*., 2006). Other factors predisposing to oral candidosis include age itself, diabetes mellitus and treatment with antibiotics and corticosteroids (Peterson, 1992; Shay *et al*., 1997; Paillaud *et al*., 2004; Fanello *et al*., 2006). Oral candidosis also appears to be related to malnutrition, and mucosal lesions resulting from candidosis may lead to reduced energy intake and further worsening of the nutritional status (Paillaud *et al*., 2004).

#### 2.1.4.1 Etiology and pathogenesis

*Candida* species are usually harmless commensals of the digestive tract, but under optimal circumstances benign colonisation of this opportunistic organism may be transformed into a pathological state (for reviews, see Peterson, 1992; Shay *et al*., 1997). Candidosis thus originates mainly from endogenous microbiota (Fanello *et al*., 2006). A normal, healthy oral mucosa protects against *Candida* infection by the clearance of micro-organisms through the shedding of the surface layer and by maintaining a chemical barrier which inhibits compounds
from penetrating the epithelium (Peterson, 1992). Antibiotic usage disturbs the resident oral microbiota, in particular anaerobes, which can compete with Candida for the same host receptor sites, resulting in the overgrowth of endogenous yeasts (Payne et al., 2003). In the oral cavity, Candida adheres to salivary proteins, which are absorbed by the oral surfaces of the host cells (for review, see Calderone et al., 2000). The conversion of yeast cells to filamentous hyphae growth is essential for virulence. Thus, the deleterious action of Candida has been attributed to the ability of the fungal hyphae to grow within the oral epithelium, releasing their virulence factors, including hydrolytic enzymes such as proteinases (for review, see Cannon et al., 1995). Candidosis is most commonly caused by Candida albicans, and to a lesser extent by C. glabrata, C. tropicalis and C. parapsilosis (Wilkieson et al., 1991; Budtz-Jørgensen et al., 2000). The normal carriage of Candida is less than 1000 cfu/mL, whereas in infected individuals counts range between 4000 and 20,000 cfu/mL (Farah et al., 2000).

The ability of Candida to colonise, penetrate and damage host tissues depends on the imbalances between Candida virulence attributes and specific defects in host immune defences. According to animal models, host resistance to Candida infection in the oral mucosa is mainly due to T-cell proliferation, cytokine production and nitric oxide production (Elahi et al., 2000; 2001; Farah et al., 2001; 2002). The essential role of CD4+ T cells in oral infection has been demonstrated in an immunodeficient mouse model (Farah et al., 2002). The reconstitution of immunodeficient mice with naive CD4+ but not CD8+ T cells reduced the oral colonisation of yeast. However, T cells alone are not able to kill Candida - phagocytic neutrophils and macrophages are needed for the clearance of oral yeasts. Their anticandidal activity, on the other hand, is dependent on Th1-type cytokines, such as IL-12 and IFN-γ, produced by T cells (Farah et al., 2001). The early production of IL-12 and IFN-γ, and also of the Th2-type cytokine, IL-4, together with the accumulation of T cells in the regional lymph nodes, correlates with the rapid elimination of Candida in mice (Elahi et al., 2000). These results suggest that a balanced Th1 and Th2 helper-cell response, characterised by the production of both IFN-γ and IL-4, is important in mucosal protection against oral infection. Vaccination against Candida in mice produces high levels of IFN-γ and IL-4 and also five times higher levels of NO in the saliva (Elahi et al., 2000). IL-4 thus appears to act through the paracrine enhancement of NO production by macrophages (Elahi et al., 2001). Nitric oxide, an antimicrobial compound, on the other hand, is associated with macrophage candidacidal activity, and is thus involved in controlling the oral colonisation of Candida (Elahi et al., 2001). Serum IgG and salivary IgA antibodies have been shown to increase in mice suffering from oral candidosis, but only after the infection has been cleared, indicating that antibodies do not play a significant role in the mucosal clearance of Candida (Elahi et al., 2000).

2.1.4.2. Clinical manifestation

Even though colonisation by Candida may be asymptomatic, heavy growth usually leads to local candidosis with various types of mucosal lesions and symptoms (for review, see Shay et al., 1997). Thus colonisation of Candida is a significant predictive factor for yeast infections (Fanello et al., 2006). Symptoms associated with infection include burning, sensitivity, altered taste and a change in the sense of smell. Oral mucosal candidosis can be classified as 1) acute, asymptomatic pseudomembranous candidosis with white patches that can be wiped off
(the classical form of candidosis, so-called “trash”); 2) acute atrophic candidosis with a burning sensation in the mouth or on the tongue, the affected tissues being bright red; 3) chronic, asymptomatic hyperplastic candidosis with non-removable white lesions in the buccal mucosa or on the tongue (“leucoplakia”); 4) chronic, atrophic candidosis usually localised in the maxillary alveolus and hard palate, which can be asymptomatic or cause burning, itching and sometimes a salty taste (“denture stomatitis”); 5) median rhomboid glossitis, often asymptomatic or associated with vague complaints of an aching tongue; and 6) angular cheilitis in the form of erythematous fissuring at the corners of the mouth, which can be asymptomatic, itching or painful (“perleche”) (Shay et al., 1997). In compromised aged persons, whose immune competence is reduced and salivary function diminished, oral mucosal lesions may enhance localised candidial growth and possibly lead to systemic consequences. In general, oral health is known to be associated with systemic diseases and thus with general well-being (Meurman and Hääläinen, 2006). Therefore, the management of oral Candida carriage in the elderly is of primary importance.

2.1.4.3 Treatment and prevention

The management of oral candidosis requires a concurrent resolution of the underlying predisposing systemic conditions. Cases of oral candidosis that appear not to be complicated by systemic factors are generally managed through the improvement of oral hygiene and the use of topical antifungal rinses (Shay et al., 1997; Farah et al., 2000). The most commonly-used rinse is nystatin. Nystatin oral suspension, four times a day for 7-14 days, is usually sufficient to allow the oral microbiota to return to normal, although it is relatively ineffective if the candidosis is complicated by diabetes, steroid use or an immunocompromised state (Farah et al., 2000). Chlorhexidine-gluconate oral rinses may be effective in uncomplicated cases when appropriate attention is paid to denture hygiene. A 6-week daily or weekly treatment with chlorhexidine solution resulted in a 20% reduction in the proportion of subjects carrying Candida (Persson et al., 1991), and treatment with chlorhexidine-xylitol chewing-gum, in a 22% reduction in Candida counts (Simons et al., 1997). Clotrimazole troches are effective in cases that do not resolve with nystatin. Systemic antifungal drugs, such as fluconazole and itraconazole, may be advantageous when topically-delivered medications are administered concurrently. A two-week treatment with fluconazole reduced Candida counts by approximately 50% in patients with denture stomatitis (Budtz-Jørgensen et al., 1988). However, partial relapse occurred 2-4 weeks after termination of the treatment. Topical or systemic antifungal therapy may result in the resolution of symptoms and lesions, but unfortunately many cases of candidosis are refractory to therapy, and lesions recur if underlying factors are not erased. Therefore, antifungal prophylaxis is indicated to prevent colonisation or multiplication, especially in susceptible subjects. However, there is only a limited number of preventive means of reducing Candida carriage. Improving oral hygiene is the most efficient non-pharmacological method. It has been possible to reduce high Candida carriage by approximately 50% by improving oral and dental care practices (Budtz-Jørgensen et al., 2000).
2.2 Probiotics in the prevention of infectious diseases

2.2.1 Probiotics and their health effects

According to the working group convened by the FAO/WHO, probiotics are defined as “Live micro-organisms which, when administered in adequate amounts, confer a health benefit to the host” (FAO/WHO, 2001). Probiotics should fulfil several basic criteria: they must be able to survive in the gastrointestinal tract and to proliferate in the gut, which means that they must be resistant to gastric juices and bile; in addition, they should exert benefits to the host through growth and/or activity in the human body. Obviously, probiotics must be non-pathogenic and non-toxic. To confer health benefits, they should protect against pathogenic micro-organisms by means of multiple mechanisms (FAO/WHO, 2001). The consumption of $1 \times 10^6$ – $1 \times 10^{10}$ viable cells per day is usually required for beneficial effects to be seen (for review, see Bernardeau et al., 2006), although higher doses of $10^9$ – $10^{10}$ are needed for faecal recovery (Saxelin et al., 1993). All existing evidence, including genomic data, suggests that health effects are strain-specific. It is therefore of importance to identify the genus and species of the probiotic strains. The probiotic species most often investigated and most widely used in food products are mainly the lactic acid bacteria, *Lactobacillus* ssp., and *Bifidobacterium* ssp., although several other species such as *Propionibacterium*, *Streptococcus*, *Bacillus*, *Enterococcus* and *E.coli*, as well as yeasts, have been used.

Probiotics have been advocated for the prevention and treatment of a diverse range of disorders. Table 1 summarises the proposed targets and possible health effects, based on recent reviews or meta-analyses. *Lactobacillus rhamnosus* GG (LGG), discovered by professors Goldin and Gorbach (Goldin et al., 1992), is by far the most extensively studied probiotic strain (research evidence of LGG is illustrated in Figure 1). Although LGG seems to have multifunctional activities against a wide range of health problems, it has become evident that multistrain probiotics (containing several strains of the same genera) or multispecies probiotics (containing strains of different genera) may have advantages over monostrains (for review, see Timmerman et al., 2004). Since probiotics are expected to control multi-factorial diseases demanding a variety of probiotic properties, the synergistic actions or additive effects of several strains may be advantageous. Recently, a multispecies combination of *Lactobacillus* GG with three other species - *L. rhamnosus* LC 705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* 99 - has been shown to be effective, especially against certain gastrointestinal disorders; for instance, in improving the eradication of *Helicobacter pylori* (Myllyluoma et al., 2005) and alleviating IBS symptoms (Kajander et al., 2005), while LGG alone has not been particularly efficient in those conditions (Cremonini et al., 2002; Bausserman and Michail, 2005). The efficacy of multistrain probiotics could be due to their more efficient ability to colonise several niches in the GI tract. For example, the adhesion of *Bifidobacterium* Bb-12 and *Propionibacterium* JS to intestinal cells was more than double in the presence of LGG (Ouwehand et al., 2000; Ouwehand et al., 2002). Furthermore, strains from different genera of *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Propionibacterium* show symbiotic relationships to each other which enhance their growth and metabolic activity (Timmerman et al., 2004). However, in some indications such as in the treatment of atopic eczema, the same probiotic combination has not been effective while LGG alone has (Viljanen et al., 2005c). It has, however, shown beneficial effects in the prevention of atopic eczema when
probiotics were administered from birth (Kukkonen et al., 2007). This supports the idea of using multispecies probiotics specifically selected for a certain application, particularly in conditions with identified microbiota aberrancies or at the time when the microbiota is still developing, or in situations where an enhanced multispecies colonisation (which creates a probiotic niche) or synergistic actions against pathogens, may have a major impact on the disease. However, this does not necessarily mean that multispecies are superior for every indication.

Figure 1. Research areas of *Lactobacillus rhamnosus* GG (ATCC 53103) during the years 1987-2007 (Modified from Bernardeau et al., 2006).
### Table 1. Proposed targets and possible health effects of probiotics.

<table>
<thead>
<tr>
<th>Target organ</th>
<th>Indication</th>
<th>Health effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>Dental health</td>
<td>Reduced risk of caries due to inhibition of salivary <em>Streptococcus mutans</em></td>
<td>(Meurman, 2005)</td>
</tr>
<tr>
<td>Stomach</td>
<td><em>Helicobacter pylori</em> / gastritis</td>
<td>Increased eradication of <em>H. pylori</em> when combined with antibiotics Diminished adverse effects of antibiotics</td>
<td>(Gotteland <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Intestine</td>
<td>Acute gastroenteritis</td>
<td>Reduced duration of rotavirus diarrhea in children</td>
<td>(Huang <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td></td>
<td>Traveller's diarrhoea</td>
<td>Prevention of traveller's diarrhoea</td>
<td>(McFarland, 2007)</td>
</tr>
<tr>
<td></td>
<td>Antibiotic-associated diarrhoea (AAD)</td>
<td>Reduced duration of ADD</td>
<td>(Hawrelak <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td></td>
<td>Irritable bowel syndrome (IBS)</td>
<td>Alleviation of IBS symptoms</td>
<td>(Kajander and Korpela, 2006)</td>
</tr>
<tr>
<td></td>
<td>Inflammatory bowel diseases</td>
<td>Enhancement of mucosal immune responses, improvement of intestinal permeability, lowered disease activity and reduced relapse rates</td>
<td>(Shanahan, 2004)</td>
</tr>
<tr>
<td></td>
<td>Lactose intolerance</td>
<td>Increased tolerance of lactose due to microbial hydrolysis</td>
<td>(Levri <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td></td>
<td>Constipation</td>
<td>Shortening of intestinal transit time through modulation of colonic microbiota</td>
<td>(Hamilton-Miller, 2004)</td>
</tr>
<tr>
<td></td>
<td>Colorectal cancer</td>
<td>Down-regulation of intestinal microbial enzyme activities and the conversion of precarcinogens to carcinogens</td>
<td>(Commann <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td></td>
<td>Microbiota deviations</td>
<td>Combating aberrant microbiota</td>
<td>(Isolauri <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>Urogenital tract</td>
<td>Urinary tract infections</td>
<td>Inhibition of the growth of <em>E. coli</em> by restoration of vaginal lactobacilli microbiota</td>
<td>(Falagas <em>et al.</em>, 2006b)</td>
</tr>
<tr>
<td></td>
<td>Bacterial vaginitis / Vaginal candidosis</td>
<td>Inhibition of the growth of pathogenic bacteria/ <em>Candida</em> by establishing the vaginal lactobacilli microbiota</td>
<td>(Falagas <em>et al.</em>, 2006a) (Reid and Bocking, 2003)</td>
</tr>
<tr>
<td>Systemic effects</td>
<td>Allergy/atopy</td>
<td>Alleviation of symptoms and reduction of risk of atopic eczema</td>
<td>(Rautava <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td></td>
<td>Respiratory infections</td>
<td>Prevention of viral respiratory infections by enhancement of immunity, reduction of the carriage of bacterial pathogens</td>
<td>(de Vrese and Schrezenmeir, 2002)</td>
</tr>
</tbody>
</table>
2.2.2 Normal microbiota against pathogens

The commensal microbiota, which consists of micro-organisms present on mucosal surfaces covered by epithelial cells in the oral, gastrointestinal and respiratory tracts, plays an important role in protecting the body against pathogenic organisms (for review, see Tlaskalova-Hogenova et al., 2004). The normal indigenous microbiota, with an intact epithelium, creates a barrier (colonisation resistance) against pathogens by competing for nutrients and available adhesion sites on the mucosa, as well as by producing metabolic and regulatory substances, such as lactic acid, short-chain fatty acids, hydrogen peroxide and bacteriocins (for review, see Lu and Walker, 2001). Commensal microbes are in close “cross-talk” with the epithelial cells, and are able to induce an immunological response which may prevent infection (for review, see Corthesy et al., 2007). Environmental pressures such as changes in the diet or the use of antimicrobials can dramatically alter the composition of microbiota (Madden et al., 2005), leading to enhanced growth of pathogens or opportunistic pathogens.

The mucosal parts of the oral cavity (lips, cheek, tongue, palate) and non-shedding surfaces (teeth) contain approximately $10^8$-$10^{10}$ bacteria/mL, consisting of facultative, micro-aerophilic and obligate anaerobic species (for review, see Marsh, 2000) (Figure 2). The development of the resident oral microbiota starts at birth and changes during the time of teeth eruption, eventually leading to a relatively stable situation in adulthood, with broad species diversity. However, transient fluctuations in the oral ecosystem are caused by aging, the type of food ingested, medication, and the composition and rate of flow of saliva. *Candida albicans* is also a commensal organism, but can be converted to a pathogen when the normal microbiota is reduced as a result of antimicrobials, or the host defence mechanisms are impaired.

A large number of bacterial species colonise the upper respiratory tract, while the lower respiratory tract is virtually free of micro-organisms. The nasopharynx of healthy children is predominantly colonised by non-pathogenic organisms, some of which have the ability to inhibit the growth of potential otitis pathogens (Tano et al., 1999) (Figure 2). These interfering organisms include the aerobic α- and γ-haemolytic streptococci (Brook and Gober, 2000). α-haemolytic streptococci have been shown to inhibit the colonisation or the growth of potential pathogens in the adenoids of otitis-prone children (Brook and Yocum, 1999), and in the nasopharynx of sinusitis-prone children (Brook and Gober, 1999; 2000) possibly by producing inhibitory substances and through pH reduction. The normal microbiota and the interfering activity of α-haemolytic streptococci against *S. pneumoniae*, *S. pyogenes*, *H. influenzae* and *Staphylococcus aureus* have been shown to be lower in children with recurrent otitis media than in healthy children (Bernstein et al., 1993; Fujimori et al., 1996; Tano et al., 2000). This may be due to the wide use of broad-spectrum antimicrobials, such as amoxicillin-clavulanate, which disturb the normal microbiota more than narrow-spectrum antimicrobials (Brook and Gober, 2005), and is associated with prolonged absence of normal microbiota and rapid colonisation with pathogens (Brook and Gober, 2005), which probably leads to recurrent infections. Restoring the normal microbiota of the nasopharynx after antibiotic treatment, by replacement of α-streptococci, has been beneficial in reducing the clinical recurrences of acute and secretory otitis (Roos et al., 2001) and streptococcal pharyngotonsillitis (Roos et al., 1996).

It has been estimated that the adult gut microbiota is composed of 400-1000 species, the majority of which are strict anaerobes (97%), and only 3% belong to aerobes (for reviews, see Salminen...
et al., 1998; Noverr and Huffnagle, 2004a). There is a high degree of variability between the small intestine and the colon in terms of numbers and bacterial species (Figure 2). The small intestine harbours bacteria in numbers of $10^4$-$10^7$ cfu/g, increasing rapidly to $10^{10}$-$10^{12}$ cfu/g in the colon (Salminen et al., 1998). However, the new metagenomics analysis, based on 16S ribosomal DNA sequence-based enumeration used for sequencing the whole human intestinal microbiome, has resulted in the total number of $10^{13}$-$10^{14}$ microorganisms (Gill et al., 2006). The composition of individual microbiota is relatively constant, but may fluctuate under some circumstances, for instance during intestinal illnesses or antibiotic treatment, and to a lesser extent by dietary changes (Noverr and Huffnagle, 2004a). The wide diversity and inter-subject variability of the intestinal microbiota have been recently highlighted by the microbial sequence analysis of gut mucosal tissue and faecal samples derived from healthy subjects (Eckburg et al., 2005). Of the 395 bacterial phylotypes, 62% of the sequences were found to be novel and 80% were from species that have not been cultivated. This indicates that the bacterial diversity within humans is far greater than previously described. The intestinal microbiota plays an important part in the development and functioning of the immune system, as is clearly shown in studies on germ-free animals (for review, see Cebra, 1999). The development of the immune system is impaired in germ-free animals, as reflected by decreased numbers of lymphocytes in gut-associated lymphoid tissues and less diversified immunoglobulins.

**Figure 2.** Composition of the microbiota in the oral cavity, in the nasopharynx and in the gut.
2.2.3 Probiotic mechanisms

2.2.3.1 Antagonism to pathogens

Mucosal epithelial surfaces cover an enormous area of the body, and those in the mouth and gastrointestinal and respiratory tracts are constantly exposed to numerous micro-organisms and serve as primary ports of entry for most infectious agents. In the gut, altered intestinal permeability of the epithelium facilitates the invasion of pathogens. Permeability is increased in inflammatory conditions as a result of mucosal dysfunction caused by viruses, bacteria or dietary antigens (for review, see Lu and Walker, 2001). One possible mechanism of probiotic therapy is the promotion of this non-immunological gut defence barrier by normalising increased permeability and disturbed gut microecology (Isolauri et al., 1993). Probiotics may strengthen the epithelial barrier by promoting the recovery of the intestinal epithelium, as suggested by a study showing the prevention of cytokine-induced apoptosis in mouse or human epithelial cells in the presence of *Lactobacillus* GG (Yan and Polk, 2002). *Lactobacillus* GG has also been shown to increase the rate of mitoses in the small intestine of rats, resulting in increased numbers of cells in the villi, suggesting that probiotics may enhance mucosal regeneration (Banasaz et al., 2002). An enhanced epithelial barrier could prevent microbial translocation into the epithelium, as shown in an experimental study in which *L. acidophilus*, *L. rhamnosus*, or *L. plantarum* administered rectally to animals reduced bacterial translocation to the liver and mesenteric lymph nodes in an acute liver injury model (Adawi et al., 2001).

A probiotic mixture (VSL#3) containing four lactobacilli, three bifidobacteria and one *Streptococcus*, and their bacterial cell lysates, also increased transepithelial resistance in HT-29 intestinal epithelial cells, stabilised the tight junctions and prevented the decrease in resistance induced by pathogenic *Salmonella* (Ottem and Podolsky, 2004).

Bacterial adhesion to host cells or mucosal surfaces is always the essential first step in the disease process, and therefore interruption of the pathogen adhesion could be of benefit to the host. On the other hand, the adhesion of probiotics to mucosal surfaces is considered one of the main properties by which probiotics exert their health effects. Adherence to mucosal surfaces can be mediated by different mechanisms, such as electrostatic, hydrophobic, hydrophilic, capsular and fimbrial ones, and a wide range of mammalian cell surface constituents, such as glycoproteins and glycolipids, can act as receptors for bacterial attachment (Lu and Walker, 2001). Specific adhesin-receptor interaction involves carbohydrate moieties on the mucosal surface and carbohydrate-binding adhesins on the bacterial surface. The adhesion of pathogens can be inhibited by steric hindrance, by which a large number of beneficial bacteria may occupy receptor sites in a non-specific manner, or by competing for specific carbohydrate receptors. It has been suggested that lactobacilli such as *Lactobacillus rhamnosus* GG are able to compete with *E. coli* and *Salmonella* through steric hindrance (Lee and Puong, 2002). Mucosal surfaces are also protected by luminal secretions such as mucus, glycolipids, protective peptides and antibiotic-like substances such as defensins. Intestinal mucus (high-molecular-weight glycoproteins secreted by epithelial goblet cells) may protect epithelial cells from pathogens by providing a physico-chemical barrier, or by binding to pathogens through specific mucus-bacterial/viral interaction, thereby inhibiting their adherence to epithelial cells (Deplancke and Gaskins, 2001). *Lactobacillus* GG, *L. plantarum* 299V and a VSL#3 combination of probiotic strains has
inhibited the *in vitro* adherence of enteropathogenic *E. coli* to HT-29 intestinal epithelial cells by inducing the epithelial expression of mucin genes (Mack *et al.*, 1999; 2003). In *in vitro* assays, a specific probiotic combination, containing *L. rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* 99 or *B. lactis* Bb12, has displaced already adhered pathogens (*E. coli*, *Bacteroides*, *Clostridium*, *Listeria*, *Salmonella* and *Staphylococcus*) and has competitively excluded these (Collado *et al.*, 2006). However, the adhesion inhibition, displacement activity and competitive exclusion ability do not correlate, suggesting that different mechanisms are responsible for these phenomena. The ability of probiotics to inhibit adhesion and to displace pathogens seems to be strain-dependent. It is likely that the differences in the adhesion of probiotics themselves - caused by different surface molecules such as carbohydrates – may play a role in the inhibition or displacement of pathogens (Gueimonde *et al.*, 2007).

In addition to the competitive inhibition of the mucosal adherence of pathogens, lactobacilli and bifidobacteria also show direct antimicrobial activity against pathogens by producing antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, short chain fatty acids, biosurfactants and bacteriocins (for review, see Servin, 2004). Bacteriocins are bactericidal proteinaceous molecules which have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the strain which produces them. Bacteriocins, produced by lactobacilli or bifidobacteria, are effective against the *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Listeria* and *Mycobacterium* species. Several *in vitro* studies have shown growth inhibition against numerous pathogens, such as *Clostridium*, *Bacteroides*, *Bacillus*, *Enterobacteriaeae*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Salmonella* and *E. coli*, achieved by different antimicrobial substances produced by probiotics (Silva *et al.*, 1987; Huttunen *et al.*, 1995; Hudault *et al.*, 1997; Ocana *et al.*, 1999; Lievin *et al.*, 2000; Lee and Puong, 2002; Hutt *et al.*, 2006). The antibacterial activity of *Lactobacillus* strains has been shown to be mainly due to lactic acid (De Keersmaecker *et al.*, 2006), but for some strains evidence of an unknown inhibitory substance exists (Makras *et al.*, 2006). The antagonistic activity of *Lactobacillus* GG against *Salmonella typhimurium* seems to be dependent on the acidic environment (Hudault *et al.*, 1997). The growth inhibition may also result from a coaggregation phenomenon. In this event, lactobacilli and pathogens, through their specific surface properties, develop large contact areas (“a microenvironment”), where the activity of inhibitory metabolites is exacerbated leading to reduced growth capacity of pathogens (Drago *et al.*, 1997).

### 2.2.3.2 Immunomodulatory effects

An optimally functioning immune system is fundamentally important for protection against infectious diseases. Stimulation of the immune system has been suggested as one possible probiotic mechanism that provides protection against infections. Probiotic immunomodulation seems to be induced by bacterial patterns such as cell wall components (e.g. peptidoglycans in gram-negative and gram-positive bacteria, capsular polysaccharides and lipoteichoic acid in gram-positive, and lipopolysaccharide present in gram-negative groups), which are recognised by Toll-like receptors expressed in intestinal and immune cells (Pena and Versalovic, 2003). Lactobacilli have been shown to up-regulate the expression of TLR-2 transcripts, suggesting that lactobacilli deliver signals in dendritic cells through TLR-2, and thereby promote the
activation of these antigen-presenting cells (Miettinen et al., 1998; Foligne et al., 2007). The activation of immune cells, such as macrophages, neutrophils and dendritic cells, by bacterial cell wall components leads to the release of different immune mediators, cytokines and chemokines.

Peripheral blood mononuclear cells (PBMC) of humans and experimental animals have been found to secrete increased amounts of cytokines following in vitro stimulation with lactobacilli or bifidobacteria (Miettinen et al., 1996; Menard et al., 2004). The effect is mediated through the activation of the transcription factors, NF-κB and STAT by lipopolysaccharides, leading to enhanced gene expression and protein synthesis (Miettinen et al., 2000; Ishida-Fujii et al., 2007). Lactobacillus GG has induced cytokine production in human macrophages (Veckman et al., 2003), but the induction in dendritic cells has only been weak (Veckman et al., 2004). The effect, although much weaker, has also been shown in non-viable lactobacilli and bifidobacteria strains (Miettinen, 1998). Lactobacilli have mainly induced pro-inflammatory cytokines (such as IL-1β, IL-6, IL-12, TNF-α and IFN-γ), and the effect on anti-inflammatory cytokines (IL-4, IL-10 or TGF-β) has been weak or non-existent (Miettinen et al., 1996; 1998) (Table 2). However, under inflammatory conditions, lactobacilli have reduced the production of pro-inflammatory cytokines and induced anti-inflammatory cytokines (Pena and Versalovic, 2003; Hart et al., 2004; Jijon et al., 2004; Menard et al., 2004; Zhang et al., 2005), although there is a wide diversity between individual strains (Hart et al., 2004). Immunomodulation also seems to be different in in vitro and ex vivo conditions (Flinterman et al., 2007). Based on in vivo trials, the probiotic effect on Th1/Th2 balance seems to be dependent on the immunological status of the subject. In healthy adults and patients with an inflammatory condition, lactobacilli have mainly reduced the production of proinflammatory cytokines (Schultz et al., 2003; Braat et al., 2004), but in healthy elderly subjects (Arunachalam et al., 2000; Matsumoto et al., 2007) and in children with atopic diseases (Prescott et al., 2005; Viljanen et al., 2005b; Flinterman et al., 2007), probiotics have mainly enhanced the Th1 response by increasing the production of proinflammatory cytokines. However, the in vivo effect on anti-inflammatory cytokines seems to be controversial.
### Table 2. Effect of different probiotic bacteria on cytokine production in experimental *in vitro* assays and in clinical interventions.

<table>
<thead>
<tr>
<th>Probiotic strains</th>
<th>Model/Study design</th>
<th>Assessment</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td><em>In vitro</em></td>
<td>Cytokine production by human PBMC</td>
<td>IL-6, TNF-α↑&lt;br&gt; IL-10 (↑)&lt;br&gt; best inducers: <em>L. rhamnosus</em> GG, <em>B. animalis</em>, <em>L. acidophilus</em></td>
<td>(Miettinen <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> E509</td>
<td><em>In vitro</em></td>
<td>Cytokine production by human PBMC</td>
<td>IL-1β, IL-6, TNF-α↑&lt;br&gt; IL-12, IFN-γ, IL-18↑&lt;br&gt; IL-10 (↑)</td>
<td>(Miettinen <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td><em>In vitro</em></td>
<td>Cytokine production by human PBMC</td>
<td>IL-10 (↑)&lt;br&gt; IL-4 ↔&lt;br&gt; Expression of co-stimulatory molecules (↑) / ↔&lt;br&gt; TNF-α (↑)&lt;br&gt; IL-2, IL-12, IL-23, IL-27 ↔&lt;br&gt;</td>
<td>(Veckman <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td><em>In vitro</em>: inflammatory conditions induced by LPS or LTA</td>
<td>Cytokine production by murine macrophages</td>
<td>TNF-α↓&lt;br&gt; IL-10 ↔&lt;br&gt;</td>
<td>(Pena and Versalovic, 2003)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG: live or heat-killed</td>
<td><em>In vitro</em>: inflammatory conditions induced by TNF-α</td>
<td>Cytokine production by Caco2 intestinal epithelial cells</td>
<td>IL-8↑ for live LGG&lt;br&gt; IL-8 (↑) for heat-killed LGG&lt;br&gt; The effect was dose-dependent.</td>
<td>(Zhang <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td><em>B. breve</em> BbC50</td>
<td><em>In vitro</em>: inflammatory conditions induced by LPS or LTA</td>
<td>Cytokine production by human PBMC and intestinal cell monolayers</td>
<td>TNF-α↓ for both strains&lt;br&gt; IL-10↑ for <em>B. breve</em>&lt;br&gt; IL-10↓ for <em>S. thermoph</em>.</td>
<td>(Menard <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>S. thermophilus</em> S065</td>
<td><em>In vitro</em>: inflammatory conditions induced by LPS or LTA</td>
<td>Cytokine production by Caco2 intestinal epithelial cells</td>
<td>TNF-α↑&lt;br&gt; IL-12↑&lt;br&gt; IL-6 ↔&lt;br&gt;</td>
<td>(Ishida-Fujii <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>L. casei</em> I-5</td>
<td><em>Ex vivo</em>: inflammatory conditions induced by <em>E. coli</em></td>
<td>Cytokine levels in rat peripheral blood</td>
<td>TNF-α, IL-12↑&lt;br&gt; IL-6 ↔&lt;br&gt;</td>
<td>(Ishida-Fujii <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td>Combination of 8 strains (VSL#3)</td>
<td><em>In vitro</em>: inflammatory conditions induced by LPS</td>
<td>Cytokine production by human blood dendritic cells and intestinal lamina propria mononuclear cells</td>
<td>IL-12, IFN-γ↓&lt;br&gt; IL-10↑&lt;br&gt; Individual bacteria displayed different effects than combination: lactobacilli: IL-10↓ / ↔&lt;br&gt; bifidobacteria: IL-10↑&lt;br&gt;</td>
<td>(Hart <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Combination of 8 strains (VSL#3)</td>
<td><em>In vitro</em>: inflammatory conditions induced by <em>E. coli</em></td>
<td>Cytokine production by murine colonic cells, splenocytes and HT-29 cells</td>
<td>IFN-γ, TNF-α↓ in colonic cells&lt;br&gt; IL-8↓ in HT-29 cells&lt;br&gt;</td>
<td>(Jijon <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Probiotic strains</td>
<td>Model/Study design</td>
<td>Assessment</td>
<td>Outcome</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>----------------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG in capsules</td>
<td>5-week oral suppl. n=10 healthy adults</td>
<td>Cytokine production by PBMC</td>
<td>TNF-α, IL-6, IFN-γ ↓ IL-10, IL-4 ↑</td>
<td>(Schultz et al., 2003)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> in lyophilised powder</td>
<td>2-week oral suppl. n=6 healthy volunteers and 6 Crohn's disease patients</td>
<td>Cytokine production by PBMC</td>
<td>IL-2, IL-4, IL-10 ↓ in healthy subjects IL-2, IFN-γ, IL-10 ↓ in Crohn's patients IL-4 ↔ in Crohn's patients</td>
<td>(Braat et al., 2004)</td>
</tr>
<tr>
<td><em>B. lactis</em> HN019 or placebo in milk</td>
<td>DBPC 6-week oral suppl. n=25 healthy elderly subjects</td>
<td>Cytokine production by PBMC</td>
<td>IFN-α ↑</td>
<td>(Arunachalam et al., 2000)</td>
</tr>
<tr>
<td><em>B. animalis</em> ssp. lactis LKMS12 in yoghurt or placebo</td>
<td>PC 2-week oral suppl. n=6 elderly subjects</td>
<td>In vitro stimulation of murine macrophage-line cells by faecal precipitates from elderly subjects after oral consumption of <em>B. animalis</em></td>
<td>TNF-α ↑ IL-1α, IL-10 ↔</td>
<td>(Matsumoto et al., 2007)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG or a probiotic mixture containing <em>L. rhamnosus</em> GG, LC705, <em>B. breve</em> 99, <em>Propionibacterium</em> JS or placebo in capsules</td>
<td>DBPC 4-week oral suppl. n=230 infants with atopic eczema</td>
<td>Plasma levels of cytokines</td>
<td>IL-6 ↑ for LGG IL-10 ↑ for mixture IL-2, IL-4, TGF-β, IFN-γ ↔</td>
<td>(Viljanen et al., 2005b)</td>
</tr>
<tr>
<td><em>L. fermentum</em> in freeze-dried powder or placebo</td>
<td>DBPC 8-week oral suppl. n=53 infants with atopic dermatitis</td>
<td>Cytokine production by PBMC</td>
<td>TNF-α, IFN-γ ↑ IL-13 ↓ IL-10 ↓ TGF-β ↔</td>
<td>(Prescott et al., 2005)</td>
</tr>
<tr>
<td>Probiotic bacteria or placebo</td>
<td>DBPC 12-week oral suppl. n=13 infants with food allergy</td>
<td><em>In vitro</em> and <em>ex vivo</em> cytokine production by PBMC</td>
<td><em>In vitro</em>: TNF-α, IFN-γ, IL-10 ↑ <em>Ex vivo</em>: TNF-α, IL-10, IL-6 ↓</td>
<td>(Flinterman et al., 2007)</td>
</tr>
</tbody>
</table>

PBMC = Peripheral blood mononuclear cells, DCs = dendritic cells
DBPC = double-blind, placebo-controlled intervention
LTA = lipoteichoic acid, LPS = lipopolysaccharide
↑ = increased
(↑) = only a slight increase
↓ = decreased
↔ = no effect
The main function of pro-inflammatory cytokines and interferons is to attract phagocytic cells to the site of infection, and to stimulate cytotoxic T cells, monocytes, macrophages and NK cells. Experimental animal studies have shown that lactobacilli and bifidobacteria enhance the ex-vivo phagocytic activity of blood leucocytes and peritoneal or pulmonary macrophages, and increase the activity of NK cells (Moineau, 1991; Lee and Lee, 2005; Ogawa et al., 2006; Ishida-Fujii et al., 2007; Sun et al., 2007) (Table 3). In healthy adults, oral consumption of different lactobacilli or bifidobacteria has increased the ex-vivo phagocytic capacity of polymorphonuclear cells, and increased the NK cell-killing activity (Schiffrin et al., 1997; Arunachalam et al., 2000; Gill et al., 2001a; Gill et al., 2001b; Sheih et al., 2001; Parra et al., 2004). However, contrary results also exist (Spanhaak et al., 1998). The immuno-stimulatory effects of probiotics thus seem to be species- and strain-dependent, and may also depend on the viability of the bacteria. The enhancement of the immune system may also depend on age, since subjects over 70 years of age have experienced greater increases in NK-cell activity (Gill et al., 2001a). The immunological status of the host may also play a role: for instance, Lactobacillus GG has down-regulated the phagocytic activity of neutrophiles in milk-hypersensitive adults, but has increased the activity in healthy subjects (Pelto et al., 1998).

Table 3. Effect of different probiotic bacteria on immune cell activity in experimental animal studies and in clinical interventions.

<table>
<thead>
<tr>
<th>Probiotic strains</th>
<th>Model/Study design</th>
<th>Assessment</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. longum L. bulgaricus L. casei L. helveticus S. cremoris S. lactis S. thermophilus</td>
<td>In vivo mice: gastric intubation of probiotics for 8 days</td>
<td>Pulmonary alveolar macrophages</td>
<td>Phagocytic activity of alveolar macrophages ↑ for L. casei, B. longum, L. helveticus</td>
<td>(Moineau, 1991)</td>
</tr>
<tr>
<td>L. fermentum L. plantarum</td>
<td>In vivo mice: gastric intubation of probiotics for 3 weeks</td>
<td>Phagocytic activity of peritoneal leucocytes</td>
<td>Ex vivo phagocytic capacity of leucocytes ↑</td>
<td>(Lee and Lee, 2005)</td>
</tr>
<tr>
<td>L. casei L. paracasei L. acidophilus</td>
<td>In vivo mice: gastric intubation of probiotics for 2 days</td>
<td>NK-cells activity in spleen mononuclear cells</td>
<td>NK-cell activity ↑</td>
<td>(Ogawa et al., 2006)</td>
</tr>
<tr>
<td>L. casei I-5</td>
<td>In vivo rats: oral administration of probiotics for 10 days</td>
<td>Phagocytic activity of rat peritoneal macrophages and NK-cell cytotoxicity</td>
<td>Phagocytic activity ↑ NK-cell activity ↔</td>
<td>(Ishida-Fujii et al., 2007)</td>
</tr>
<tr>
<td>9 lactobacillus strains of Lactobacillus ssp. L. plantarum L. bulgaricus Rat small intestinal mucus incubated with lactobacilli</td>
<td>Phagocytic activity of Peyer’s patches’ macrophages</td>
<td>Strain specific enhancement of phagocytic activity of macrophages, which was correlated with the ingestion of lactobacilli by macrophages.</td>
<td>(Sun et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Probiotic strains</td>
<td>Model/Study design</td>
<td>Assessment</td>
<td>Outcome</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------</td>
<td>------------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>L. acidophilus La1 or B. bifidum Bb12 in fermented milk</td>
<td>3-week oral suppl. n=28 healthy volunteers</td>
<td>Lymphocyte subsets and leucocyte phagocytic activity</td>
<td>Lymphocyte subsets ↔, Phagocytic activity of granulocytes and monocytes ↑</td>
<td>(Schiffrin et al., 1997)</td>
</tr>
<tr>
<td>L. casei DN114001 or placebo in fermented milk</td>
<td>DBPC 8-week oral suppl. n=45 healthy volunteers</td>
<td>Lymphocyte subsets, NK-cell tumoricidal activity, phagocyte functions</td>
<td>Lymphocyte subsets ↔ NK-cell activity ↑ Oxidative burst ↑ capacity of monocytes ↑</td>
<td>(Parra et al., 2004)</td>
</tr>
<tr>
<td>B. lactis HN019 or placebo in milk</td>
<td>DBPC 6-week oral suppl. n=25 healthy elderly volunteers</td>
<td>Phagocytic activity of PMN</td>
<td>Phagocytic capacity ↑</td>
<td>(Arunachalam et al., 2000)</td>
</tr>
<tr>
<td>L. rhamnosus HN001 or B. lactis HN019 in milk</td>
<td>3-week oral suppl. n=27 healthy elderly volunteers</td>
<td>Proportion of CD56+ lymphocytes, ex vivo tumoricidal activity of PBMC</td>
<td>PBMC tumoricidal activity ↑: 101% for L. rhamnosus, 62% for B. lactis NK-cell activity ↑</td>
<td>(Gill et al., 2001a)</td>
</tr>
<tr>
<td>B. lactis HN019 in milk</td>
<td>3-week oral suppl. n=30 healthy elderly volunteers</td>
<td>Ex vivo leucocyte phagocytic activity, NK cell tumour-killing activity, leucocyte subsets</td>
<td>Phagocytic activity of phagocytes ↑ NK-cell activity ↑ CD4+ and CD25+ cells ↑</td>
<td>(Gill et al., 2001b)</td>
</tr>
<tr>
<td>L. rhamnosus HN001 or placebo in powder</td>
<td>DBPC 3-week oral suppl. n=52 healthy volunteers</td>
<td>Ex vivo phagocytic activity of PMN, NK-cell tumour-killing activity</td>
<td>Phagocytic activity of phagocytes ↑ NK-cell activity ↑</td>
<td>(Sheih et al., 2001)</td>
</tr>
<tr>
<td>L. casei Shirota or placebo in fermented milk</td>
<td>DBPC 4-week oral suppl. n=20 healthy volunteers</td>
<td>Lymphocyte subsets, NK-cell activity, phagocyte functions</td>
<td>Lymphocyte subsets ↔ NK-cell activity ↔ Phagocytosis ↔</td>
<td>(Spanhaak et al., 1998)</td>
</tr>
<tr>
<td>L. rhamnosus GG or placebo in milk</td>
<td>DBPC 1-week oral suppl. n=17 volunteers (9 healthy, 8 milk-hypersensitive)</td>
<td>Expression of phagocytosis receptors on neutrophiles and monocytes</td>
<td>Receptor expression in neutrophiles ↑ in healthy Receptor expression in neutrophiles ↓ in hypersensitivnesubjects</td>
<td>(Pelto et al., 1998)</td>
</tr>
</tbody>
</table>

PMN = Peripheral polymorphonuclear leucocyte
PBMC = Peripheral blood mononuclear cells
DBPC = double-blind, placebo-controlled intervention
↑ = increased
↓ = decreased
↔ = no effect
Lactobacilli have also been shown to enhance cell-mediated immune responses and T cell
development through the modulation of dendritic cell functions (Smits et al., 2005). Human
myeloid DCs, activated by live or heat-killed lactobacilli, clearly skewed to T helper I and Tc1
polarisation, leading to the enhancement of proliferation of CD4+ and CD8+ T cells (Miettinen
et al., 1998) (Table 4). In animal studies, the proliferative activity of T and B lymphocytes
has increased in murine lymphocytes after feeding with lactobacilli or Propionibacterium
(Kirjavainen et al. 1999). In humans, the effect of oral supplementation with lactobacilli on T
and B cell proliferative activity seems to depend on the immunological status of the subject:
duodenal B lymphocyte numbers and CD4+ T lymphocytes seem to increase in healthy
subjects (Schultz et al., 2003) and ileostomy patients (Valeur et al., 2004). However, in patients
with an inflammatory condition such as Crohn’s disease, an oral supplementation of lactobacilli
has resulted in decreased T cell proliferation (Braat et al., 2004).

Table 4. Effect of different probiotic bacteria on the proliferative activity of B and T lymphocytes
in experimental animal studies and in clinical interventions.

<table>
<thead>
<tr>
<th>Probiotic strains</th>
<th>Model/ Study design</th>
<th>Assessment</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG, <em>Propionibacterium freudenreichii</em> JS</td>
<td><em>In vivo</em> mice: peroral treatment with probiotics for 7 days</td>
<td>Proliferative activity of murine lymphocytes</td>
<td>B cell proliferation ↑ +57% for LGG / B cell proliferation ↑ +82% for PJS / T cell proliferation ↑ + 69% for LGG / T cell proliferation ↑ + 84% for PJS</td>
<td>(Kirjavainen et al. 1999)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>5-week oral suppl. n=10 healthy volunteers</td>
<td>CD4+ T lymphocyte activation</td>
<td>CD4+ T cell activation ↑</td>
<td>(Schultz et al., 2003)</td>
</tr>
<tr>
<td><em>L. reuteri</em></td>
<td>4-week oral suppl. n=10 healthy volunteers and 9 ileostomy patients</td>
<td>Cell counts in mucosal biopsy specimens</td>
<td>Duodenal B lymphocytes ↑ ileal CD4+ T cell ↑ Duodenal and stomach T cells ↔</td>
<td>(Valeur et al., 2004)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>2-week oral suppl. n=6 healthy volunteers and 6 Crohn’s patients</td>
<td>T cell proliferation</td>
<td>Expression of CD4+ T cells ↓</td>
<td>(Braat et al., 2004)</td>
</tr>
</tbody>
</table>

↑ = increased  
↓ = decreased  
↔ = no effect

To summarise, probiotic bacteria are able to stimulate the immune system in several ways. Probiotics induce cytokine release from immune cells, mainly towards Th1 polarization.
Through increased cytokine production, probiotics are able to stimulate different immune cells, including phagocytic cells, NK cells, and T and B cells. However, the effects are highly strain-dependent, and a combination of several strains may show synergistic as well as antagonistic effects. In addition, a probiotic effect *in vivo* seems to rely largely on the immunological status and possibly on the age of the subject.
2.2.3.3 Mucosal defence mechanisms

Oral ingestion of antigens produces IgA synthesis locally in the gut. After antigenic stimulation, T and B lymphocytes activated in the Peyer’s patches travel via the blood and enter the secondary lymphatic organs in the distal mucosal effector sites of the GI and respiratory tracts, or in the salivary glands (so-called mucosa-associated lymphoid tissue, MALT), where B cells differentiate into immunoglobulin-producing plasma cells. This provides one logical mechanism whereby orally-ingested probiotic bacteria may initiate an immune response in the gut, which then leads to enhanced responses at other mucosal surfaces.

Data from animal studies strongly indicate that several specific lactic acid and bifidobacteria strains induce the synthesis of antigen-specific IgA in intestinal secretions, in Peyer’s patch cells and in serum (Perdigon et al., 1990). It has been proposed that the possible underlying mechanism in triggering antibody secretion may be the enhanced secretion of IL-6 (He et al., 2005), which stimulates the growth of antibody-producing B lymphocytes. Metabolites of lactic acid bacteria, such as peptides derived from milk fermented with *L. helveticus*, have also increased the number of IgA-producing B cells in the lamina propria, and enhanced the secretory and systemic IgA levels in mice following *E. coli* challenge (Leblanc et al., 2004). In clinical studies, oral bacteriotherapy with *Lactobacillus* GG has increased the numbers of specific antibody-secreting cells against dietary β-lactalbumin and casein in patients with Crohn’s disease (Malin et al., 1996), antibody-secreting cells producing rotavirus-specific IgA antibodies in children with rotavirus gastroenteritis (Kaila et al., 1992; Majamaa et al., 1995), and faecal IgA in children with cow’s milk allergy (Viljanen et al., 2005a). In breast-fed infants, whose mothers consumed *Lactobacillus* GG before childbirth, and who were themselves supplemented with LGG after the birth, the total number of circulating IgM-, IgA- and IgG-secreting cells in peripheral blood was higher than in children without probiotic supplementation (Rinne et al., 2005). The results suggested that early treatment with probiotics with breastfeeding beneficially influences the maturation of gut humoral immunity. However, traditional yoghurt strains (*Streptococcus thermophilus* and *L. bulgaricus*) induced only a weak activity of salivary IgA to yoghurt cultures (Carlsson and Bratthall, 1985). Nor did the consumption of a fermented milk product containing *L. johnsonii* La1 increase the secretion of jejunal IgA against La 1, although serum IgA concentrations increased slightly (Marteau et al., 1997). *L. plantarum* 299v had no effect on the concentration of plasma cells, IgA or IgM positive cells in the lamina propria in patients undergoing abdominal surgery (Woodcock et al., 2004). The effectiveness of probiotics in enhancing the immunogenicity of oral vaccines in healthy humans has also been reported (Link-Amster et al., 1994). In these studies, *Lactobacillus* GG or *L. fermentum* have increased the formation of poliovirus-specific IgA and IgG in serum (de Vrese et al., 2005a), the numbers of IgA antibody-secreting cells against *Salmonella typhi* (He et al., 2000) the numbers of rotavirus-specific IgM-producing cells (Isolauri et al., 1995) and the level of influenza-specific IgA in serum (Olivares et al., 2007), suggesting that orally-ingested lactic acid bacteria have an adjuvant-like effect on the humoral responses.

The possible mechanisms by which probiotics may influence the growth of pathogens or stimulate mucosal innate and acquired immunity are presented in Figure 3.
Figure 3. Probiotics may prevent infections through several mechanisms:

1. By inhibiting the adhesion of pathogens to the epithelium in a non-specific manner or by competing for specific receptors and nutrients,
2. by producing antimicrobial agents against pathogens,
3. by inducing mucin (MUC2 and -3) production in the epithelial cells,
4. by strengthening the mucosal barrier by promoting the regeneration of epithelial cells and by reducing permeability,
5. by modulating the immune system through the antigen-presenting cells (macrophages and dendritic cells),
6. by inducing pro-inflammatory cytokine production from epithelial and immune cells, resulting in enhanced cell-mediated immune responses and the activation of cytotoxic T cells, phagocytic cells and NK cells,
7. by increasing the proliferation of B cells through the induction of Th2 cytokines, which travel to secondary lymphatic organs in mucosa-associated lymphoid tissue (MALT) and differentiate into immunoglobulin-producing plasma cells and may return to gut-associated lymphoid tissue (GALT),
8. by inducing the production of specific antibodies such as secretory IgA.
2.2.4 Clinical effects of probiotics against infectious diseases

2.2.4.1 Respiratory infections

The mucosal surfaces of the upper respiratory tract are functionally linked to other mucosal surfaces of the common mucosal-associated lymphoid tissue, and the respiratory tract is therefore an appropriate area for probiotic immuno-stimulation. There are some clinical studies that have examined the effects of probiotics on respiratory infections in basically healthy subjects. No positive effect of probiotics on the rate or duration of respiratory infections was seen in infants who consumed an infant formula with *Lactobacillus reuteri* or *Bifidobacterium lactis* for 12-weeks (Weizman *et al.*, 2005) (Table 5). The probiotic groups, however, had fewer febrile and diarrhoea episodes than the placebo group, and *L. reuteri* seemed to be more effective than *Bifidobacterium*. In schoolchildren, continuous consumption of *Lactobacillus casei* for 20 weeks reduced the occurrence of lower respiratory tract infections, while no effect was seen on the duration of the respiratory infections (Cobo Sanz *et al.*, 2006). However, the same probiotic did not reduce the incidence of either respiratory or gastrointestinal infections in independent elderly subjects during a 3-week consumption, but the duration of all infections, including gastrointestinal infections, was 20% shorter in the probiotic group than in the control group (Turchet *et al.*, 2003).

In healthy adults, the consumption of a dietary supplement containing *L. gasseri, B. longum* and *B. bifidum* combined with several vitamins and minerals, over a period of at least three months, reduced the incidence of respiratory tract infections, the number of days with fever and the symptoms of influenza, but did not have any effect on the duration of infections (Winkler *et al.*, 2005). However, on the basis of the results, it is difficult to separate the effect of probiotics from the possible beneficial effects of vitamins and minerals. When the effect of the supplement containing probiotics with vitamins and minerals was compared with the vitamin-mineral supplement alone, the probiotics significantly reduced the severity of symptoms, the duration of the common cold episodes and the number of days with fever, although no influence was seen on the overall incidence of respiratory tract infections (de Vrese *et al.*, 2005b). Consumption of a *Lactobacillus reuteri* drink for 80 days reduced the frequency of days off sick in Swedish employees, but no data were available on the actual incidence of infections (Tubelius *et al.*, 2005). Some studies suggest that probiotics might also be used as a supportive therapy in more serious conditions. In a non-controlled, pilot study among 7 paediatric patients suffering from respiratory distress, synbiotic therapy (containing *B. breve, L. casei* Shirota and galactooligosaccharides) from between 8 months and several years showed improvement in the intestinal microbiota which probably led to the improvement of intestinal functions and the suppression of bacterial translocation and thereby lessened the risk of severe bacterial infections in the lungs (Kanamori *et al.*, 2006). In patients with cystic fibrosis, colonised by *Pseudomonas*, a 6-month therapy with *Lactobacillus* GG reduced pulmonary exacerbation episodes, suggesting that probiotics might delay respiratory impairment in these patients (Bruzzese *et al.*, 2007).

Although there are some clinical data suggesting that probiotics may reduce either the occurrence or the duration of respiratory infections, only a few trials have made an attempt to clarify the mechanisms behind the positive effects. The groups of de Vrese and colleagues (2005, 2006), and Winkler and colleagues (2005) have shown that the acute cellular immune response before and 14 days after the probiotic supplementation increased the cell counts.
### Table 5. Clinical interventions on probiotics in the prevention of respiratory infections.

<table>
<thead>
<tr>
<th>Study design and settings</th>
<th>Subjects</th>
<th>Probiotics used and length of the intervention</th>
<th>Main findings: probiotic vs. placebo*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDBPC in day-care centres</td>
<td>201 healthy infants age: 4-10 mo</td>
<td><em>L. reuteri</em> SD 2112 or <em>B. lactis</em> BB-12 or placebo in infant formula for 12 weeks</td>
<td>Rate and duration of ARI ↔ Febrile episodes ↓ 0.11 for <em>L. reuteri</em> vs. 0.27 <em>B. lactis</em>, 0.41 placebo Absences from day care ↓ 0.14 for <em>L. reuteri</em> vs. 0.41, 0.43 Antibiotics ↓ 0.06 for <em>L. reuteri</em> vs. 0.21, 0.19</td>
<td>(Weizman et al., 2005)</td>
</tr>
<tr>
<td>RDBPC in schools</td>
<td>251 healthy children age: 3-12 y</td>
<td><em>L. casei</em> DN-114 001 or placebo in fermented milk for 20 weeks</td>
<td>Incidence of ARI ↔ Duration of ARI ↔ Incidence of LRI ↓ 32 vs. 49%, Incidence of fatigue ↓ 3 vs. 13%</td>
<td>(Cobo Sanz et al., 2006)</td>
</tr>
<tr>
<td>RC free-living subjects</td>
<td>260 subject age: &gt; 60 y</td>
<td><em>L. casei</em> DN-114 001 in fermented milk for 3 weeks vs. a control group</td>
<td>Incidence of influenza or GI inf. ↔ Duration of ARI and GI inf. ↓ 7 vs. 8.7 days Fever ↓ 38.3 vs. 38.5</td>
<td>(Turchet et al., 2003)</td>
</tr>
<tr>
<td>RDBPC free-living subjects</td>
<td>477 healthy adults age: 18-70 y</td>
<td><em>L. gasseri</em> PA16/8+, <em>B. longum</em> SP07/3+, <em>B. bifidum</em> MF 20/5 + vitamin. + miner. or placebo in tablet for 3-5.5 mo</td>
<td>Incidence of ARI ↔ 13.6% (n.s.) Duration of ARI ↔ (n.s.) ARI symptoms ↓ 19% (n.s.) Days with fever ↓ 0.3 vs. 0.7</td>
<td>(Winkler et al., 2005)</td>
</tr>
<tr>
<td>RDBPC free-living subjects</td>
<td>479 healthy adults age: 18-67 y</td>
<td><em>L. gasseri</em> PA16/8+, <em>B. longum</em> SP07/3+, <em>B. bifidum</em> MF 20/5 or placebo in tablet for 3 mo</td>
<td>Incidence of ARI ↔ Duration of ARI ↓ 7.0 vs. 8.9 Symptom score ↓ 79 vs. 103 Days with fever ↓ 0.2 vs. 1.0</td>
<td>(de Vrese et al., 2005b)</td>
</tr>
<tr>
<td>RDBPC free-living subjects</td>
<td>479 healthy adults age: 18-67 y</td>
<td><em>L. gasseri</em> PA16/8+, <em>B. longum</em> SP07/3+, <em>B. bifidum</em> MF 20/5 + vitamin. + miner. or placebo in tablet for 3-5 mo</td>
<td>Incidence of ARI ↔ Duration of ARI ↓ 7.0 vs. 8.9 Symptom score ↓</td>
<td>(de Vrese et al., 2006)</td>
</tr>
<tr>
<td>RDBPC work-place</td>
<td>262 employees age: 18-65 y</td>
<td><em>L. reuteri</em> or placebo through a drinking straw for 80 days</td>
<td>Sick-leave ↓ 10 vs. 26%, Percentage of sick-days ↓ 0.4 vs. 0.9</td>
<td>(Tubelius et al., 2005)</td>
</tr>
<tr>
<td>Pilot study</td>
<td>7 pediatric patients with severe respiratory distress</td>
<td><em>B. breve</em> + <em>L. casei</em> Shirota + galactooligosaccharides admin. orally or via a nasogastric tube for varying time periods (8 mo-6 y)</td>
<td>Improvement of the intestinal microbiota Antibiotics treatments ↓ Risk of bacteremia ↓</td>
<td>(Kanamori et al., 2006)</td>
</tr>
<tr>
<td>RPC</td>
<td>19 children with cystic fibrosis</td>
<td><em>L. rhamnosus</em> GG dissolved in ORS for 6 mo</td>
<td>Pulmonary exacerbations ↓ Hospital admissions ↓ Pulmonary function ↑</td>
<td>(Bruzzese et al., 2007)</td>
</tr>
</tbody>
</table>

RDBPC = Randomised, double-blind, placebo-controlled; RPC = Randomised, placebo-controlled; RD = Randomised, controlled
ARI= Acute respiratory infection; LRI= Lower respiratory infection; GI= Gastrointestinal
* significant difference unless otherwise stated
↑ = increased
↓ = decreased
↔ = no effect
of total leucocytes, lymphocytes, CD4+ T helper cells, cytotoxic and suppressor CD8+ T cells, and monocytes. Another probiotic mechanism suggested is their antagonistic effects against pathogens. A combination of *Lactobacillus* GG, *Bifidobacterium*, *L. acidophilus* and *S. thermophilus* given in fermented milk reduced the nasal colonisation of potentially pathogenic bacteria in healthy adults (Glück and Gebbers, 2003). The mechanism has been postulated to be mediated by the stimulation of B lymphocytes in gut-associated lymphoid tissue. This may have resulted in lymphocyte migration to the upper respiratory immune system, resulting in the production of antibodies.

To sum up the probiotic effect on respiratory infections: there is increasing evidence that specific probiotics may reduce infections outside the gastrointestinal tract, too. However, the effect seems to be mild or modest, and is mainly seen in the reduction of the severity or duration of infections. The probiotic mechanisms are probably mediated through the systemic immune system, but growth inhibition of pathogens may also occur locally in the upper respiratory tract.

### 2.2.4.2 Infectious diarrhoea

The most extensively studied health benefits of probiotics are to be found in diarrhoeal studies. The rationale for using probiotics to treat and prevent diarrhoeal diseases is based on the assumption that they modify the colonic microbiota and act against enteric pathogens. Several studies among children, using different *Lactobacillus* species (such as *L. rhamnosus* and *reuteri*), have shown a reduced duration of acute diarrhoea mainly caused by rotavirus, reduced excretion of rotavirus and reduced length of hospitalisation (Guarino *et al.*, 1997; Rosenfeldt *et al.*, 2002a; Szymanski *et al.*, 2006). In a multicentre trial conducted by the Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition, with 287 hospitalised children suffering from acute diarrhoea, *Lactobacillus* GG administered with an oral rehydration solution reduced the duration of diarrhoea from 71 to 58 hours, and in rotavirus-positive patients even more markedly, from 77 to 56 hours (Guandalini *et al.*, 2000). The number of watery stools and the risk of a prolonged course of disease were also reduced. The effect was evident among rotavirus-positive children and among those with diarrhoea of unknown etiology, but not among those with invasive diarrhoea caused by *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* or *Entamoeba* (Guandalini *et al.*, 2000). Nor did *Lactobacillus* GG administered with an oral rehydration solution reduce the duration of diarrhoea in Brazilian or Peruvian children with severe dehydrating diarrhoea (Costa-Ribeiro *et al.*, 2003; Salazar-Lindo *et al.*, 2004), when EPEC, *Vibrio cholera*, *Campylobacter* and *Shigella* or mixed species were causative pathogens in addition to rotavirus. It thus seems that the effect of probiotics in treating acute rotavirus diarrhoea is well established, but the efficacy in ameliorating invasive bacterial enteritis is not. Differences between studies might also result from the different degrees of severity of the disease. The beneficial effects of probiotics in the treatment of acute diarrhoea have also been shown in systematic reviews and meta-analyses carried out among hospitalised children (Szajewska and Mrukowicz, 2001; Huang *et al.*, 2002) (Table 6). The risk of prolonged diarrhoea has been reduced by 57% by the probiotic therapy (Szajewska and Mrukowicz, 2001).

Several systematic reviews or meta-analyses of probiotic therapy for preventing or treating *Clostridium difficile*-associated diarrhoea (CDAD), or antibiotic-associated diarrhoea (AAD)
<table>
<thead>
<tr>
<th>Design</th>
<th>Studies included</th>
<th>Inclusion criteria</th>
<th>Subjects</th>
<th>Probiotics used</th>
<th>Main findings: probiotic vs. placebo*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systematic review</td>
<td>13 RDBPC studies: 10 treatment, 3 prevention</td>
<td>Acute diarrhoea defined as &gt; 3 loose or watery stools/d</td>
<td>Children or infants n=731 / 773</td>
<td>*L. rhamnosus GG, L. reuteri, L. acidophilus, S. thermophilus, Saccharomyces boulardii</td>
<td>RR = 0.43 for diarrhoea lasting &gt; 3 days. Only LGG showed a consistent effect. Duration of diarrhoea ↓ -18.2 h for all -24.8 h for LGG and L. reuteri. Efficacy in prevention IRR: 0.17 0.23 (n.s.) 0.97 (n.s.)</td>
<td>(Szajewska and Mrukowicz, 2001)</td>
</tr>
<tr>
<td>Meta-analysis</td>
<td>18 RPC treatment studies</td>
<td>Acute diarrhoea, duration of diarrhoea reported</td>
<td>Healthy children &lt; 5 years n=1917</td>
<td>Coadministration of *L. rhamnosus GG, L. reuteri, L. acidophilus, L. bulgaricus, S. thermophilus, Sacch. boulardii, Enterococcus, B. subtilis with rehydration therapy</td>
<td>Duration of diarrhoea ↓ -0.8 day -1.2 days with LGG -0.6 day with other lactobacilli</td>
<td>(Huang et al., 2002)</td>
</tr>
<tr>
<td>Systematic review</td>
<td>8 RPC prevention or treatment studies: 4 CDAD 4 AAD</td>
<td>CDAD or AAD Adults n=not known</td>
<td>Sacch. boulardii, L. rhamnosus GG, L. plantarum, L. acidophilus + B. bifidum</td>
<td>RR ↓ in 2/8 studies: 0.13 0.17 (n.s.) 0.29 0.32 (n.s.) Number of days with diarrhoea ↓ 2 vs. 8 days</td>
<td>(Dendukuri et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Systematic review</td>
<td>6 RPC prevention studies</td>
<td>AAD</td>
<td>Children and adults n=692</td>
<td>*L. rhamnosus GG</td>
<td>RR ↓ in 4/6 studies: 0.13 0.17 (n.s.) 0.29 0.32 (n.s.) for AAD</td>
<td>(Hawrelak et al., 2005)</td>
</tr>
<tr>
<td>Meta-analysis</td>
<td>6 RPC prevention trials</td>
<td>AAD, incidence of diarrhoea available</td>
<td>Children n=707</td>
<td>*L. rhamnosus GG, L. acidophilus + B. infantis, L. acidophilus + L. bulgaricus, L. sporogens, Sacch. boulardii</td>
<td>RR = 0.43 for AAD</td>
<td>(Johnston et al., 2006)</td>
</tr>
<tr>
<td>Meta-analysis</td>
<td>25 RPC studies</td>
<td>AAD defined as &gt;3 loose or watery stools/d for at least 2 days</td>
<td>Children and adults n=3164</td>
<td>*L. rhamnosus GG, L. acidophilus, B. longum, C. butyricum, Bacillus clausi, Sacch. boulardii, Enterococcus faecium</td>
<td>RR = 0.43 for AAD RR = 0.59 for CDAD (only Sacch. boulardii) RR = 0.31 for LGG RR = 0.37 for Saccharomyces</td>
<td>(McFarland, 2006)</td>
</tr>
<tr>
<td>Meta-analysis</td>
<td>6 RPC treatment studies</td>
<td>AAD or CDAD</td>
<td>Children n=766</td>
<td>*L. rhamnosus GG, L. acidophilus + B. infantis, L. acidophilus + L. bulgaricus, B. lactis + S. thermophilus, Sacch. boulardii</td>
<td>RR = 0.44 for AAD RR = 0.48 for B. lactis + S. thermop. RR = 0.30 for LGG RR = 0.20 for Saccharomyces RR = 0.38 (n.s.) for CDAD</td>
<td>(Szajewska et al., 2006)</td>
</tr>
</tbody>
</table>
have been performed among children and adult patients (Dendukuri et al., 2005; Hawrelak et al., 2005; Johnston et al., 2006; McFarland, 2006; Szajewska et al., 2006) (Table 6). The pooled risk ratio (RR) for ADD has been around 0.4 in the probiotic treatment groups, and for the most effective strains, such as L. rhamnosus GG or Saccharomyces boulardii, even lower, around 0.2-0.3. The incidence of ADD, for example, has varied between 3 and 8% in the LGG groups, while in the placebo groups it was 16-30%, indicating a 0.17-0.32 relative risk (Hawrelak et al., 2005).

Although there is fairly convincing evidence of the therapeutic benefits of probiotics in treating viral and antibiotic-associated diarrhoea, especially in children, the evidence of probiotics in actually preventing infectious diarrhoea is still sparse. According to a recent meta-analysis of 34 randomised, placebo-controlled diarrhoeal prevention trials carried out among both children and adults, probiotic treatment showed a significant protective effect - a 35% reduction in the risk of diarrhoea (Sazawal et al., 2006) (Table 6). The effect varied according to the type of diarrhoea: it was most pronounced in preventing ADD, while the reduction of traveller’s diarrhoea was non-significant, although a separate meta-analysis on traveller’s diarrhoea showed a 15% reduction in the incidence (McFarland, 2007). According to Sazawal and colleagues, the effect was higher in children than in adults, probably as a result of the differences between the colonisation and gut microbiota of children and adults. A few studies that examined the preventive effect of probiotics on hospital-acquired diarrhoea in children during their hospital stay and shortly after discharge reported the incidence of nosocomial diarrhoea as being approximately 7% in the probiotic groups compared
to 31-33% in the control groups, indicating a relative risk of 0.20 (Saavedra et al., 1994; Szajewska et al., 2001). However, there are also studies reporting no preventive effects (Mastretta et al., 2002).

Apart from hospital-acquired diarrhoea, only a few studies have examined the preventive effect of probiotics on the occurrence of community-acquired diarrhoea among a healthy population (Oberhelman et al., 1999; Pedone et al., 1999; 2000; Chouraqui et al., 2004; Thibault et al., 2004; Weizman et al., 2005) (Table 7). Three studies have examined the effect of an infant formula containing bifidobacteria or lactobacilli on the incidence of diarrhoea in infants. Consumption of an infant formula containing *Bifidobacterium* or *Lactobacillus* has reduced

<table>
<thead>
<tr>
<th>Study design and settings</th>
<th>Subjects</th>
<th>Probiotics used and length of the intervention</th>
<th>Main findings: probiotic vs. placebo*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDBPC Peri-urban Peruvian town</td>
<td>204 under-nourished infants and children age: 6-24 mo</td>
<td><em>L. rhamnosus</em> GG or placebo in gelatine capsules for 15 mo</td>
<td>Diarrhoea episodes ↓ 5.2 vs. 6.0  Incidence of diarrhoea ↓ in nonbreastfed 4.7 vs. 5.9  Duration of diarrhoea ↔</td>
<td>(Oberhelman et al., 1999)</td>
</tr>
<tr>
<td>RPC Day-care centres</td>
<td>287 infants and children age: mean 19 mo</td>
<td><em>L. casei</em> DN-114001 or placebo in fermented milk for 6 mo</td>
<td>Incidence of diarrhoea ↔  Duration of diarrhoea ↓ 4.3 vs. 8.0 days</td>
<td>(Pedone et al., 1999)</td>
</tr>
<tr>
<td>RDBPC Multicentre</td>
<td>928 infants and children age: 6-24 mo</td>
<td><em>L. casei</em> DN-114001 or placebo in fermented milk for 4 mo</td>
<td>Incidence of diarrhoea ↓ 16% vs. 22%</td>
<td>(Pedone et al., 2000)</td>
</tr>
<tr>
<td>RDBPC Multicentre residential care settings</td>
<td>90 infants age &lt; 8 mo</td>
<td><em>B. lactis</em> Bb-12 or placebo in infant formula for 4.5 mo</td>
<td>Incidence of diarrhoea ↔  Duration of diarrhoea ↓ 1.2 vs. 2.3 days</td>
<td>(Chouraqui et al., 2004)</td>
</tr>
<tr>
<td>RDBPC Pediatric centres</td>
<td>971 infants age: 4-6 mo</td>
<td><em>B. breve</em> + <em>S. thermophilus</em> or placebo in infant formula for 5 mo</td>
<td>Incidence or duration of diarrhoea, hospital admissions ↔  Medical consultations ↓ 46% vs. 57%  Need for dehydration ↓ 2.5% vs. 6.1%  ORS prescription ↓ 42% vs. 52%</td>
<td>(Thibault et al., 2004)</td>
</tr>
<tr>
<td>RDBPC Day-care centres</td>
<td>201 infants age: 4-10 mo</td>
<td><em>B. lactis</em> Bb-12 or <em>L. reuteri</em> or placebo in infant formula for 3 mo</td>
<td>Diarrhoeal episodes ↓: 0.02 for <em>B. lactis</em> group 0.13 for <em>L. reuteri</em> group 0.31 for controls  Duration of episodes ↓: 0.15 vs. 0.37 vs. 0.59</td>
<td>(Weizman et al., 2005)</td>
</tr>
<tr>
<td>RPC Military camps</td>
<td>502 military men</td>
<td><em>L. casei</em> DN-114001 or placebo in yogurt for 2 mo</td>
<td>Incidence of diarrhoea ↔  Duration of diarrhoea ↔</td>
<td>(Pereg et al., 2005)</td>
</tr>
</tbody>
</table>

RDBPC = Randomised, double-blind, placebo-controlled; RPC = Randomised, placebo-controlled  
* significant difference unless otherwise stated  
↑ = increased  
↓ = decreased  
↔ = no effect
diarrhoeal episodes and the duration of diarrhoea, but no differences were seen in stool pathogens as regards rotavirus or bacterial cultures (Weizman et al., 2005). However, studies by Thibault et al., (2004) and Chouraqui et al., (2004) could not show any beneficial effect of a Bifidobacterium infant formula on the incidence or duration of diarrhoea, but the severity of infections (e.g. the need for rehydration), was reduced (Thibault et al., 2004). Lactobacillus GG (Oberhelman et al., 1999) and L. casei (Pedone et al., 1999; 2000) have reduced the incidence and the severity of diarrhoea in infants and small children but not in young military men (Pereg et al., 2005). Although some studies suggest beneficial effects of probiotics in the prevention of diarrhoea in healthy infants and children, the data on adults is sparce, and virtually no data among the elderly exists so far.

Taken together, specific probiotics seem to be effective in reducing the duration of acute diarrhoea, especially rotavirus diarrhoea in children. Specific probiotic strains may protect from antibiotic-associated and nosocomial diarrhoea in both children and adults. Probiotics have also shown a modest effect in preventing community-acquired infectious diarrhoea in healthy infants and children, but the evidence is weak on the prevention of diarrhoea among adults and non-existant among the elderly.

### 2.2.4.3 Candida infections

It has been shown in animal studies that Candida albicans colonises germ-free animals more easily than conventional animals, suggesting that normal anaerobic microbiota in the oral cavity, digestive tract or vagina suppresses the growth of Candida in vitro (for review, see Kerr, 1999). The importance of the normal gut microbiota in suppressing the growth of C. albicans has been clearly demonstrated in an in vitro model of the human colon (Payne et al., 2003). In that particular model, tetracycline disrupted the composition of the normal bacterial population by decreasing the total numbers of anaerobes, bifidobacteria, lactobacilli, bacteroides and clostridia, while the amount of yeasts increased. The addition of Lactobacillus plantarum to the model noticeably reduced the growth of Candida.

Clinical evidence of the antifungal properties of probiotics in humans is sparce, and comes mainly from studies examining the prophylactic effects of lactobacilli on vaginal candidosis (Hilton et al., 1992; Shalev et al., 1996). Long-term consumption of yoghurt containing Lactobacillus acidophilus reduced both vaginal candidal colonisation and infection compared to the period without yoghurt consumption (Hilton et al., 1992). The mechanism in the prevention of vaginal candidosis has been postulated as being due to the production of hydrogen peroxide by the lactobacilli, since the most effective strains have been H₂O₂-producers (Hilton et al., 1992; Shalev et al., 1996). Normalisation of vaginal microbiota has also been postulated as a possible mechanism, since Lactobacillus species are the predominant micro-organisms in the vaginal microbiota, and orally-consumed strains have been found in faeces and vaginal secretions (Hilton et al., 1992; Shalev et al., 1996). However, yeasts and lactobacilli can co-exist in the vagina, and there are also contradictory results on the effect of lactobacilli on vaginal candidosis. No beneficial effects on candidal vaginitis were observed when a 4-month consumption of yoghurt containing live L. acidophilus was compared to pasteurised yoghurt (Shalev et al., 1996). Nor did a short-term treatment of lactobacilli simultaneously with antibiotics reduce the incidence of post-antibiotic vulvovaginitis (Pirotta et al. 2004). Although
the oral administration of different *Lactobacillus* strains can result in restoration of normal vaginal microbiota (Reid *et al.*, 2003), and some probiotic strains can be recovered from the vagina after oral consumption (Vasquez, 2005), the protective role of lactobacilli in preventing *Candida* vaginitis still remains unproven. There are only a very few clinical trials showing the beneficial effects of probiotics on oral or gastrointestinal *Candida*. The consumption of cheese containing *Lactobacillus* GG and *L. rhamnosus* LC705 showed a tendency to reduce salivary yeasts in healthy adults (Ahola *et al.*, 2002). Recently, *Lactobacillus* GG has also been shown to reduce the enteric colonisation of *Candida* in very-low-birth-weight preterm neonates, measured by colonies isolated from oropharyngeal, gastric aspirate, stool and faecal specimens (Manzoni *et al.*, 2006).

*In vivo* animal studies have shown that the oral administration of live *L. acidophilus* and *fermentum* has shortened the duration of oral colonisation by *Candida* in mice (Elahi *et al.*, 2005). Different *Lactobacillus* species (*L. acidophilus*, reuteri, and *rhamnosus* GG) and *Bifidobacterium animalis* have also reduced the incidence of systemic candidiasis in immunodeficient mice (Wagner *et al.*, 1997a). Probiotics also reduced the number of *Candida* in the alimentary tract, but *Bifidobacterium* was the only one to reduce the severity of orogastric candidiasis. All the strains were seen to colonise and persist in the alimentary tract (Wagner *et al.*, 1997b). However, the viability of probiotics does not seem necessary for reducing the number of *Candida*, since also heat-killed *Lactobacillus* GG, too, induces limited protection against candidiasis in immunodeficient mice; LGG suppresses the severity of candidiasis in the stomach, oesophagus, tongue and hard palate of mice with dysfunctional phagocytic cells, deficient NK-cell activity, and missing T cells (Wagner *et al.*, 2000). Heat-killed *Lactobacillus* GG and *L. acidophilus* were both able to inhibit systemic candidiasis in *Candida*-resistant mice; it was hypothesised that the adherence of *Candida* was blocked by the surface components of the heat-killed lactobacilli which stimulated the immune system (Wagner *et al.*, 2000).

To summarise, lactobacilli may have antagonistic effects against *Candida*. The clinical evidence on the effect of probiotics comes mainly from vaginal candidosis studies. However, several animal trials suggest that lactobacilli are able to suppress the growth of *Candida* in different parts of the alimentary tract.

### 2.2.5 Safety of probiotics

The increasingly widespread use of probiotics, in addition to the fact that probiotics are live bacteria, has raised the question of their possible health risks. Although probiotic therapy is generally considered harmless, rare case reports have been published on the ability of lactobacilli to cause endocarditis and bacteraemia (for review, see Boyle *et al.*, 2006b). Few cases have been linked to the ingestion of probiotic supplements, or else bacteria found in blood cultures could not have been distinguished from the strains used in food or probiotic supplements. However, all the cases have occurred in patients with underlying severe chronic diseases (Boyle *et al.*, 2006b). Immunosuppression, prior prolonged hospitalisation and surgical operations have been identified as major predisposing factors for *Lactobacillus* bacteremia (Salminen *et al.*, 2004a). However, no reports have described bacteremia related to probiotic use in otherwise healthy people. Nor does published evidence suggests that the consumption of probiotics containing lactobacilli or bifidobacteria increases the risk of opportunistic
infections in immunocompromised subjects. In fact, probiotics have been proved to be safe and well-tolerated in immunocompromised patients with HIV (Wolf et al., 1998; Salminen et al., 2004b) and also in children, including pre-term neonates (Agarwal et al., 2003; Bin-Nun et al., 2005).

In Finland, the safety of *L. rhamnosus* GG, a widely used probiotic strain, has been followed nationwide since the year 1990, when the probiotic LGG was introduced to the market. Despite the marked increase in the use of probiotic foods containing LGG since then, no significant increase in *Lactobacillus* bacteremia attributable to probiotics has been observed (Salminen et al., 2002). During the years 1990-2000, all *Lactobacillus* isolates from blood culture specimens were collected, and lactobacilli were isolated in 0.02% of all the blood cultures and 0.2% of all the positive blood cultures. The annual incidence of *Lactobacillus* bacteremia in the Finnish population was 0.3 cases / 100,000 inhabitants (Salminen et al., 2002). Lactobacilli and bifidobacteria belong to the normal intestinal microbiota; lactobacillemia may thus occur naturally, without exogenous administration. There is also no evidence that ingested probiotic lactobacilli or bifidobacteria pose any higher risk of infection than the risk associated with commensal strains (Borriello et al., 2003). A workshop panel consisting of experts on probiotics has concluded that on the basis of current scientific and medical evidence, the consumption of probiotic products presents a negligible risk to consumers, including immunocompromised subjects (Borriello et al., 2003). The European Food Safety Authority (EFSA) has also proposed a QPS status (Qualified Presumption of Safety) for *Lactobacillus* (including *L. rhamnosus*), *Bifidobacterium* and *Propionibacterium* (particularly *P. freudenreichii* ssp. *shermanii*) species (EFSA, 2006). EFSA concludes that “there are apparently no specific safety concerns regarding a number of *Lactobacillus*, *Bifidobacterium* and *Propionibacterium* species, which have a long history of safe use in food.”
3 AIMS OF THE STUDY

The main objective of this thesis was to evaluate whether long-term treatment with a specific probiotic or a specific probiotic combination would prevent the clinical appearance of common infections in children, and also in the elderly, who are at increased risk of infection. To resolve the main question, four studies among children and the elderly were performed, in which the specific aims were:

To examine whether a specific probiotic supplementation would reduce the risk /occurrence of:

1. acute respiratory infections and their complications in healthy children (I), in otitis-prone children (II), and in the institutionalised elderly (III), and to consider the possible mechanisms of probiotics by investigating whether probiotics reduce the prevalence of common viral respiratory pathogens (II),

2. acute otitis media in healthy children (I) and in otitis-prone children (II), and to consider the possible mechanisms of probiotics by investigating whether probiotics reduce the carriage of the main bacterial otitis pathogens (II),

3. diarrhoea in healthy children (I) and in the institutionalised elderly (III) and

4. high oral yeast carriage in independent elderly subjects (IV).
4 MATERIALS AND METHODS

4.1 Subjects and study designs (I-IV)

The description of the study settings, subjects and interventions in each study are shown in Table 8, the individual study designs, in Figure 4.

All the studies were randomised, double-blind, placebo-controlled clinical intervention studies with two parallel groups (probiotic and placebo). The required sample size for each study was calculated on the basis of previous probiotic studies examining the incidence of respiratory infections (II) or diarrhoea (III). If there were no previous probiotic studies available, other non-pharmacological intervention studies were used as a reference (I, IV). Estimated sample sizes were increased by 10-30%, depending on the study population, in order to compensate for the anticipated drop-out rates. A total of 500 (I), 380 (II), 216 (III) and 216 (IV) subjects completing the study were needed for the expected differences between the groups to be detected. The exact sample size calculations are presented in the original articles.
## MATERIALS AND METHODS

### 4.1.1 Respiratory and gastrointestinal infections in healthy children (I)

Healthy children aged 1-6 years and attending day-care centres, were recruited through meetings with parents. Those with severe chronic diseases, cow’s milk allergy, lactose intolerance or severe food allergy were excluded. The children were considered to have a slightly increased risk of infections because of their day-care attendance.

During the 7-month intervention (October 1998 to April 1999), data on the occurrence and duration of respiratory and gastrointestinal infections, medication, absences from day-care centre and doctor’s diagnoses, were gathered by means of symptom diaries filled in daily by the parents. Information on the demographic data, home environment, the child’s nutrition habits and history of illnesses was collected at baseline. No scheduled clinical examinations were carried out during the study. The children used either municipal health care services, or private doctors whenever necessary. All the diagnoses and visits to doctors were reported in the study diaries either by the doctor or by the parents. Faecal samples were collected at the beginning, in the middle and at the end of the study to determine the recovery of *Lactobacillus GG*. The parents were also instructed to contact the researchers each time their child had

<table>
<thead>
<tr>
<th>Study</th>
<th>Settings</th>
<th>Subjects</th>
<th>Numbers starting/completing the study (%)</th>
<th>Intervention</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>18 day-care centres</td>
<td>Healthy children attending day-care centres: slightly increased risk</td>
<td>571 / 513 (90%)</td>
<td>Low-fat milk containing LGG</td>
<td>7 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low-fat milk without probiotics</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1 study centre</td>
<td>Otitis-prone children: moderately increased risk</td>
<td>309 / 269 (87%)</td>
<td>Capsules containing LGG, LC705, PJS, Bb99</td>
<td>6 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Placebo capsules</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2 old people’s homes</td>
<td>Institutionalised elderly subjects: moderately increased risk</td>
<td>265 / 226 (85%)</td>
<td>Capsules containing LGG, LC705, PJS, Bb99</td>
<td>5 mo</td>
</tr>
<tr>
<td></td>
<td>1 hospital</td>
<td></td>
<td></td>
<td>Placebo capsules</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12 old people’s homes</td>
<td>Independent elderly subjects: slightly increased risk</td>
<td>276 / 192 (70%)</td>
<td>Low-fat cheese containing LGG, LC705, PJS</td>
<td>4 mo</td>
</tr>
<tr>
<td></td>
<td>37 sheltered housing units</td>
<td></td>
<td></td>
<td>Low-fat cheese without probiotics</td>
<td></td>
</tr>
</tbody>
</table>

* *LGG* = *Lactobacillus rhamnosus* GG (ATCC 53103)  
  LC705 = *Lactobacillus rhamnosus* LC705 (DSM 7061)  
  PJS = *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7067)  
  Bb99 = *Bifidobacterium breve* 99 (DSM 13692)
diarrhoea, and to collect a faecal sample for pathogen analysis. However, although there were 179 diarrhoeal episodes during the study, only 31 faecal samples were obtained, and therefore these results have been omitted.

4.1.2 Respiratory infections and acute otitis media in otitis-prone children (II)

Otitis-prone children, aged 10 months to 6 years, who had experienced at least four episodes of acute otitis media during the preceding 12 months or at least three episodes during the preceding 6 months, were recruited by advertisements in newspapers and through primary healthcare and day-care centres. These children were considered to be at moderately increased risk of infection because of their history of recurrent otitis media. Children on regular medication, with chronic illnesses, Down's syndrome, lip or palatal cleft, otitis media with effusion, or who were scheduled for tympanostomy or adenoidectomy during the study were excluded.

The study consisted of a four-week run-in period followed by a 6-month intervention (carried out between September 2001 and April 2002), during which period the occurrences of acute respiratory infections and AOM episodes were registered. The children were examined by a full-time physician, a senior resident in otorhinolaryngology, three times during the study. These visits were made at baseline, in the middle (3 months) and at the end (6 months) of the study, and scheduled at times when the child was free of respiratory symptoms and free of AOM. At each visit, the physician carried out a general physical examination, including inspection of the child's ears with a pneumatic otoscope and a tympanometry (GSI 37 Auto-Tymp, Grason-Stadler, Milford, NH, USA). In addition, a nasopharyngeal sample for the culture of potential bacterial pathogens (S. pneumoniae, H. influenzae, M. catarrhalis and S. pyogenes) and the detection of viral pathogens (rhinovirus and enterovirus), and a saliva sample for analysis of anti-pneumococcal IgA antibodies were taken during these "healthy visits". Each time the parents suspected AOM, they were instructed to bring their child to the study clinic. During these illness visits, a physical examination, a pneumatic otoscope and a tympanometry were carried out by the same doctor, but no samples were taken. If the child visited any doctor other than the study physician, the diagnosis was double-checked as soon as possible by the study physician. The parents kept a daily diary of the symptoms of acute respiratory infections, the use of medication, and visits to doctors other than the study physician.

4.1.3 Respiratory and gastrointestinal infections in the institutionalised elderly (III)

Institutionalised elderly people aged 65 years or over were recruited from the long-term care units in the municipal hospital of the City of Jyväskylä and from two old people's homes in Jyväskylä. Patients with chronic gastrointestinal disorders and those who were expected to move to other residential care units during the study were excluded. Because nurses participated actively in the study, no attempt was made to exclude patients with dementia. Subjects were considered to have moderately increased risk of infectious diseases because of their frail health status and the need for long-term care.
MATERIALS AND METHODS

The study consisted of a two-week run-in period followed by a 5-month intervention (January to May 2001). During both the run-in period and the intervention, personal nurses evaluated the overall health status of the subjects daily. Information on the occurrence of respiratory and gastrointestinal infections, fever (≥ 38°C) and the use of antimicrobial agents was reported in a study diary by nurses on a daily basis. The number of bowel movements, stool consistency, and the use of laxatives were also recorded daily. The health status of the patients was evaluated at a routine doctor’s examination, five times a week in the hospital and once a week in the old people’s homes. Baseline information on medical history was obtained from the hospital medical records. Functional capacity was evaluated on the basis of the Barthel Index score (Mahoney and Barthel, 1965), weight and height were measured, and the body mass index (BMI) was calculated at baseline.

4.1.4 Oral Candida in the independent elderly (IV)

Independent elderly people aged 65 years or over living in old people’s homes or sheltered housing units and who were mentally able to understand and follow the study protocol were recruited through informational meetings about the study. Those with current oral yeast medication, or dementia according to the Mini Mental State (MMS) test (Folstein et al., 1975), were excluded. The subjects who participated were considered to represent the average elderly population with a slightly increased risk of infections due to age-related diseases and medication.

The study consisted of a three-week run-in period followed by a 4-month intervention and was carried out during different periods between January 2001 and March 2002. Baseline information on health status was gathered by interviewing the subjects, and on the use of medication, from medication prescriptions. Oral yeast samples were taken by research assistants four times during the study. The first sample at the beginning of the run-in period was taken in order to form a stratifying factor for the randomisation. The next three samples were taken on scheduled clinical examinations (at 0, 2 and 4 months). Stimulated and unstimulated salivary secretion rates and salivary buffering capacity were measured (by the Dentobuff® Strip method; Orion Diagnostica, Espoo, Finland) at baseline and at the end of the intervention. The subjects were interviewed five times (at 0, 1, 2, 3 and 4 months) about their general health, changes in medication and subjective feelings of oral pain and dry mouth. The oral and dental status of the subjects was examined according to WHO criteria (WHO, 1997) by an experienced dentist, at baseline and at the end of the intervention. Periodontal conditions, the presence of mucosal lesions and the number of decayed, missing and filled teeth or the number and type of prosthetic appliances were recorded. Mucosal lesions were recorded as leukoplakia (white oral lesions, hyperkeratotic areas that are not removable by scraping), erythroplakia (erythematous, red oral lesions), hyperplasia (white plaques, not easily removed), ulceration and pigmented oral lesions.
MATERIALS AND METHODS

Figure 4. Study designs.

**STUDY I**
- Probiotic group (n=282)
- Placebo group (n=289)
- 0 mo
- 4 mo
- 7 mo

**STUDY II**
- Probiotic group (n=155)
- Placebo group (n=154)
- Run-in period 4 wk
- 0 mo
- 3 mo
- 6 mo

**STUDY III**
- Probiotic group (n=135)
- Placebo group (n=130)
- Run-in period 2 wk
- 5 mo

**STUDY IV**
- Probiotic group (n=136)
- Placebo group (n=140)
- Run-in period 3 wk
- 0 mo
- 2 mo
- 4 mo

= reporting infection symptoms in a study diary (Study I, II, III)
= faecal sample (Study I)
= nasopharyngeal and saliva samples (Study II)
= yeast and saliva samples (Study IV)
4.2 Administration and doses of probiotics (I-IV)

The subjects were randomly allocated to the probiotic or placebo groups according to a computer-generated random permuted block method. A block size of four was used and the randomisation was stratified according to the suspected factors affecting the susceptibility of infections: in Study I, according to age and the individual day-care centre; in Study II, according to age, gender and the form of day care; in Study III, according to the health-care unit; and in Study IV, according to the baseline yeast count.

Probiotic treatment was given either in food (Studies I and IV) or in capsules (Studies II and III). In Study I, the probiotic *Lactobacillus rhamnosus* GG (ATCC 53103) was given in milk (Gefilus®, Valio Ltd, Riihimäki, Finland). The children drank the milk during their attendance at day care, five days a week and three times a day, at an average of 260 mL/day. The milk contained 1% of fat and 5-10 x 10^5 cfu/mL (colony-forming units) of strain LGG®. Thus the average amount of LGG was approximately 1-3 x 10^8 cfu/day. The control milk was similar, but without the LGG. In Study IV, a probiotic combination of the strains *L. rhamnosus* GG, *L. rhamnosus* LC705 (DSM 7061) and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7067) was given in low-fat cheese. The daily dose of cheese was 50g, containing 15% of fat and 10^7 cfu/g of each probiotic strain. The average quantity of probiotics was 1.5 x 10^9 cfu/day. The placebo cheese contained 17% of fat and no probiotics. The placebo products were blinded by using coded packages.

The gelatine capsules consumed in Studies II and III contained a mixture of probiotic strains (*L. rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* 99 (DSM 13692), *Propionibacterium JS*, Valio Ltd, Helsinki, Finland), 8-9 x 10^9 cfu/capsule of each strain. The placebo capsules were identical in appearance, with cellulose microcrystalline as a filling material. Otitis-prone children (II) consumed one capsule (daily dose of 3-6 x 10^10 cfu) and the institutionalised elderly (III), two capsules per day (daily dose of 6 x 10^10 – 1.2 x 10^11 cfu). The subjects were instructed to consume the capsules in the morning with breakfast. They were allowed to swallow the capsules, or if they were not able to swallow, the capsules were opened and the powder was blended with a cold drink or food, preferably a milk product.

The amount of milk or cheese eaten, or the number of probiotic capsules consumed, was recorded in the study diary. Compliance (%) was measured as 100*(the amount of milk or cheese or capsules eaten/ total amount). The use of other products containing probiotic bacteria was forbidden for one month preceding the interventions (I and II), during the run-in periods (III and IV) and throughout the interventions. The study products were obtained from the Research and Development Centre of Valio, Ltd, Helsinki, Finland.
4.3 Diagnostics of infectious diseases

4.3.1 Acute respiratory tract infections (I-III)

In Studies I and II, the symptoms of acute respiratory infections (ARI) (fever, rhinitis, sore throat, cough, wheezing, earache, otorrhoea), and other illness-related symptoms such as irritability, night-restlessness and poor appetite, were recorded on a daily basis in a symptom diary by the children's parents. Acute respiratory infection was defined when at least one respiratory symptom was present for at least two consecutive days or at least two symptoms were present for at least one day. In Study III, “the common cold” was recorded in a study diary by a personal nurse on the basis of common respiratory symptoms (rhinitis, cough, sore throat and wheezing). The duration of ARI episodes was counted from the study diaries. In all the studies, a new ARI episode was defined as occurring after at least 7 asymptomatic days.

Respiratory infections with complications (bronchitis, pneumonia, sinusitis) were diagnosed by a doctor on the basis of commonly accepted clinical criteria. In Study I, the diagnoses were made by municipal or private physicians, in Study II, by a study doctor, and in Study III, by hospital doctors. A new bronchitis and sinusitis episode was defined as one after at least 7 symptom-free days, and a new pneumonia episode, after at least 14 symptom-free days. The use of antimicrobials for treating respiratory infections was recorded in the study diaries.

4.3.2. Acute otitis media (I-II)

In Study I, the diagnosis of acute otitis media (AOM) was made by municipal or private doctors on the basis of commonly accepted criteria. In Study II, 69% of all AOM diagnoses were made by a study doctor, a senior resident in otorhinolaryngology, and the remainder by municipal or private doctors.

In Study II, AOM was defined as the simultaneous presence of middle ear effusion, abnormalities of the tympanic membrane indicating an inflammation, and at least one symptom of an acute infection (fever, earache, otorrhoea, cough, rhinitis, sore throat, wheezing, irritability, poor appetite, vomiting or diarrhoea). The presence of middle ear effusion was detected in pneumatic otoscopy by a combination of abnormal colour (haemorrhagic, pale or yellow), reduced or absent mobility or a bulging position of the tympanic membrane, and a B-type, flat tympanogram curve. The duration of AOM was calculated on the basis of the doctor's diagnosis, and the presence of infection symptoms recorded in the study diaries. The AOM episode was considered to have lasted as long as the child had respiratory infection symptoms after the AOM diagnosis. A new episode of AOM was diagnosed when there was at least one day without any respiratory symptoms since the previous episode. If the child visited the doctor again because of the extension of the symptoms and the criteria of AOM were fulfilled but the child had been asymptomatic for at least one day in between, the AOM was diagnosed as a new episode. Otherwise, if the child had suffered from symptoms continuously, the “newly” diagnosed AOM was considered to be part of the same episode. Otitis media was treated on the basis of the audit of the Finnish Medical Societies, amoxicillin being the first-line
antibiotic (Puhakka et al., 1999), and the use of non-steroidal anti-inflammatory drugs being recommended when needed. The use of antimicrobials for treating AOM was recorded in the study diaries (I, II).

4.3.3 Diarrhoea and gastrointestinal infections (I, III)

Gastrointestinal symptoms - diarrhoea, vomiting and abdominal pain - were recorded on a daily basis in the symptom diaries by the children's parents (I) or by personal nurses (III). In the children (I), diarrhoea was defined as abnormal loose or watery stools during at least one day. In the elderly (III), personal nurses evaluated the consistency of stools (hard, normal/soft, loose, watery). Loose or watery stools indicated diarrhoea. The definition of diarrhoea also included abstention from the use of laxatives on the same or on the previous day. In both children and the elderly, gastrointestinal (GI) infection was defined as diarrhoea and/or vomiting lasting at least one day. A new diarrhoea or GI-infection episode was defined as one occurring after at least 14 days without any gastrointestinal symptoms. The duration of diarrhoea and GI infections was assessed from the study diaries.

4.4 Bacteriological and serological methods

4.4.1 Detection of nasopharyngeal rhino- and enteroviruses (II)

Nasopharyngeal samples were taken three times during the study. A senior resident in otorhinolaryngology took the samples from the nasopharynx with a flexible calcium alginate swab via the nostril. The swab was immediately immersed in a tube containing 1 mL of STGG transport medium (20 g skimmed milk powder, 30 g Oxoid tryptone soya broth, 5 g glucose, 100 mL glycerol, 1 L distilled water). The STGG tube containing the swab was vortexed immediately, stored in a refrigerator for not more than 8 hours, and frozen at –70°C until analysis. Samples were sent in dry ice for analysis to the Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland. They were thawed and a multiplex reverse-transcription PCR-hybridisation assay for rhino- and enteroviruses was carried out as described in earlier studies (Blomqvist et al., 1999).

4.4.2 Cultivation of nasopharyngeal bacterial pathogens (II)

Nasopharyngeal samples were cultured for potential otitis pathogens (Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Streptococcus pyogenes) at the Laboratory for Chlamydia and Respiratory Tract Bacteria, National Public Health Institute, Oulu, Finland. The samples were thawed and vortexed before being inoculated on sheep's blood agar, sheep's blood agar with gentamicin (5 mg/L), and chocolate agar plates. The plates were incubated overnight in 5% CO₂ at +37°C, and colonies suspected of being S. pneumoniae, H. influenzae, M. catarrhalis and S. pyogenes were identified using generally accepted methods (Kilpi et al., 2001). The number of bacterial colonies were recorded semiquantitatively: negative
(no colonies), + (1-10 colonies), ++ (11-100 colonies) and +++ (>100 colonies). The serotyping of pneumococcal isolates was performed by latex agglutination and counterimmunoelectrophoresis and if necessary confirmed by a capsular swelling test using commercial pneumococcal antisera purchased from the Statens Serum Institute, Copenhagen, Denmark (Kilpi et al., 2001).

4.4.3 Salivary pneumococcal IgA antibodies (II)

Saliva samples were aspirated with a plastic pipette into an Eppendorf tube. The samples were stored in a refrigerator for not more than 8 hours and then frozen at -70°C. After thawing, the samples were centrifuged at 15,000 rpm for 10 minutes and the supernatant was used for assay. The salivary IgA antibodies against pneumococcal proteins (PspA1, PspA2 and PhtD) and polysaccharides (6B and 19F) were measured by enzyme immunoassay (EIA), as described by Nurkka et al., (2004), with a few modifications. The antigens used were PspA family 1 (Pneumococcal surface protein A) recombinant protein from the Rx1 strain and PspA family 2 from the V24 strain (University of Alabama, Birmingham, USA), the full-length protein of PhtD (Pneumococcal histidine triad) (GSK, Rixensart, Belgium), and polysaccharides 6B and 19F (ATCC, Rockville, Maryland, USA). Analyses were performed in the Vaccine Immunology Laboratory, National Public Health Institute, Helsinki, Finland.

4.4.4 Cultivation of oral Candida (IV)

Oral yeast samples were taken three times during the study. The research assistant obtained the yeast samples by rotating a cotton stick in the oral mucosa of the cheeks and tongue, and the gingival margin (dentate subjects) or alveolar ridge (edentate subjects), and immediately inoculating it onto a Dentocult® CA slide (Orion Diagnostica, Espoo, Finland). After incubation for 48 hours at +37°C, the growth was assessed semiquantitatively according to the manufacturer’s visual scale, using the following categories: negative (no visible colonies), + (equivalent to a colony density of 10³cfu/mL), ++ (equivalent to 10⁴cfu/mL), +++ (equivalent to 10⁵cfu/mL), and ++++ (equivalent to 10⁶cfu/mL). The growth category “+” was used to distinguish between patients with low (<10⁴cfu/mL) and high (≥10⁴cfu/mL) counts. Further identification of the yeast species was made by subculturing them onto CHROMAgar Candida (Becton Dickenson, Franklin Lakes, New Jersey, USA), which permitted the presumptive identification of C. albicans, C. glabrata, C. krusei and C. tropicalis. The cultivations were made in the Department of Bacteriology and Immunology, University of Helsinki, Finland.

4.4.5 Lactobacillus GG in faeces (I)

Faecal samples, taken three times during the study, were delivered from the day-care centres to Valio Research & Development Centre, where they were immediately frozen at -45°C until analysis. One hundred samples (51 probiotic + 49 placebo) were randomly selected to study the recovery of Lactobacillus GG in the faeces in order to confirm compliance. The thawed samples were diluted in phosphate buffered saline (pH 7.2, 10 mM phosphate) and the dilutions were
spread on MRS agar supplemented with vancomycin (50 mg/L). The plates were incubated aerobically for 48 hours at +37°C, and the typical colonies of *Lactobacillus* GG were purified to check for cell morphology and lactose fermentation. Analyses were performed in the microbiological laboratory of Valio Ltd, R&D.

**4.5 Statistical methods**

Statistical analyses were performed by using SPSS for Windows versions 9.0-14.0 (SPSS Inc, Chicago, IL, USA). All the analyses were based on the intention to treat (ITT) population. However, to avoid any inaccuracy in estimating missing values for the number of episodes and other variables describing the counts of incidents, only those children who completed the study were included in the primary analysis. Baseline health status and demographic factors were compared between the probiotic and the placebo groups by the *t* test, or in the case of skewed distribution, by the non-parametric Mann-Whitney U test or by the χ²-test, when the proportions of the groups were being compared (I-IV).

The occurrence of infections (ARI, AOM, diarrhoea), recurrent infections, complicated infections and the proportion of subjects on antimicrobial treatment, were dichotomised (at least one / at least two, etc.), and the differences between the intervention groups were analysed by the χ²-test, or by Fisher’s exact test when appropriate (I-III). A logistic regression analysis was used to study the association between the intervention and the outcome; the factors that based on previous studies were strongly associated with the outcome, and possible confounding factors were included as covariates. In Study I, age (as years) for the analysis of respiratory infections, and age, gender and the number of diarrhoea or GI infections during the preceding 12 months for the analysis of GI infections, were included as covariates. The corresponding factors in Study II were: age (<3; ≥3 years), gender, form of day care (home care/small group; large group/day-care centre), and the number of AOM episodes during the preceding 6 months, and in Study III, the number of chronic diseases, type of care, Barthel-index and chronic respiratory diseases (analysis of respiratory infections), and the number of chronic diseases and type of care (analysis of GI infections). All the results of the logistic regression models are presented as odds ratios (OR) with 95% confidence intervals (95% CI).

The number and duration of infection episodes and the days with respiratory symptoms were skewed to the right, and differences between the intervention groups were tested by the Mann Whitney U test (I-III). The results are presented as medians with inter-quartile range (IQR), except for the number of ARI episodes, which was almost normally distributed, and are presented as means (SD). In Study I, the days of absence from day-care centre were skewed to the right and were logarithmically transformed before analysis, and the geometric mean was tested using the *t* test. To control for the confounding factor (age), analysis of covariance (ANCOVA) was carried out, and age was included as a continuous covariate.

The Kaplan-Meier method was used to estimate the time free of the first infection episode (ARI, AOM, diarrhoea) (I-III), and the Log rank test was used to compare the groups. In these analyses all the randomised subjects were included, and the missing values due to premature
withdrawal were treated as censored observations. Cox’s regression analyses were performed to adjust for other explanatory factors. For respiratory infection-free time these were: age (I); age, gender and AOM episodes during the preceding 6 months (II); chronic diseases and antibiotic treatments during the preceding 12 months (III); for AOM-free time: age, gender, AOM episodes during the preceding 12 months (I); age, gender, AOM episodes during the preceding 6 months (II); and for diarrhoea-free time: age (I); type of care (III). Results are presented as Hazard ratios (HR) with 95% confidence intervals (95% CI).

The association between the intervention and the frequency of nasopharyngeal viruses and bacterial carriage, as well as the proportion of subjects positive to anti-pneumococcal IgA, were analysed by logistic regression analysis (II). Age, gender, form of day care, the number of AOM during the preceding 6 months, and the corresponding baseline pathogen carriage or IgA were included as covariates. The levels of IgA antibody concentrations were analysed by the repeated measures of variance (ANOVA), in which the baseline concentration was included as a covariate. The IgA distributions were skewed to the right and were logarithmically transformed before the analysis; the concentrations are presented as geometric means. The results are given as ratios (Probiotic/Placebo) with 95% confidence intervals. The interaction between the probiotic treatment, virus positivity and the occurrence of recurrent ARI, as well as between the probiotic treatment, bacterial positivity and the occurrence of AOM, was studied by the Mantel-Haenszel method. The homogeneity of the odds ratios for the occurrence of infections among pathogen-negative (all samples negative for all the pathogens) and pathogen-positive (at least one pathogen-positive sample during the study) subjects was tested by the Breslow-Day test.

In Study IV, logistic regression analysis was used to study the association between the intervention and the proportion of subjects with high yeast counts. The baseline yeast count (positive/negative) was included as a categorical covariate. The other explanatory factors (e.g. age, gender, baseline diseases such as diabetes, the amount of medication, salivary flow rate and buffering capacity, denture wearing, and the type of housing) were introduced to the stepwise multivariable regression model with an entry criterion of p<0.15. The results were adjusted with salivary buffering capacity and denture wearing, which reached the statistical significance level of p<0.15. Changes in the yeast counts between the baseline and the end were analysed by the χ²-test among those who carried low counts and high counts at baseline. The effect of probiotics on the salivary flow rate and mucosal lesions was analysed using the separate logistic regression analysis, including the corresponding baseline value as a categorical covariate.

4.6 Ethics

The study protocols were approved by the Ethics Committee of Helsinki City Health Department (Study I), the Ethics Committee of Helsinki University Central Hospital (Studies II and IV), and the Ethics Committee of Central Finland Health Care District (Study III). The subjects themselves (III, IV), or their parents (I, II), or near relatives (III) gave their written informed consent. All the data were treated confidentially.
5 RESULTS

5.1 Baseline characteristics (I-IV)

The baseline characteristics of the children participating in Studies I and II are presented in Table 9. No statistically significant differences were seen in demographic characteristics or health status between the intervention groups within each study. However, the prognostic value of each baseline variable was evaluated and if, according to previous studies, the variable was related to the outcome, it was included as a covariate. Overall, the otitis-prone children in Study II were younger and had had more infectious diseases and antimicrobial treatments during the preceding 12 months than the basically healthy children in Study I. Of the healthy children, 96% had experienced at least one respiratory infection, 43% at least one AOM, and 15% at least one bronchitis episode during the preceding 12 months. The corresponding figures in otitis-prone children were 98% for respiratory infections, 100% for AOM, and 30% for bronchitis. The children in Study I are considered as having a slightly increased risk of infection because of day-care attendance, whereas the children in Study II, as having a moderately increased risk of infection.

Baseline characteristics of the elderly subjects participating in Studies III and IV are presented in Table 10. No significant differences were seen between the intervention groups in either study, with the exception of chronic respiratory diseases in the institutionalised elderly (III). By chance, the prevalence of chronic respiratory diseases was higher in the probiotic group. Again, the prognostic value of each baseline variable was evaluated, and if the variable was considered a possible confounding factor, it was included as a covariate. Overall, the institutionalised subjects were older, had lower BMI, used antimicrobials, and had mental and neurological diseases more often than the independently-living elderly subjects. The institutionalised elderly are thus considered to have moderately increased risk of infection. Cardiovascular, respiratory, gastrointestinal and metabolic diseases seemed to be more common among the independent than the institutionalised elderly. The independent elderly are considered to have slightly increased risk of infection because of age-related chronic diseases.
## Table 9. Baseline characteristics of healthy children (I) and otitis-prone children (II).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Study I</th>
<th>Study II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy children: slightly increased risk of infection</td>
<td>Otitis-prone children: moderately increased risk of infection</td>
</tr>
<tr>
<td></td>
<td>Probiotic (n=282)</td>
<td>Placebo (n=289)</td>
</tr>
<tr>
<td>Boys, %</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>4.6 (1.3-6.8)</td>
<td>4.4 (1.3-6.7)</td>
</tr>
<tr>
<td>&lt; 3 years, %</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>≥ 3 years, %</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td>Siblings (%)</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td>Median duration of total breast-feeding, mo (range)</td>
<td>6.0 (0-32)</td>
<td>7.0 (0-30)</td>
</tr>
<tr>
<td>Parental smoking, %</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>Atopic diseases, %*</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>Food allergy, %†</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>History of infectious diseases‡¹</td>
<td>2.9 (0-12)</td>
<td>3.2 (0-20)</td>
</tr>
<tr>
<td>Mean no of AOM (range)</td>
<td>0.9 (0-12)</td>
<td>1.0 (0-11)</td>
</tr>
<tr>
<td>Mean no of GI infections (range)</td>
<td>0.9 (0-3)</td>
<td>1.0 (1-10)</td>
</tr>
<tr>
<td>Median number of antimicrobial treatments† (range)</td>
<td>1 (0-12)</td>
<td>1 (0-9)</td>
</tr>
<tr>
<td>0%</td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td>1-3%</td>
<td>50</td>
<td>51</td>
</tr>
<tr>
<td>≥ 4%</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Tympanostomy, %</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Adenoidectomy, %</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>Regular use of probiotic products, %</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>Use of pacifier, %</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Regular use of xylitol, %</td>
<td>43</td>
<td>38</td>
</tr>
</tbody>
</table>

*Atopic diseases diagnosed by a doctor, including atopic eczema /asthma /allergic rhinitis /allergic eye symptoms.
† Food allergies diagnosed by a doctor, including milk allergy /cereal allergy / other food hypersensitivities
‡ During preceding 12 months
NA = not analysed
**RESULTS**

Table 10. Baseline characteristics of institutionalised (III) and independent elderly subjects (IV).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Study III: Institutionalised elderly subjects:</th>
<th>Study IV: Independent elderly subjects:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic (n=135)</td>
<td>Placebo (n=130)</td>
</tr>
<tr>
<td>Male, %</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>83 (65-102)</td>
<td>83 (69-103)</td>
</tr>
<tr>
<td>Type of housing, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheltering housing unit</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Old people’s home</td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td>Hospital</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>Median duration of residence, years</td>
<td>1.7 (0-16)</td>
<td>1.6 (0-16)</td>
</tr>
<tr>
<td>BMI, mean (range)</td>
<td>24.4 (15.0-43.7)</td>
<td>25.1 (16.3-37.8)</td>
</tr>
<tr>
<td>&lt; 20%</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>20-24.9%</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>25-29.9%</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>≥ 30%</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoking</td>
<td>51</td>
<td>53</td>
</tr>
<tr>
<td>Former smoker</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No information available</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Chronic diseases, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Respiratory diseases*</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Gastrointestinal diseases</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Mental disorders</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Neurological disorders</td>
<td>41</td>
<td>32</td>
</tr>
<tr>
<td>Metabolic disorders</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>Musculoskeletal diseases</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>Median number of diseases (range)</td>
<td>3 (1-8)</td>
<td>3 (1-7)</td>
</tr>
<tr>
<td>Median number of medications (range)</td>
<td>6 (0-19)</td>
<td>6 (0-15)</td>
</tr>
<tr>
<td>Use of prophylactic antimicrobials, %</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Use of probiotic products, %</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mean Barthel index (range)</td>
<td>22.3 (0-90)</td>
<td>24.2 (0-95)</td>
</tr>
</tbody>
</table>

* Difference between groups: 15 subjects (11%) in probiotic vs. 1 subject (1%) in placebo group
NA = not analysed
5.2 The effect of probiotics on acute respiratory infections (ARI) (I-III)

5.2.1 Occurrence of ARI (including unpublished results)

The occurrence of acute respiratory infections (ARI) during probiotic interventions in healthy children, otitis-prone children and the institutionalised elderly is presented in Table 11, and the corresponding adjusted odds ratios (with 95% confidence intervals) resulting from logistic regression models are shown in Figure 5. No significant differences between the probiotic and placebo interventions were seen in the occurrence of at least one ARI episode or recurrent ARI episodes in healthy children (I) or in the institutionalised elderly (III), although the odds ratios were constantly in favour of the probiotic group (OR less than 1) (Figure 5).

In otitis-prone children (II), the occurrence of recurrent ARI episodes (at least 4) was reduced in the probiotic group (72%) compared to the control group (82%) (OR=0.56, 95% CI 0.31 to 0.99, p=0.046, adjusted OR=0.55, 95% CI 0.30 to 1.02, p=0.06). The occurrence of at least six episodes was also almost significantly reduced (20% vs. 30%; OR=0.59, 95% CI 0.34 to 1.03, p= 0.06, adjusted OR=0.58, 95% CI 0.32 to 1.04, p=0.07). As a secondary observation, it was noted that the preventive effect of probiotics in reducing recurrent ARI episodes was more pronounced in the allergic children. In the probiotic group, 12% (4/34) of those with a history of allergic disease or with a current allergic disease experienced at least 6 ARI episodes, while the corresponding figure in the placebo group was 33% (14/42) (p=0.03).

Table 11. Occurrence of acute respiratory infections during interventions in Studies I-III. Only subjects who completed the study are included in the analysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Healthy children</th>
<th>Otitis-prone children</th>
<th>Institutionalised elderly subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of ARI</td>
<td>Probiotic n=252</td>
<td>Placebo n=261</td>
<td>Probiotic n=135</td>
</tr>
<tr>
<td>≥ 1</td>
<td>95</td>
<td>96</td>
<td>0.44</td>
</tr>
<tr>
<td>≥ 2</td>
<td>85</td>
<td>89</td>
<td>0.13</td>
</tr>
<tr>
<td>≥ 3</td>
<td>56</td>
<td>61</td>
<td>0.21</td>
</tr>
<tr>
<td>≥ 4</td>
<td>3</td>
<td>6</td>
<td>0.18</td>
</tr>
<tr>
<td>≥ 6</td>
<td>20</td>
<td>30</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Adjusted for:
* age
† age, gender, form of day care, number of AOM episodes during preceding 6 months
‡ number of chronic diseases, type of care, Barthel index
NA = not analysed
RESULTS

In otitis-prone children (II), the mean number of ARI episodes was 4.3 days in the probiotic group compared to 4.6 days in the placebo group (p=0.08; Table 12). In healthy children (I) on probiotic treatment, the number of days with fever was reduced (p=0.03, age-adjusted p=0.07) and fewer antimicrobials for respiratory infections were needed than in the placebo group (p=0.03, age-adjusted OR=0.72, 95% CI 0.50 to 1.03, p=0.08). In addition, the healthy children had 16% fewer days of absence from day care because of illness (4.9 vs. 5.8 days, p=0.03; 11% less when age-adjusted, p=0.09) than the placebo group. In the elderly, the duration of respiratory infections was significantly longer in the probiotic group (8.8 days) than in the placebo group (4.3 days) (p=0.01).

Probiotic treatment postponed the first respiratory infection in healthy children (I): the median infection-free time from the beginning of the study was 5 weeks (95% CI 4.1-5.9) in the probiotic group and 4 weeks (95% CI 3.5 to 4.6) in the placebo group (Log-rank test p=0.03; adjusted HR=0.86, 95% CI 0.70 to 1.06, p=0.16) (Figure 6). Probiotic treatment did not significantly affect the infection-free time in otitis-prone children (II) (probiotic: 13 days vs. placebo: 11 days, Log-rank test p=0.55), nor in the elderly (III) (107 vs. 104 days respectively, Log-rank test p=0.71).

Figure 5. Adjusted odds ratios (with 95% confidence intervals) for the occurrence of at least one, at least two, and recurrent (≥4 in Studies I and II; ≥3 in Study III) respiratory infections in healthy children (I), otitis-prone children (II) and institutionalised elderly subjects (III) are shown on a logarithmic scale.
Table 12. Number and duration of acute respiratory infections (ARI), number of days with respiratory symptoms, and proportion of subjects with at least one antimicrobial treatment during interventions in Studies I-III. Only subjects who completed the study are included in the analysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>ARI episodes</th>
<th>Respiratory symptoms</th>
<th>Antimicrobial courses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number, mean (SD)/median (IQR)</td>
<td>Number, mean (SD)/median (IQR)</td>
<td>Subjects with ≥1 course, %</td>
</tr>
<tr>
<td></td>
<td>Study I Healthy children</td>
<td>Study II Otitis-prone children</td>
<td>Study III Institutionalised elderly subjects</td>
</tr>
<tr>
<td></td>
<td>Probiotic n=252</td>
<td>Placebo n=261</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>ARI episodes</td>
<td>Duration, median (IQR)</td>
<td>Days with symptoms, median (IQR)</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>3.8 (2.1)</td>
<td>6.6 (4.0, 9.5)</td>
<td>23 (11, 43)</td>
</tr>
<tr>
<td>Placebo</td>
<td>4.0 (2.0)</td>
<td>6.8 (4.6, 10.0)</td>
<td>25 (13, 45)</td>
</tr>
<tr>
<td>p</td>
<td>0.24</td>
<td>0.41</td>
<td>0.24</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Otitis-prone children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>4.3 (1.4)</td>
<td>12.3 (8.4, 18.8)</td>
<td>51 (33, 74)</td>
</tr>
<tr>
<td>Placebo</td>
<td>4.6 (1.4)</td>
<td>11.6 (8.0, 15.9)</td>
<td>49 (34, 72)</td>
</tr>
<tr>
<td>p</td>
<td>0.08</td>
<td>0.33</td>
<td>0.96</td>
</tr>
<tr>
<td>Study III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Institutionalised elderly subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>0* (0, 1)</td>
<td>8.8 (2.0, 12.0)</td>
<td>0 (0, 7.5)</td>
</tr>
<tr>
<td>Placebo</td>
<td>0* (0, 1)</td>
<td>4.3 (1.5, 6.6)</td>
<td>0 (0, 4.0)</td>
</tr>
<tr>
<td>p</td>
<td>0.85</td>
<td>0.01</td>
<td>0.76</td>
</tr>
</tbody>
</table>

NA = not analysed

Figure 6. Cumulative incidence of the first ARI episode during the 7-month intervention with healthy children (I), during the 6-month intervention with otitis-prone children (II), and during the 5-month intervention with the institutionalised elderly (III).
5.2.2 Prevalence of rhinovirus and enterovirus (II, unpublished results)

The prevalences of rhino- and enterovirus-positive children are presented in Table 13. Rhinovirus positivity decreased in both intervention groups from the baseline to 3 months, and increased again from 3 to 6 months. The prevalence of enterovirus positivity at the baseline in the probiotic group was by chance more than double that of the placebo group. The prevalence decreased in the probiotic group from the baseline to 3 and 6 months, and in the placebo group from the baseline to 6 months. No significant differences between the groups were seen in virus positivity during the study.

Table 13. Prevalence of rhinovirus and enterovirus positivity in the nasopharynx of otitis-prone children during the 6-month intervention. Only children with all measurements (0, 3 and 6 months) are included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Probiotic n=94–96 %</th>
<th>Placebo n=126 %</th>
<th>OR*</th>
<th>Probiotic vs. Placebo 95 % CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhinovirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>20</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>3</td>
<td>8</td>
<td>0.27</td>
<td>0.05 to 1.37</td>
<td>0.11</td>
</tr>
<tr>
<td>6 months</td>
<td>16</td>
<td>18</td>
<td>0.93</td>
<td>0.43 to 2.01</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>Enterovirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>55</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>34</td>
<td>22</td>
<td>1.70</td>
<td>0.85 to 3.38</td>
<td>0.13</td>
</tr>
<tr>
<td>6 months</td>
<td>26</td>
<td>11</td>
<td>2.09</td>
<td>0.91 to 4.78</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Adjusted for age, gender, form of day care, number of AOM during preceding 6 months, and virus positivity at baseline.

5.2.3 Interaction of virus positivity and probiotic treatment on the occurrence of ARI (II, unpublished results)

In order to examine whether the effect of probiotic treatment on the occurrence of ARI was different among children who were virus positive or virus negative, the interaction between virus positivity/negativity and the probiotic treatment was tested by the Mantel-Haenszel method. No interaction was observed when it was a question of 1-3 respiratory infections (data not shown). However, probiotic treatment reduced the recurrence of ARI episodes (≥ 4 ARI episodes) more if the children were negative to both rhinovirus and enterovirus (44% in the probiotic vs. 85% in the placebo group) than if they were positive (78% vs. 83%) (p=0.02, Table 14). Correspondingly, a significant interaction (p=0.04) was observed in the number of ARI episodes. Probiotic treatment reduced the mean number of ARI episodes more among the virus-negative children (3.5 vs. 4.6) than among the virus-positive (4.5 vs. 4.7).
Table 14. Interaction between virus positivity/virus negativity, probiotic treatment and the occurrence of acute respiratory infections in otitis-prone children (II).

<table>
<thead>
<tr>
<th></th>
<th>Virus-negative¹</th>
<th>Virus-positive¹</th>
<th>Interaction (virus x treatment) p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic</td>
<td>Placebo</td>
<td>Probiotic</td>
</tr>
<tr>
<td>Rhino-negative</td>
<td></td>
<td></td>
<td>Rhino-positive</td>
</tr>
<tr>
<td>n=63</td>
<td></td>
<td></td>
<td>n=33</td>
</tr>
<tr>
<td>≥ 4 ARI (%)</td>
<td>68</td>
<td>83</td>
<td>79</td>
</tr>
<tr>
<td>Entero-negative</td>
<td></td>
<td></td>
<td>Entero-positive</td>
</tr>
<tr>
<td>n=24</td>
<td></td>
<td></td>
<td>n=72</td>
</tr>
<tr>
<td>≥ 4 ARI (%)</td>
<td>58</td>
<td>82</td>
<td>76</td>
</tr>
<tr>
<td>Rhino and Entero-negative</td>
<td></td>
<td></td>
<td>Rhino or Entero-positive</td>
</tr>
<tr>
<td>n=18</td>
<td></td>
<td></td>
<td>n=40</td>
</tr>
<tr>
<td>≥ 4 ARI (%)</td>
<td>44</td>
<td>85</td>
<td>78</td>
</tr>
</tbody>
</table>

¹ negative if all the measurements (0, 3, 6 months) negative; positive if at least one positive measurement

5.2.4 ARI with complications (I-III)

The occurrences of ARI episodes with complications are shown in Table 15, and the corresponding adjusted odds ratios for the occurrences are presented in Figure 7. In healthy children (I), probiotics reduced the occurrence of at least one episode of complication: of bronchitis, pneumonia, sinusitis or otitis media combined (probiotic: 39% vs. placebo: 47%, p=0.05; adjusted OR=0.75, 95% CI 0.52 to 1.09, p=0.13). No significant differences in the occurrence of bronchitis, pneumonia or sinusitis separately were seen in either study (I-III).

Table 15. Occurrence of ARI with complications other than AOM during interventions in Studies I-III. Only subjects who completed the study are included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Study I Healthy children</th>
<th>Study II Otitis-prone children</th>
<th>Study III Institutionalised elderly subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic n=252  %</td>
<td>Placebo n=261 %</td>
<td>Probiotic n=135 %</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>5.6</td>
<td>7.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1.2</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Sinusitis</td>
<td>3.2</td>
<td>3.8</td>
<td>0.7</td>
</tr>
<tr>
<td>≥1 complication</td>
<td>9.5</td>
<td>11.5</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Adjusted for
*age
† age, gender, form of day care, number of AOM episodes during preceding 6 months
‡ number of chronic diseases, type of care, Barthel index
NA = not analysed
RESULTS

Figure 7. Adjusted odds ratios (with 95% confidence intervals) for the occurrence of acute respiratory infections with complications other than AOM, in healthy children (I), otitis-prone children (II), and in institutionalised elderly subjects (III) are shown on a logarithmic scale.

5.3 The effect of probiotics on acute otitis media (AOM) (I-II)

5.3.1 Occurrence of AOM episodes (I-II)

In healthy children (I), at least one AOM episode occurred in 31% of the probiotic group and in 39% of the placebo group, corresponding to a relative reduction of 21% in the prevalence (p=0.08). When age-adjusted, the relative reduction was 22% (OR=0.78, 95% CI 0.53 to 1.14, p=0.19, Table 16). Probiotic treatment postponed the first AOM episode by an average of 12 days: the mean infection-free time was 171 days (95% CI 163 to 179) in the probiotic group compared to 159 days (95% CI 150 to 167) in the placebo group (Log-rank test p=0.04, adjusted HR=0.78, 95% CI 0.59 to 1.05, p=0.09) (Figure 8).
In the otitis-prone children (II), probiotics did not affect the occurrence or recurrence of AOM episodes in the group as a whole (Table 16). However, in the allergic children (those with a history of allergic diseases or a current allergic disease), probiotics reduced the occurrence of recurrent AOM episodes: none of the allergic children in the probiotic group vs. 14% (6/42) of those in the placebo group experienced recurrent AOM episodes (p=0.03). Probiotic treatment did not postpone the first otitis episode (85 vs. 99 days; Log-rank test p=0.23) (Figure 8). No differences were seen in the need for tympanostomy: 8.5% in the probiotic group and 8.8% in the control group needed tympanostomy during the study.

Table 16. Occurrence and duration of AOM episodes in Studies I-II. Only children who completed the study are included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Study I Healthy children</th>
<th>Study II Otitis-prone children</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic n=252</td>
<td>Placebo n=261</td>
</tr>
<tr>
<td>≥ 1 AOM (%)</td>
<td>31</td>
<td>39</td>
</tr>
<tr>
<td>≥ 3 AOM (%)</td>
<td>4.4</td>
<td>6.9</td>
</tr>
<tr>
<td>Number of episode,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>(0, 1.0)</td>
<td>(0, 1.0)</td>
</tr>
<tr>
<td>Duration of episodes,</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 1 one antimicrobial course for AOM (%)</td>
<td>30</td>
<td>36</td>
</tr>
</tbody>
</table>

Adjusted for
* age
† age, gender, form of day care, number of AOM episodes during preceding 6 months
NA = not analysed

Figure 8. Cumulative incidence of the first AOM episode during the 7-month intervention with healthy children (I), and during the 6-month intervention with otitis-prone children (II).
RESULTS

5.3.2 Carriage of otitis pathogens (II)

Probiotic treatment did not have any significant effect on the carriage of *Streptococcus pneumoniae* or *Haemophilus influenzae* compared to the placebo (Table 17). During the study, the prevalence of *S. pneumoniae* increased slightly in both intervention groups, and the prevalence of *H. influenzae* decreased slightly in both groups. However, at 6 months, the prevalence of *Moraxella catarrhalis* was significantly higher in the probiotic group. Probiotic treatment increased the prevalence of *M. catarrhalis*, particularly in children under 3 years of age (OR= 2.44, 95% CI 1.34 to 4.44, p=0.004). However, in children of over 3 years, the odds ratio was in favour of the probiotic group (OR=0.54, 95% CI 0.16 to 1.82, p=0.32). The overall carriage of at least one potential otitis pathogen (*S. pneumoniae* or *H. influenzae* or *M. catarrhalis*) was 76% in the probiotic group at baseline and 75% at the end, and 68% in the placebo group both at baseline and at the end. At the end of the treatment, the difference between the groups was non-significant (adjusted OR=1.42, 95% CI 0.80 to 2.53, p=0.23).

Table 17. Prevalence of potential otitis pathogens in the nasopharynx of otitis-prone children, at baseline and at the end of the 6-month intervention. Only children with both measurements (0 and 6 months) are included in the analysis.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Baseline Probiotic</th>
<th>Placebo</th>
<th>6 months Probiotic</th>
<th>Placebo</th>
<th>OR*</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic n=128 %</td>
<td>Placebo n=126 %</td>
<td>Probiotic n=128 %</td>
<td>Placebo n=126 %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>41</td>
<td>39</td>
<td>47</td>
<td>47</td>
<td>1.02</td>
<td>0.61 to 1.70</td>
<td>0.94</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>27</td>
<td>26</td>
<td>23</td>
<td>17</td>
<td>1.43</td>
<td>0.76 to 2.70</td>
<td>0.27</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>43</td>
<td>40</td>
<td>52</td>
<td>39</td>
<td>1.79</td>
<td>1.06 to 3.00</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Adjusted for age, gender, form of day-care, and pathogen carriage at baseline.

5.3.3 Interaction of pathogen carriage and probiotic treatment on the occurrence of AOM (II, unpublished results)

In order to examine whether the effect of probiotic treatment on the occurrence of AOM was associated with bacterial pathogen carriage, the interaction between bacterial pathogen positivity/negativity and probiotic treatment was tested by the Mantel-Haenszel method. The probiotic treatment reduced the occurrence of AOM among pathogen-negative children (63% in the probiotic group vs. 83% in the placebo group), but increased the occurrence among the pathogen-positive (74% vs. 62%) (p=0.05, Table 18). No interaction was observed in recurrent AOMs.
Table 18. Interaction between bacterial pathogen positivity/pathogen negativity, probiotic treatment and the occurrence of acute otitis media in otitis-prone children (II).

<table>
<thead>
<tr>
<th>Pathogen-negative</th>
<th>Pathogen-positive</th>
<th>Interaction (pathogen x treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic</td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>n=50</td>
<td>n=49</td>
</tr>
<tr>
<td></td>
<td>n=84</td>
<td>n=84</td>
</tr>
<tr>
<td>≥ 1 AOM (%)</td>
<td>72</td>
<td>78</td>
</tr>
<tr>
<td>≥ 3 AOM (%)</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. influenzae</td>
<td>n=81</td>
<td>n=85</td>
</tr>
<tr>
<td>negative</td>
<td>n=53</td>
<td>n=48</td>
</tr>
<tr>
<td>≥ 1 AOM (%)</td>
<td>69</td>
<td>68</td>
</tr>
<tr>
<td>≥ 3 AOM (%)</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. catarrhalis</td>
<td>n=38</td>
<td>n=59</td>
</tr>
<tr>
<td>negative</td>
<td>n=96</td>
<td>n=74</td>
</tr>
<tr>
<td>≥ 1 AOM (%)</td>
<td>71</td>
<td>68</td>
</tr>
<tr>
<td>≥ 3 AOM (%)</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathogen-negative</td>
<td>n=16</td>
<td>n=18</td>
</tr>
<tr>
<td></td>
<td>n=118</td>
<td>n=115</td>
</tr>
<tr>
<td>≥ 1 AOM (%)</td>
<td>63</td>
<td>83</td>
</tr>
<tr>
<td>≥ 3 AOM (%)</td>
<td>25</td>
<td>33</td>
</tr>
</tbody>
</table>

1negative, if all potential pathogens negative at 0 and 6 months; positive, if at least one positive pathogen at 0 or 6 months.
5.3.4 Pneumococci-specific antibodies (II, unpublished results)

The most prevalent pneumococcal serotypes found among a whole group of otitis-prone children during the study were serotypes 19F (ranging from 13-18% of the positive samples), 15 (10-15%), 23F (10-13%), 6A (9-12%) and 6B (7-13%). No significant changes between the probiotic and placebo groups were seen in the total prevalence of serotypes 19F, 15, 23F, 6A and 6B: these serotypes accounted for 59% of the positive samples in the probiotic group at baseline and 60% at the end. The corresponding figures for the placebo group were 64% and 54%.

According to the logistic regression analysis, the proportion of subjects with IgA-positive samples to pneumococcal serotype 6B tended to increase in the probiotic (62%) compared to the placebo group (51%) in the middle of the study (p=0.08). The proportion of IgA-positive samples to serotype 19F or to protein antigens PspA1 and PhtD did not differ significantly between the groups (Table 19).

Table 19. Proportions of samples with detectable salivary IgA antibodies to pneumococcal polysaccharide (6B and 19F) and protein (PspA-1 and PhtD) antigens in otitis-prone children, at baseline, in the middle (3 months) and at the end (6 months) of the intervention. Only children with all the three measurements are included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Probiotic</th>
<th>Placebo</th>
<th>OR</th>
<th>95 % CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B Baseline</td>
<td>58</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6B 3 months</td>
<td>62</td>
<td>51</td>
<td>1.73</td>
<td>0.93 to 3.20</td>
<td>0.08</td>
</tr>
<tr>
<td>6B 6 months</td>
<td>63</td>
<td>57</td>
<td>1.26</td>
<td>0.70 to 2.28</td>
<td>0.44</td>
</tr>
<tr>
<td>19F Baseline</td>
<td>65</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19F 3 months</td>
<td>63</td>
<td>61</td>
<td>0.99</td>
<td>0.52 to 1.86</td>
<td>0.96</td>
</tr>
<tr>
<td>19F 6 months</td>
<td>65</td>
<td>64</td>
<td>1.00</td>
<td>0.55 to 1.80</td>
<td>0.99</td>
</tr>
<tr>
<td>PspA-1 Baseline</td>
<td>64</td>
<td>68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PspA-1 3 months</td>
<td>70</td>
<td>67</td>
<td>1.46</td>
<td>0.71 to 3.01</td>
<td>0.31</td>
</tr>
<tr>
<td>PspA-1 6 months</td>
<td>74</td>
<td>70</td>
<td>1.36</td>
<td>0.72 to 2.57</td>
<td>0.34</td>
</tr>
<tr>
<td>PhtD Baseline</td>
<td>74</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhtD 3 months</td>
<td>86</td>
<td>77</td>
<td>1.50</td>
<td>0.65 to 3.47</td>
<td>0.34</td>
</tr>
<tr>
<td>PhtD 6 months</td>
<td>91</td>
<td>85</td>
<td>1.37</td>
<td>0.55 to 3.44</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Corresponding baseline value included as a covariate.

In addition, the level of the anti-6B-IgA concentration after 3 months was 1.3 times higher in the probiotic group (5.4 ng/mL) than in the placebo group (4.3 ng/ml) (p=0.04). When the anti-6B-IgA concentrations at 3 months and 6 months were combined by repeated measures of ANOVA and the baseline concentration was included as a covariate, the corresponding ratio probiotic/placebo was 1.25 (95% CI 1.00 to 1.58, p=0.06). No significant differences in the levels of anti-19F-IgA, anti-PspA1-IgA, and anti-PhtD-IgA concentrations were seen between the groups.
5.4 The effect of probiotics on diarrhoea and gastrointestinal infections (I, III)

Probiotic treatment did not reduce the occurrence of diarrhoea or GI infections (including diarrhoea and/or vomiting) either in healthy children (I) or in the institutionalised elderly (III) (Table 20). The adjusted odds ratios (with 95% confidence intervals) resulting from logistic regression models for the occurrence of diarrhoea and GI infections are shown in Figure 9. In the elderly (III), the duration of diarrhoea seemed to be 3.7 days shorter in the probiotic group, but the difference did not reach statistical significance.

Table 20. Occurrence and duration of diarrhoea and gastrointestinal infection episodes during probiotic treatments in Studies I and III. Only subjects who completed the study are included in the analysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Occurrence</th>
<th>Study</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy children</td>
<td></td>
<td>Institutionalised elderly subjects</td>
</tr>
<tr>
<td>Probiotic</td>
<td>Placebo</td>
<td>p</td>
<td>Probiotic</td>
</tr>
<tr>
<td>n=252</td>
<td>n=261</td>
<td></td>
<td>n=117</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 1 episode (%)</td>
<td>33</td>
<td>38</td>
<td>0.25*</td>
</tr>
<tr>
<td>Duration, median (IQR)</td>
<td>2.0 (1.0, 3.7)</td>
<td>2.0 (1.0, 3.0)</td>
<td>0.59</td>
</tr>
<tr>
<td>Gastrointestinal infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 1 episode (%)</td>
<td>69</td>
<td>67</td>
<td>0.75*</td>
</tr>
<tr>
<td>Duration, median (IQR)</td>
<td>1.2 (1.0, 2.6)</td>
<td>1.5 (1.0, 2.5)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Adjusted for
*age, gender, number of diarrhoea or GI infections during preceding 12 months
†number of chronic diseases, type of care

Figure 9. Adjusted odds ratios (with 95% confidence intervals) for the occurrence of diarrhoea and gastrointestinal infections in healthy children (I), and in the institutionalised elderly (III) are shown on a logarithmic scale.
RESULTS

Probiotic treatment did not postpone the first episode of diarrhoea (Figure 10). The mean diarrhoea-free time in children (I) was 168 days in the probiotic and 162 days in the placebo group (Log-rank test p=0.18). The corresponding figures for the elderly (III), were 122 vs. 119 days (Log-rank test p=0.57).

Figure 10. Cumulative incidence of the first diarrhoea episode during the 7-month intervention with healthy children (I) and during the 5-month intervention with the institutionalised elderly (III).

5.5 The effect of probiotics on oral yeast (IV)

5.5.1 Prevalence of high Candida carriage

At baseline, a total of 74% of all the randomised subjects (n=276) carried yeast; 41% carried low counts (< $10^4$ cfu/mL), and 33% high counts ($\geq 10^4$ cfu/mL). Salivary secretion, low buffering capacity and denture wearing were associated with the baseline yeast count. The prevalence of a high yeast count was 49%, 38%, 25% and 16% in the quartiles of salivary secretion, from the lowest to the highest secretion ($p<0.001$). The prevalence of high yeast counts was 24%, 35% and 51%, when the buffering capacity was high, medium and low respectively ($p=0.003$), and 36% and 25% respectively in subjects with and without dentures ($p=0.09$). Thus, the results were adjusted for salivary secretion, buffering capacity and denture wearing. It was also noted that those who had regularly used lactic acid bacteria-containing products before the intervention carried high yeast counts less often than those who had not (25% vs. 38%; $p=0.03$).

The prevalence of high yeast counts ($\geq 10^4$ cfu/mL) in the probiotic group at baseline was 30%, and 28% in the control group. After 2 and 4 months of the intervention, the prevalence of high yeast counts in the probiotic group had diminished to 25% and 21% respectively. In the placebo group the prevalence increased to 31% and 34% respectively. At the end of the intervention, the risk of having high yeast counts was reduced by 61% in the probiotic group compared to the placebo group (OR=0.39, 95% CI 0.18 to 0.83, $p=0.01$), and by 75% when adjusted for other explanatory factors (adjusted OR=0.25, 95% CI 0.10 to 0.65, $p=0.004$).
The changes occurring between the low and high yeast carriage during the 4-month intervention are shown in Figure 11. Among subjects belonging to the low yeast group ($< 10^4$ cfu/mL) at baseline, yeast counts increased in 9% of the probiotic group and in 19% of the placebo group, thus the risk of yeast increase tended to be reduced in the probiotic group (OR=0.43, 95% CI 0.15 to 1.19, p=0.10). Among those subjects who carried high yeast counts ($\geq 10^4$ cfu/mL) at baseline, the counts were reduced in 54% of the probiotic group compared to 29% of the placebo group. The risk of belonging to a high yeast group at the end was reduced by 65% in the probiotic group (OR=0.35, 95%CI 0.12 to 1.05, p=0.06).

![Figure 11. Changes (between baseline and the end of the intervention) in yeast carriage among those with low ($< 10^4$ cfu/mL, total n=184) and high ($\geq 10^4$ cfu/mL, total n=92) yeast counts at baseline. An increase indicates a change from low counts to high counts, and a decrease indicates a change from high counts to low counts.](image)

Probiotic treatment did not affect the distribution of the different yeast species; *Candida albicans* was the most dominating species, found in 94% of the positive subjects at the beginning of the study and in 90% at the end. In the control group, two subjects carried *C. glabrata* at the beginning, and seven at the end. In the probiotic group, *C. glabrata* was found in two subjects at the end. *Saccharomyces cerevisiae* was found in two subjects in the control group and *C. tropicalis* in only one subject in the probiotic group.
RESULTS

5.5.2 Salivary secretion and oral mucosal lesions

The prevalence of hyposalivation decreased in the probiotic group and increased in the control group (Table 21). Probiotic treatment reduced the risk of hyposalivation by 56%, and tended to reduce the subjective feeling of dry mouth and high buffering capacity. Oral mucosal lesions were clinically diagnosed as erythroplakia, leukoplakia, hyperplasia, ulceration and pigmentation. No significant differences between the groups were seen in mucosal lesions or the subjective feeling of oral pain.

Table 21. Effect of probiotics on hyposalivation, dry mouth, buffering capacity, mucosal lesions and oral pain after a 4-month intervention. Only subjects with both measurements (baseline and 4 months) are included in the analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Probiotic n=85–92</th>
<th>Placebo n=88-100</th>
<th>OR*</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyposalivation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>24</td>
<td>19</td>
<td>0.44</td>
<td>0.19 to 1.01</td>
<td>0.05</td>
</tr>
<tr>
<td>4 mo</td>
<td>18</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry mouth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>52</td>
<td>62</td>
<td>0.54</td>
<td>0.27 to 1.06</td>
<td>0.07</td>
</tr>
<tr>
<td>4 mo</td>
<td>53</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High buffering capacity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>65</td>
<td>59</td>
<td>0.51</td>
<td>0.24 to 1.09</td>
<td>0.08</td>
</tr>
<tr>
<td>4 mo</td>
<td>62</td>
<td>71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>25</td>
<td>24</td>
<td>0.35</td>
<td>0.04 to 3.44</td>
<td>0.37</td>
</tr>
<tr>
<td>4 mo</td>
<td>26</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11</td>
<td>15</td>
<td>0.58</td>
<td>0.25 to 1.34</td>
<td>0.20</td>
</tr>
<tr>
<td>4 mo</td>
<td>12</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Corresponding baseline value included as a categorical covariate.

5.6 Compliance (I-IV)

Compliance, according to the consumption of milk, cheese or capsules, was good in all the studies. The average milk consumption by the healthy children (I) was 260 ml/day, the aim having been 200 ml/day. Mean compliance was 60% in both groups. Compliance was also measured by the faecal recovery of *Lactobacillus GG*. At the beginning, 12% of the children in the probiotic group and 4% in the placebo group carried *Lactobacillus GG*-like bacteria (p=0.29). After four months and at the end of the study the respective percentages were 97% and 9% (p<0.0001), and 91% and 15% (p<0.0001). The median *Lactobacillus GG* count in the probiotic group was $5-8 \times 10^4$ cfu/ml (range 0-1x10^7).

In the independent elderly (IV), 61% of the subjects in both intervention groups showed cheese-eating compliance of at least 80%, while the median cheese-eating compliance was 93%. The mean compliance of eating capsules among the otitis-prone children (II) was 96% in both intervention groups; in the institutionalised elderly (III), it was 98% in the probiotic and 99% in the placebo group.
5.7 Summary of results (I-IV)

Table 22 summarises the main results of the effect of probiotics on acute respiratory infections, acute otitis-media, diarrhoea and oral Candida.

Table 22. Summary of the main results in Studies I-IV.

<table>
<thead>
<tr>
<th></th>
<th>Study I Healthy children</th>
<th>Study II Otitis-prone children</th>
<th>Study III Institutionalised elderly subjects</th>
<th>Study IV Independent elderly subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute respiratory infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence/Recurrence</td>
<td>↔</td>
<td>(↓) in atopics ↓</td>
<td>↔</td>
<td>NA</td>
</tr>
<tr>
<td>Duration</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Symptoms</td>
<td>(↓)</td>
<td>↔</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Day of absence</td>
<td>(↓)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Infection-free time</td>
<td>(↓)</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>Complications</td>
<td>(↓)</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>Virus positivity</td>
<td>NA</td>
<td>↔</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>(↓)</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td><strong>Acute otitis media</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence/Recurrence</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>NA</td>
</tr>
<tr>
<td>Duration</td>
<td>NA</td>
<td>↔</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Infection-free time</td>
<td>(↓)</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>Pathogen carriage</td>
<td>NA</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diarrhoea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>NA</td>
</tr>
<tr>
<td>Duration</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>Infection-free time</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td><strong>Oral Candida</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High carriage</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Mucosal lesions</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>Hyposalivation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

↑ = Increased risk (adjusted p < 0.05)
↓ = Decreased risk (adjusted p < 0.05)
(↓) = Marginally decreased risk (crude p < 0.05; adjusted 0.05 < p < 0.20)
↔ ↔ = No evidence of effect
NA = not analysed
6 DISCUSSION

This series of studies comprised four independent clinical trials performed as randomised, double-blind, placebo-controlled interventions with two parallel groups. The study designs thus fulfilled the quality criteria for clinical trials. The total number of subjects included in the studies was 1421, and 1200 subjects completed them. On the basis of sample size estimations, the sample sizes in Studies I, III and IV, even after the drop-outs, were sufficient for the expected differences between the groups in the primary outcomes to be detected. However, in Study II, 380 children completing the study would have been needed in order to show the statistically significant difference between the groups. Unfortunately, possibly due to very strict inclusion criteria, we were able to recruit only 309 children, of whom 269 completed the study. Thus, the results approaching statistical significance (p ≤ 0.05) in Study II should be evaluated by taking into account the insufficient sample size.

6.1 Prevention of respiratory infections and acute otitis media

The hypothesis that probiotics might reduce the risk of respiratory infections was partly confirmed among children, but no effect was seen in the institutionalised elderly. It was seen in healthy children (I), that although probiotics did not reduce the total occurrence of respiratory infections, they postponed the first ARI episode by one week, and reduced the number of days with fever and the rate of complicated infections. This led secondarily to reduced child-care absences and less need for antibiotic treatment, suggesting that probiotics might reduce the severity of respiratory infections. However, these effects were weakened when the results were age-adjusted. Our findings accord with a previous study showing that the 12-week consumption of an infant formula containing either *Lactobacillus reuteri* or *Bifidobacterium lactis* BB-12 did not reduce the rate of respiratory illnesses but reduced febrile episodes, child-care absences and antibiotic prescriptions in 4-10-month-old infants (Weizman et al., 2005). The same effects
have also been observed in basically healthy adults: a combination of Lactobacillus gasseri with Bifidobacterium longum and B. bifidum given in capsule form reduced the number of days with fever and the total symptom score (Winkler et al., 2005), as well as the duration of infections (de Vrese et al., 2005b), although the incidence of ARI was not reduced during an intervention lasting 3-5.5 months.

Otitis-prone children (II) suffered an average of 4-5 respiratory infections during the 6-month study. Due to their greater susceptibility to infections (almost all the children suffered at least two ARI episodes and nearly 80%, at least 3 episodes) probiotics could not reduce the overall occurrence of ARI. However, when there were extremely recurrent infections (at least 4 or 6), probiotics tended to reduce recurrences, suggesting again that they might reduce the severity of infections. In addition, it was noted that the protective effect was more pronounced in children with a history of allergic diseases. This observation is extremely interesting, since Lactobacillus GG has also prevented early atopic diseases (Kalliomäki et al., 2001), and alleviated atopic eczema in IgE-sensitised infants (Viljanen et al., 2005c), through the augmentation of the Th1 cytokine, IFN-\(\gamma\), secretion in peripheral blood mononuclear cells (Pohjavuori et al., 2004). However, a probiotic combination, the same as that used with otitis-prone children (II), has not been effective in alleviating present atopic diseases (Viljanen et al., 2005c), probably because of a slightly different cytokine profile; the combination induced the production of IL-4 along with IFN-\(\gamma\), which may have deviated immunity towards a Th2 response (Pohjavuori et al., 2004). However, the same combination has prevented the development of atopic eczema when the supplementation was started before the birth (Kukkonen et al., 2007), suggesting that the effect of probiotics depends on the immunological status of the subject. It is also known that oral treatment with probiotic LGG modulates the immune response differently in healthy subjects than in hypersensitive ones (Pelto et al., 1998). It can thus be hypothesised that the probiotic treatment prevented recurrent respiratory infections in the children whose immune system was skewed towards a Th2 response. This assumption is supported by a previous study in which a combination of LGG and Bifidobacterium lactis BB-12 reduced the incidence of recurrent respiratory infections during the first 7 months of age when probiotics were given to infants from the age of one month, at a time when the neonatal Th2 response still dominates (Rautava, 2005).

In healthy children (I), probiotics also reduced the total occurrence of complicated infections, the majority of these being otitis media. However, no beneficial effects on the occurrence of individual complications such as bronchitis, pneumonia and sinusitis were seen in either study (I-III). This is understandable, since the prevalence of complications was low (0-7.3% depending on the study). In healthy children (I), probiotics also showed a tendency to reduce the occurrence of otitis media and prolonged the otitis-free time. As viral respiratory infections usually precede bacterial otitis and otitis can be of viral origin (Kleemola et al., 2006; Ruohola et al., 2006), presumably the reduced occurrence of AOM was a secondary consequence of the reduced severity of ARI. However, although probiotics tended to reduce recurrent ARIs in otitis-prone children (II), the effect was insufficient to prevent the development of AOM in children with a history of recurrent AOMs and a high carriage of bacterial pathogens. The duration of respiratory symptoms has been shown to increase among children carrying bacterial pathogens (Kristo et al., 2006), suggesting that infections might be more severe in children carrying pathogenic bacteria. To be effective against the development of AOM, probiotics should have reduced the nasopharyngeal carriage of bacteria, which was not the case in the present study.
However, in contrast to the results obtained in children, probiotics showed no beneficial effect on respiratory infections in the institutionalised elderly, and the duration of ARI episodes actually increased in the probiotic group. This finding is also the opposite of that of a previous study which showed that probiotic \textit{L. casei} reduced the duration of winter infections by 1.7 days in free-living elderly subjects (Turchet \textit{et al.}, 2003). One major difference between Study \textbf{III} and the study by Turchet and colleagues (2003) is the health status of the subjects. The institutionalised elderly (\textbf{III}) were very frail, suffered an average of three chronic diseases, and regularly used an average of six medications. Compared to the independent elderly, the institutionalised elderly are more susceptible to infections because of multiple co-morbid underlying diseases, the use of several medications, and different invasive procedures such as catheters, feeding tubes or tracheostomies (Garibaldi, 1999). Probiotics thus seem ineffective in subjects with high vulnerability to infection. In addition, irrespective of the random allocation to the treatment groups, the prevalence of chronic respiratory diseases, mainly asthmatic disorders, was more common in the probiotic group. This uneven distribution may have increased the susceptibility of the probiotic group to infections. When subjects with chronic respiratory diseases were excluded from the analyses, the occurrence of ARI in the probiotic group was slightly reduced, but the duration of ARI was not affected. Thus the reason for the extended duration of ARI remains unclear.

\subsection*{6.1.1 Methodological considerations}

The diagnoses of ARI in Studies \textbf{I-III} were based on the subjective evaluation of infection symptoms, and thus the method cannot be independently validated. Measuring can be difficult when a carer evaluates a child’s symptoms or nurses evaluate symptoms of the elderly, who may be unable to communicate their own symptoms or whose symptoms may be atypical, diminished or even absent (Garibaldi, 1999). It is thus possible that the prevalence of infections among the elderly may have been underestimated. In children, on the other hand, the symptoms of allergic rhinitis can sometimes mimic the common cold, leading to an overestimation of the respiratory symptoms. However, the prevalences of respiratory infections in Studies \textbf{I-III} seem to give a fair representation of the average disease prevalence in children and the elderly, on the basis of population-based cross-sectional studies (Pönkä \textit{et al.}, 1991; Nafstad \textit{et al.}, 1999; Engelhart \textit{et al.}, 2005).

Defining the exact duration of an infection episode is also vague, because there is no valid definition of an episode. Three asymptomatic days between two separate ARI episodes is quite commonly used as a definition in intervention trials (Uhari and Möttönen, 1999), although in cohort studies even 30 asymptomatic days have been used (Nokso-Koivisto \textit{et al.}, 2002b). We defined an ARI episode as lasting as long as the subjects had respiratory symptoms, and 7 asymptomatic days were needed to separate two episodes. The duration of an AOM episode was also defined on the basis of the presence of respiratory symptoms after AOM diagnosis, and one asymptomatic day separated two episodes. The diagnostic accuracy of otitis media among paediatric residents has shown only moderate correlation to the diagnoses of otolaryngologists (Steinbach \textit{et al.}, 2002; Blomgren \textit{et al.}, 2004), suggesting that the incidence of otitis media is frequently overdiagnosed. Because AOM diagnoses among healthy children (I) were made by both municipal and private doctors, again, overdiagnosing, which is common among general physicians, is possible. However,
even though there may have been misdiagnoses, it can be assumed that these occurred equally in both intervention groups. Among the otitis-prone children (II), the diagnosis was made mainly by one study physician, which increases the reliability of the diagnosis.

6.1.2 Effect on respiratory viruses

The primary end-points in the clinical trials were the occurrences of infections. An attempt was also made to hypothesise on the possible mechanisms of probiotics by examining their effects on the prevalence of two common respiratory viruses. These effects were evaluated only in the infection-prone children (II), because these children were supposed to be frequently virus-positive. Although the prevalence of both viruses decreased during the six-month study, the changes seemed to follow seasonal patterns. The prevalence of rhinovirus decreased from the baseline (measured in the autumn) to the middle of the study (winter), and increased again at end of the study (spring), corresponding to rhinovirus predominance in the early autumn and spring (Nokso-Koivisto et al., 2006). The reduction of enterovirus positivity from baseline to the end of the study can also be explained by an enterovirus peak in the autumn and winter (Nokso-Koivisto et al., 2006).

Probiotics did not reduce the presence of rhinovirus or enterovirus. There are no comparative clinical data available, but animal studies have shown that oral and intranasal administration of probiotic *Lactobacillus casei* Shirota has reduced viral titres in nasal washings of mice infected with an influenza virus (Hori et al., 2001; Yasui et al., 2004). This effect has been postulated as being due to the activation of innate immunity, as shown by the production of IL-12, TNF-α and IFN-γ from medistinal lymph node cells (Hori et al., 2001), and the enhancement of pulmonary NK-cell activity (Yasui et al., 2004). Oral feeding with *B. breve* has also augmented antibody response to an oral influenza vaccination (Yasui et al. 1999). In humans, an oral consumption of fermented milk containing *Lactobacillus* GG or *L. acidophilus* accompanied by a polio vaccine increased the poliovirus neutralizing antibody titres and the formation of poliovirus-specific IgA and IgG (de Vrese et al., 2005a).

A significant interaction between probiotics and viruses on recurrent ARI episodes was seen. Probiotics reduced both the recurrence and the number of recurrent ARI episodes in the virus-negative children, but did not have any effect on the virus-positive children. This finding is astonishing, because ARIs are of viral origin, and it could be expected that probiotic effects would be seen particularly in the virus-positive subjects. However, the most reasonable explanation might be that other viruses, in addition to rhino- and entero-virus, may have been involved. Overall, the only possible way for probiotics to reduce viral respiratory infections is by immunological means. However, we did not measure any immunological parameters, leaving the immunological mechanisms to be merely speculated on.

6.1.3 Antagonism to otitis pathogens

Nasopharyngeal colonisation by *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* is associated with increased risk of AOM (Aniansson et al., 1994). It also seemed clear in our study that
these bacterial pathogens played an important role in the development of AOM in the otitis-prone children (II), since the pathogen carriage was high, reaching up to 75%, while in healthy children the prevalence has varied from 20 to 50% (Ejlertsen et al., 1994; Gunnarsson et al., 1998; Principi et al., 1999). The probiotic combination did not reduce the carriage of pathogens, in contrast to a previous study in which a 3-week consumption of fermented milk containing *L. rhamnosus* GG, *Bifidobacterium*, *L. acidophilus* and *S. thermophilus* reduced the growth of potential pathogens on nasal mucosa in healthy adults (Glück and Gebbers, 2003). If probiotics reduced the growth of pathogens by direct antagonism, by producing antimicrobial compounds or by blocking adhesion, the method of administering probiotics would be of great importance. To achieve a local effect on the oro/nasopharyngeal microbiota, probiotics should be taken in the form of food. The administration of the probiotics in capsules in Study II may thus partly explain their ineffectiveness. Although it was also permissible to mix the probiotic powder with food or drink, the older children at least may have swallowed the capsules whole, and no direct contact between the probiotics and the oropharyngeal microbiota will have occurred. Animal studies also show that intranasally-inoculated *Lactobacillus* strains have enhanced the clearance of *S. pneumoniae* (Cangemi de Gutierrez et al., 2001; Villena et al., 2005) and of *Pseudomonas aeruginosa* (Alvarez et al., 2001) from the respiratory tract of mice. However, these effects were mediated through the induction of broncho-alveolar antibody production, and through the increased number and capacity of alveolar macrophages. In humans, consumption of fermented milk containing *L. johnsonii* La1 for 5 weeks, in addition to vaccination against *H. influenzae* also showed the enhancement of the antibody titre against *H. influenzae* (Yamori, 2004).

Another factor affecting the efficacy of probiotics might derive from the use of multispecies probiotics. It is widely believed that combining several probiotic species will show synergistic effects against pathogen adhesion (Timmerman et al., 2004). However, the immunomodulatory effects of probiotics are highly strain-dependent (Moineau, 1991), and the interference of immunomodulating effects between individual strains might also take place (Hart et al., 2004). Although probiotics did not affect the carriage of *S. pneumoniae* or *H. influenzae* in Study II, the prevalence of *M. catarrhalis* increased in the probiotic group, which might have resulted from the possible antagonistic effects of the probiotic strains in the combination. On the other hand, the prevalence of *Moraxella* increased, particularly in the children of under 3 years of age, but not in the older children. This might also result from age-dependent factors, since the colonisation rate of *Moraxella* is highest among 1-4-year-old children (54%), while it is relatively low (7%) in children of 4-15 years of age (Ejlertsen et al., 1994).

To evaluate whether probiotics may enhance mucosal immunity in otitis-prone children (II), salivary IgA-antibodies against the most common otitis pathogen, *S. pneumoniae* (Kilpi et al., 2001), were measured. On the basis of different capsular polysaccharides, at least 90 different pneumococcal serotypes are known. In Study II, the most frequent serotypes were 6A, 6B, 15, 19F and 23F, which are also known to be the most prevalent serotypes in Finnish children, and to be related to the majority of cases of AOM (Syrjänen et al., 2001). Antibodies formed against the capsule are type-specific, and we measured antibodies against serotypes 6B and 19F. The level of anti-6B-IgA concentration was higher in the probiotic group and the proportion of IgA-positive samples to serotype 6B also tended to be higher, suggesting that
probiotics might have enhanced mucosal immunity. However, no effect was seen on the level of anti-19F-IgA. Several surface proteins of *Pneumococcus* can also be used as immunogens, such as pneumococcal surface protein A (PspA), which is a virulence factor of *S. pneumoniae*, expressed on the surface of most clinical isolates (Crain *et al*., 1990). The oral and intranasal immunisation of mice with PspA with an adjuvant toxoid conjugate has induced mucosal and systemic antibody responses and protected against pneumococcal carriage and infections (Wu *et al*., 1997; Yamamoto *et al*., 1997). In healthy humans, the intranasal inoculation of pneumococcal 23F isolate has also induced the development of serum IgG and sIgA to PspA (McCool *et al*., 2002). However, in Study II, probiotics did not affect the levels of salivary IgA to the pneumococcal surface proteins, PhtD or PspA-1. Thus the results do not provide convincing evidence of the enhancement of mucosal immunity by probiotics, although anti-6B antibodies suggested a positive influence.

Although probiotics did not reduce the overall occurrence of AOM, a significant interaction was found between the probiotics and pathogen carriage on the occurrence of AOM. Probiotics increased the occurrence of AOM in the pathogen-positive subjects. This again highlights the strong influence of otitis pathogens on the occurrence of AOM, and that probiotics cannot compete with pathogen carriage in children who are highly susceptible to AOM because of disturbances in the nasopharyngeal microbiota. However, probiotics reduced the occurrence of AOM when the child was free of all bacterial pathogens. This indicates that probiotics may reduce the risk of AOM, which is of viral origin.

### 6.2 Prevention of diarrhoea

Probiotic treatment did not prevent diarrhoeal illnesses in healthy day-care children. This finding partly conflicts with the results of previous prevention studies. A slight decrease in the incidence of diarrhoea was seen when small infants and toddlers were supplemented with yoghurt containing *Lactobacillus casei* (Pedone *et al*., 2000). However, most of the prevention studies with children have not reduced the incidence of diarrhoea, although the severity of the diarrhoea, in terms of dehydration (Thibault *et al*., 2004), the duration of diarrhoea (Pedone *et al*., 1999; Chouraqui *et al*., 2004; Weizman *et al*., 2005) and the number of diarrhoeal episodes (Oberhelman *et al*., 1999) has been reduced. One possible explanation for the ineffectiveness of probiotics in Study I might be the age of the children. The children in previous studies who found beneficial effects on diarrhoea, were 4 months to 2 years old (Oberhelman *et al*., 1999; Pedone *et al*., 2000; Chouraqui *et al*., 2004; Thibault *et al*., 2004; Weizman *et al*., 2005), while the children in Study I were older - 1-6 years. Our finding also accords with another preventive study performed among older day-care children: a 6-month consumption of *L. casei* did not reduce the incidence of diarrhoea, either (Pedone *et al*., 1999). It can be speculated that the microbiota of infants and toddlers can be influenced more effectively (Guerin-Danan *et al*., 1997; Waligora-Dupriet *et al*., 2007) than those of older children. The duration of diarrhoea among the healthy children (I) was short, lasting an average of only two days, while the average duration of diarrhoea among Finnish day-care children was 3.6 days (Pönkä *et al*., 1991). The short duration probably reflects the mild nature of the diarrhoeal episodes, which could also explain the ineffectiveness of the probiotics.
DISCUSSION

As with the children in Study (I), no beneficial effect of probiotics on the prevention of diarrhoea was seen in the institutionalised elderly (III). However, the average duration of diarrhoea was 3.7 days shorter in the probiotic group than in the placebo group. This difference might have clinical relevance, although it did not reach statistical significance because of the low incidence of diarrhoea (only 22 subjects in the probiotic and 21 in the placebo group experienced diarrhoea). According to a recent meta-analysis, the treatment effect of probiotics has been found to be more evident in children than among adults, which may result from the differences between the gut microbiota of children and adults, and the differences in the colonisation ability of probiotics (Sazawal et al., 2006). On the other hand, it can also be speculated that the gut microbiota of frail elderly subjects (III) suffering from several chronic diseases and consuming a wide range of medicines may have been severely unbalanced (Hebuterne, 2003) and therefore more responsive to beneficial changes induced by probiotics. In healthy, elderly subjects it has been possible to induce beneficial changes in the intestinal microbiota (reflected by an increase in bifidobacteria, lactobacilli and enterococci, and a decrease in enterobacteria counts) by the 4-week consumption of a milk containing bifidobacteria (Ahmed et al., 2007). However, there are only very limited data on the preventive effects of probiotics on community- or hospital-acquired diarrhoea in adults, and it is surprising that no previous data exist on the elderly, apart from data on antibiotic-associated diarrhoea, against which probiotics have been ineffective (Lewis et al., 1998).

On the basis of a recent meta-analysis of preventive studies, the effective size of probiotics seems to vary accordingly to the type of diarrhoea, being highest for antibiotic-associated diarrhoea and lowest for traveller's diarrhoea (Sazawal et al., 2006). This indicates that the effect of probiotics is dependent on the causal pathogens: LGG has been found to be effective against viral gastroenteritis, but not against invasive agents such as Salmonella, Shigella, Campylobacter, Yersinia or Entamoeba (Guandalini et al., 2000). There are no data available on the causative agents in Studies I and III, and therefore we cannot confirm that the origin of the diarrhoeal episodes was infectious. It is well known that stress and changes in diet or medication may also cause diarrhoea. If these non-infectious causes had confounded the results, it would have lessened the effect of the probiotics. The probiotic effects are also known to be strain- and species-dependent. Combining two or more probiotic strains/species has been marginally more effective than using single strains in preventing diarrhoea (Sazawal et al., 2006). This might also explain why a single strain of Lactobacillus GG was not effective in preventing mild diarrhoea in children (I), but a combination of four probiotic strains showed a clinical difference in the duration of diarrhoea in the elderly (III). The specific combination seems to be especially beneficial in gastrointestinal disorders, since it has reduced IBS-induced gastrointestinal symptoms (Kajander et al., 2005), as well as symptoms caused by Helicobacter pylori eradication treatment (Myllyluoma et al., 2005).

6.2.1 Methodological considerations

The definition of diarrhoea might have affected the outcome. Among the healthy children (I), diarrhoea was defined as "abnormally loose stools compared to the child's normal stool consistency". We thought that this definition would be understandable to parents, and that the parents would be reliable observers in distinguishing changes in the usual stool consistency of
their own children. However, in most of the diarrhoea treatment studies (e.g. Guarino et al., 1997; Szajewska et al., 2001; Mastretta et al., 2002; Costa-Ribeiro et al., 2003; Salazar-Lindo et al., 2004; Pereg et al., 2005), diarrhoea has been defined as “three or more loose or watery stools per 24 hours” following the World Health Organization (WHO) criteria, although different modifications, e.g. “three or more bowel movements looser than normal” (Szymanski et al., 2006) or “two or more loose stools” (Rosenfeldt et al., 2002a), have also been used. It is, however, possible that such things as changes in the diet may have caused looser stools, and this may have been wrongly interpreted as infectious diarrhoea. This would have led to overestimation of the incidence of diarrhoea, which seems unlikely, because in our study the prevalence of diarrhoea was even lower than the average prevalence among day-care children (Hjelt et al., 1987b; Rosenfeldt et al., 2005).

In the institutionalised elderly (III), the definition of diarrhoea was more accurate: stool consistency was recorded as hard, normal/soft, loose, watery. Because the use of laxatives is extremely common among the intstitutionalised elderly and may cause functional diarrhoea on the same or the following day, the definition included abstention from laxatives on the same or the previous day. Personal nurses were responsible for recording stool frequency and consistency, which makes the results more reliable. However, the incidence of diarrhoea in the elderly was even lower than in the children. It is thus possible that diarrhoeal episodes of very short duration may have gone unnoticed. There might also be some methodological weaknesses in reporting the duration of diarrhoea. All the treatment studies have measured the duration in hours, in order to detect differences of less than one day (e.g. Guandalini et al., 2000; Rosenfeldt et al., 2002a; Rosenfeldt et al., 2002b). The duration in Studies I and III was counted in whole days, and we might therefore have missed small differences in duration between the groups.

6.3 Prevention of oral carriage of Candida

The mouth represents the initial part of the gastrointestinal tract. It could therefore be hypothesised that probiotic antagonism to pathogens could act the same way in the mouth as in other distal parts of the gastrointestinal tract. During the past few years, evidence has been accumulating on the ability of probiotics to influence the composition of oral microbiota in adults and children, in terms of increasing the salivary Lactobacillus population (Montalto et al., 2004), or reducing Streptococcus mutans counts (Näse et al., 2001). However, Study IV was the first study to show that probiotics may modify the oral microbiota of the elderly by suppressing the growth of Candida. Probiotics reduced the risk of high Candida carriage by 75%, which can be considered a remarkable reduction. The overall prevalence of yeast carriage was 74%, which is a fair representation of the average yeast carriage among the elderly (Wilkieson et al., 1991; Meurman et al., 1997; Budtz-Jørgensen et al., 2000; Fanello et al., 2006), and thus the results can be generalised for an average elderly population.

Only a very few clinical studies have been performed to study probiotic effects on Candida overgrowth, either in the oropharyngeal area or in the gastrointestinal tract. In an open, non-randomised clinical study, consumption of both fermented milk containing L. casei
Shirota and buttermilk containing *L. lactis cremoris* as a control product, decreased the quantity of yeasts in the voice prosthesis (Schwandt *et al.*, 2005). An 8-week consumption of traditional yogurt containing *S. thermophilus* and *L. bulgaricus* did not affect the amount of salivary *Candida* in young adults, although the amount of *S. mutans* was reduced compared to the control ice-cream (Petti *et al.*, 2001). However, cheese containing *Lactobacillus* GG and *L. rhamnosus* LC705 showed a tendency to reduce salivary *Candida* in healthy young adults (Ahola *et al.*, 2002). In Study IV, *L. rhamnosus* GG and LC705 strains were combined with a *Propionibacterium* strain, because a combination of *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS has previously been shown effectively to prevent the growth of food spoilage yeasts and moulds in fermented milk products (Suomalainen and Mäyrä-Mäkinen, 1999). Thus, the addition of *Propionibacterium* probably improved the effectiveness of the probiotic mixture. *Candida albicans* was the most common species found, followed by *C. glabrata* and *C. tropicalis*, which accords with several other studies (Wilkieson *et al.*, 1991; Paillaud *et al.*, 2004; Fanello *et al.*, 2006). No significant switches between different *Candida* species were found during the intervention. However, the probiotic treatment did not affect the subjective feeling of oral pain or the prevalence of mucosal lesions. Previous data on the effects of probiotics on oral mucosal disorders are very limited, though a few studies have reported some beneficial effects of lactobacilli and bifidobacteria on gingivitis and periodontal diseases - the inflammations of the gum tissue caused by plaque (Morozova *et al.*, 1996; Grudianov *et al.*, 2002; Volozhin *et al.*, 2004).

### 6.3.1 Methodological considerations

Several possible confounding factors which might have affected the yeast growth should be considered. No major differences in denture wearing, number of chronic diseases or medication were seen between the groups, but the prevalence of diabetes and the use of antibiotics were slightly more common in the probiotic group. However, no association between diabetes and yeast carriage was detected. The prevalence of yeast was slightly more common among antibiotic-users compared to non-users. However, when antibiotic use was introduced into a stepwise multivariable regression model, it did not reach statistical significance, suggesting that antibiotics did not confound the treatment effect of probiotics. Low salivary secretion and buffering capacity, in addition to denture wearing, are known risk factors for oral candidiasis (Närhi *et al.*, 1993; Ikebe *et al.*, 2006), and the results were therefore adjusted to these factors. Hyposalivation, on the basis of unstimulated secretion, was exhibited in 27% of the subjects, and in 45% when based on stimulated secretion, which accords with previous studies (Närhi *et al.*, 1993; Ikebe *et al.*, 2006). Interestingly, probiotics reduced the risk of hyposalivation, which may indirectly have affected the yeast growth. The mechanism behind this finding is unclear. It can be hypothesised that probiotics might have affected the composition of the saliva - such as the concentrations of mucins or salivary immunoglobulins, as has been shown in animal (Negretti *et al.*, 1997), and in *in vitro* studies (Mack *et al.*, 2003) - and thereby affected the amount of saliva secreted. Saliva plays an important role in the preservation of oral health. Salivary proteins, such as histatin, defensins and secretory IgA, protect the soft tissue of the oral cavity (Shay *et al.*, 1997). Thus, alterations in the quality or quantity of saliva might affect the host resistance to candidal infection.
6.3.2 Antagonism to *Candida*

Antifungal activity is a relatively common characteristic of many bacterial species, including lactobacilli (for review, see Kerr, 1999). Several *Lactobacillus* species (e.g. *L. acidophilus, paracasei* and *rhamnosus*), isolated from milk products, the female genital tract or the oral cavities of healthy volunteers, produce different metabolites, such as organic acids, bacteriocins, hydrogen peroxide (Strus et al., 2005) and antifungal cyclic peptides (Ström et al., 2002), which inhibit the *in vitro* growth of yeasts. It has been suggested that hydrogen peroxide, together with peroxidase and hypothiocyanite, may be involved with the inhibition of *Candida* by lactobacilli (Jack et al., 1990) It has also been postulated that lactobacilli, in the presence of the peroxidase system, converts thiocyanate to hypothiocyanate, which is toxic to microorganisms. However, the mechanism of the probiotics in this clinical trial (IV) remains uncertain. Direct antagonism to *Candida* might partly explain the effects, especially since the probiotics were given in cheese. Lengthy chewing of cheese has, without doubt, prolonged the persistence of probiotics in the mouth, and thereby facilitated their functional ability in the oral cavity. In addition, lactobacilli are known to suppress the adhesion of oral micro-organisms such as *Streptococcus mutans* on saliva-coated hydroxyapatite (Wei et al., 2002), and several *Lactobacillus* strains can also inhibit *C. albicans* hyphal transformation, which is a key step towards epithelial invasion (Noverr and Huffnagle, 2004b). However, there are no comparative data available on the ability of probiotics to reduce the adhesion of *Candida* in the mouth, although *in vitro* studies have proved the ability of lactobacilli to displace *Candida* and prevent its adhesion to uroepithelial or vaginal cells (Reid et al., 1995; Strus et al., 2005). Coaggregation of probiotics with *Candida* may also explain the probiotic effect, as has been shown to happen between *Candida* and oral microbes, such as *Fusobacteria* (Jabra-Rizk et al., 1999).

The prolonged persistence of probiotics in the mouth may also have promoted the adhesion of the probiotics themselves to the mucosal surface, although previous colonisation studies fail to show any persistent colonisation of *Lactobacillus GG* in the mouth (Meurman et al., 1994; Yli-Knuuttila et al., 2006). LGG can be found in the saliva two weeks after the cessation of consumption (Meurman et al., 1994), but a recent study shows that even one third of the subjects do not carry LGG on the first follow-up day after consumption (Yli-Knuuttila et al., 2006). The presence of probiotics in the saliva or oral mucosa was not determined in Study IV.

If probiotics have the ability to colonise the oral cavity only temporarily, it is again tempting to assume that in addition to straight antagonistic mechanisms, systemic effects might also play a role in reducing *Candida* - a hypothesis supported by several animal studies. For example, the administration of live *L. acidophilus and fermentum* by a feeding tube straight into the stomach has shortened the duration of oral *Candida* colonisation in mice (Elahi et al., 2005). Because of the method of administration, the enhanced clearance of *Candida* did not involve direct interference by lactobacilli. In fact, the clearance was only detected two weeks after the treatment with lactobacilli, and it correlated with an early mRNA gene expression for IL-4 and IFN-γ, and with high levels of IFN-γ and NO in the saliva (Elahi et al., 2001). NO is involved in controlling the colonisation of *Candida* in the oral mucosa (Elahi et al., 2001), and *Candida* infections are associated with increased systemic and local NO production induced by the NO-synthase isoform (NOS2). NOS2, on the other hand, can be stimulated by TNF-α and IFN-γ, or by bacterial cell wall components, such as LPS, peptidoglycan and lipoteichoic acid.
Lactobacilli, including *Lactobacillus* GG, are known to induce the production of TNF-α and IFN-γ (Miettinen et al., 1996; 1998) and also of NO in murine macrophages and human colon epithelial cells (Korhonen et al., 2001). In addition, heat-killed *Lactobacillus* GG and *L. acidophilus* have been able to inhibit systemic candidiasis in mice, suggesting that the effect is mediated through the systemic stimulation of the immune system (Wagner et al., 2000). It thus seems that probiotics may exert their *Candida*-inhibiting effect by producing antifungal metabolites, by inhibiting the adhesion of *Candida*, through the displacement of already existing *Candida*, and by enhancing host immunity.
7 CONCLUSIONS

On the basis of the results presented in this thesis, the following conclusions as to the efficacy of *Lactobacillus* GG and the combination of four probiotic bacteria in reducing common infections, can be drawn:

1. Specific probiotics may alleviate the severity of acute respiratory infections and prevent recurrent infections in certain subgroups of children. In healthy children, *Lactobacillus* GG slightly attenuates respiratory infections by postponing their appearance and by reducing complicated infections. Reduction of the complications may lead to reduced need for antimicrobial treatments, and to the reduction of child-care absences. In infection-prone children, the probiotic combination relieves infections by reducing recurrent episodes, especially in children with allergic diseases. Presumably, these effects are mediated through the stimulation of the immune system. In frail elderly subjects, a probiotic combination does not reduce respiratory infections.

2. In healthy children, the alleviation of acute respiratory infections by *Lactobacillus* GG may lead to a marginal reduction in the occurrence of acute otitis media. In children prone to recurrent otitis media and with disturbed nasopharyngeal microbiota, the probiotic combination is not effective in preventing otitis, except in the case of children with allergic diseases. Probiotics taken in capsules do not reduce the carriage of bacterial pathogens, suggesting that no direct antagonism between probiotics and pathogens occurs in the nasopharynx. Specific probiotics may be effective against viral but not against bacterial acute otitis media.

3. The long-term consumption of *Lactobacillus* GG or a probiotic combination, either taken in food or as capsules, is not effective in preventing acute diarrhoea in healthy children or in frail, institutionalised elderly subjects. This result, which is contrary to previous findings, needs to be clarified in further studies with a sufficient number of subjects and diarrhoea events with known causative pathogens.

4. The long-term consumption of specific probiotics in a food matrix modifies the oral microbiota of elderly subjects by reducing the prevalence of high oral yeast carriage, possibly related to the reduced risk of hyposalivation. Probiotics can thus be considered beneficial to the oral health of the elderly.

The specific probiotic, *Lactobacillus* GG, and a probiotic combination containing LGG both seem to be beneficial for children in terms of alleviating the severity of respiratory infections, and for elderly subjects, in balancing their oral microbiota. Since probiotics are considered to be food and are easy to include in the everyday diet, they can be considered a suitable, easy and safe method for balancing the body's natural defences against infections.
ACKNOWLEDGEMENTS

This study was carried out between the years 1998 and 2007 at Valio R&D, Helsinki, at the Division of Infectious Diseases, Helsinki University Central Hospital, and at the Institute of Dentistry, University of Helsinki.

I have been fortunate to be supervised by two experts in the field of Nutrition and Infectious Diseases, Docent Riitta Korpela and Professor Ville Valtonen. I wish to express my deepest gratitude to Riitta Korpela, who has believed in me throughout these years. I am particularly grateful for her warm-hearted guidance, her encouragement, and her never-ending enthusiasm in inspiring me for the scientific work, and her wonderful flexibility during the final stages of the thesis. I am especially indebted to Ville Valtonen for “adopting” me into his clinic and for supervising my work. His warm and encouraging support and wide knowledge of infectious diseases has been invaluable.

I would also like to express my appreciation to Professor Tiina Mattila-Sandholm, Senior Vice President of Valio R&D, for giving her support and making it possible to finish this thesis alongside my permanent work.

I am grateful to all my co-authors and collaborators. Without their expertise this work would not have been possible. I have had the privilege of collaborating with experienced specialists Professor Jukka Meurman, Professor Anne Pitkaranta and Professor Erkki Savilahti, who are warmly thanked for their most valuable and constructive advice in the planning and conducting of the clinical trials. I owe my sincere thanks to Maija Saxelin PhD for introducing me to the area of probiotics, and for sharing my work responsibilities during the last, intensive year when I was finishing this thesis. I offer my warmest thanks to Professor Heikki Vapaatalo for his valuable and constructive criticism on the writing of this thesis. Anne Nyberg MSc, Aila Ahola MSc and Sara Pohjauvori MSc are warmly thanked for their invaluable help in running the trials, and Karin Blomgren MD, PhD and Heli-Yli-Knuuttila DDS for examining the patients. Tuija Poussa MSc is gratefully acknowledged for her valuable work with the statistical analyses and her patient guidance in the world of statistics, and Hannu Kautiainen PA for his professional statistical advices.

Thanks are also due to Docent Antti Pönkä, Leena Näse DDS, Pia Laukkanen MD, PhD, Maija Rummukainen MD, Arja Lyytikäinen MSc and Sirkka Keikkala MD for sharing their experience in planning and running the clinical trials. Associate Professor Malcolm Richardson, Tarja Kaijalainen PhD and Professor Maija Leinonen are acknowledged for their expertise in microbiology, and for carrying out the microbial analyses at their laboratories. Professor Helena Käyhty’s laboratory at the National Public Health Institute, and in particular Anu Nurkka PhD and Tiina Salomäki MSc, are thanked for analysing the salivary antibodies, and Professor Tapani Hovi’s laboratory at the National Public Health Institute for performing the viral assays.

Professor Seppo Salminen and Docent Risto Vuento are acknowledged for the rapid and fluent review process and for their constructive comments for improving this thesis. Mimi Ponsonby MA is gratefully acknowledged for the revision of the language, and Taina Ilomäki-Virta MA for the visual image.
All the children and their families, the elderly subjects as well as the personnel of the day care centres, old people’s homes and hospitals are warmly thanked for participating in these studies and for making this work possible.

My present and former workmates at Valio, Kajsa, Riina, Tiina, Tuula, Annikki, Leena, Netta, Anu, Anna, Minna, Riikka, Katarina and Mirka and “The Nutraceutical girls”, Eveliina, Laura and Taru are thanked for sharing the probiotic research and for creating an intensive research-oriented atmosphere and for many stimulating conversations. All my dear good old friends, Ulla & Aki, Mepa & Pertti, Mari & Erkka, Jonna, Raika & Harri, Kati & Ari and Elina & Marcus, are thanked for the numerous cheerful moments spent together during all these years. Their friendship reminded me of life outside science, and kept me sane!

My parents-in-law Marketa and Kalevi Hatakka deserve special thanks for participating so intensively in our family life, taking good care of our children, being always available, and for their interest in my scientific work. I am truly grateful to my parents Eira and Jouko Hardén for their encouragement and support, not just during this process but all through my life, and thanks to my brother Henkka for being there for me and listening to sis’ joys and sorrows.

Finally, my deepest love and gratitude belong to my husband Tomi, for his love, understanding and support during these hardest years of my life, and to our marvellous children, Enni and Olli, for bringing us joy and happiness, and reminding us of the most precious things in this life!

This study was financially supported in part by a grant from the Finnish Academy and the University of Helsinki.

Helsinki, May 2007

Katja Hatakka
REFERENCES


REFERENCES


Corthesy B, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. J Nutr 2007; 137: 781S-90S.


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Ouwehand AC, Isolauri E, Kirjavainen PV, Tolkko S, Salminen SJ. The mucus binding of Bifidobacterium lactis Bb12 is enhanced in the presence of Lactobacillus GG and Lact. delbrueckii subsp. bulgaricus. Lett Appl Microbiol 2000; 30: 10-3.


REFERENCES


REFERENCES


Ström K, Sjögren J, Broberg A, Schnurer J. Lactobacillus plantarum MilAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 2002; 68: 4322-7.


REFERENCES


Volozhin AI, Il’in VK, Maksimovskii Iu M, Sidorenko AB, Istranov LP, Tsarev VN, et al. [Development and use of periodontal dressing of collagen and *Lactobacillus casei* 37 cell suspension in combined treatment of periodontal disease of inflammatory origin (a microbiological study)]. *Stomatologiya (Mosk)* 2004; 83: 6-8.


