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ADMINISTRATION OF *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BB-12 AND XYLITOL WITH A NOVEL PACIFIER IN EARLY CHILDHOOD

by

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ABSTRACT

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Administration of *Bifidobacterium animalis* subsp. *lactis* BB-12 and xylitol with a novel pacifier in early childhood

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Probiotic bifidobacteria are used in the prevention and treatment of childhood diseases. On the other hand, these bacteria are also connected to dental caries. The purpose of the present work was to test a food supplement containing *Bifidobacterium animalis* subsp. *lactis* BB-12 (*B. lactis* BB-12) and xylitol, and to investigate its health effects, properties and safety when used in a novel pacifier in early childhood.

In a double-blind, placebo-controlled trial, newborn infants (n=163) were assigned randomly to receive *B. lactis* BB-12, xylitol, or sorbitol from the age of 1–2 months to 2 years with a pacifier or a spoon. Children were followed up to four years of age. A part of the parents participating in the clinical trial evaluated the feasibility of the novel administration method. The pattern of tablet release from the pouch of the pacifier was tested in adults.

The food supplement tablet containing *B. lactis* BB-12 and xylitol could be delivered in a safe and controlled way with the novel pacifier. The early administration of *B. lactis* BB-12 did not result in permanent oral colonization of this probiotic or affect the colonization of mutans streptococci in early childhood. Moreover, *B. lactis* BB-12 did not increase the occurrence of caries. Controlled administration of *B. lactis* BB-12 significantly reduced the incidence of respiratory infections during the first eight months of life in a Finnish population with breastfed infants.

To conclude, administration of *B. lactis* BB-12 in early childhood is safe with regard to the future dental health of the child. In addition, *B. lactis* BB-12 may add to the protection against respiratory infections provided by human breast milk in infancy.

Key words: bifidobacteria, caries, mutans streptococci, pacifier, probiotics, xylitol

TIIVISTELMÄ

Teemu Taipale

***Bifidobacterium animalis* subsp. *lactis* BB-12:n ja ksylitolin annostelu varhaislapsuudessa uudella taskututilla**

Sosiaalihammaslääketiede, Hammaslääketieteen laitos, Turun yliopisto. Annales Universitatis Turkuensis, Turku, Finland, 2012

Probioottisia bifidobakteereja käytetään lasten sairauksien ennaltaehkäisyssä ja hoidossa. Bifidobakteerit on toisaalta myös liitetty hampaiden reikiintymiseen. Tämän työn tarkoitus oli testata *Bifidobacterium animalis* subsp. *lactis* BB-12:a (*B. lactis* BB-12) ja ksylitolia sisältävän ravintolisäpuristeen annostelua uudella taskututilla, sekä tutkia annostelun turvallisuutta ja vaikutusta lasten terveyteen.

Tutkimuksessa vastasyntyneille lapsille (n=163) annosteltiin tutilla tai lusikalla *B. lactis* BB-12:a, ksylitolia tai sorbitolia 1–2 kuukauden iästä lähtien kahteen ikävuoteen saakka. Lapsia seurattiin neljän vuoden ikään asti. Osa tutkimusperheistä osallistui jatkotutkimukseen, jossa arvioitiin uuden annostelumenetelmän käyttökelpoisuutta. Lisäksi puristeen liukenemista tutin taskusta tutkittiin aikuisilla.

Tutkimus osoitti, että *B. lactis* BB-12:a ja ksylitolia sisältävää ravintolisäpuristetta voitiin annostella turvallisesti ja luotettavasti taskututilla. *B. lactis* BB-12 ei kolonisoitunut suuhun, eikä se vaikuttanut mutans streptokokkien kolonisaatioon varhaislapsuudessa. *B. lactis* BB-12 ei myöskään lisännyt hampaiden reikiintymistä. *B. lactis* BB-12-ryhmässä esiintyi kahdeksan ensimmäisen ikäkuukauden aikana merkitsevästi vähemmän hengitystieinfektioita kuin kontrolliryhmässä. Tämä todettiin siitä huolimatta, että tutkimukseen osallistuneet lapset saivat rintamaitoa, mikä sinänsä parantaa lasten vastustuskykyä.

Johtopäätöksenä voidaan todeta, että *B. lactis* BB-12:n annostelu varhaislapsuudessa on suun terveyden kannalta turvallista. Lisäksi *B. lactis* BB-12 saattaa lisätä rintamaitoa saaneiden lasten vastustuskykyä hengitystieinfektioita vastaan.

Avainsanat: bifidobakteerit, karies, ksylitoli, mutans streptokokit, probiootit, tutti

TABLE OF CONTENTS

ABBREVIATIONS

AOM	Acute otitis media
ATCC	American Type Culture Collection
CFU	Colony forming unit
CI	Confidence interval
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
GI	Gastrointestinal tract
ICDAS	International caries detection and assessment system
MRS	de Man, Rogosa and Sharpe medium
MS	Mutans streptococci
OR	Odds ratio
PCR	Polymerase chain reaction
RR	Risk ratio
SD	Standard deviation
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–IV. In addition, some unpublished data are presented. The original publications are reproduced with the permission of the copyright holders.

I Taipale T, Pienihäkkinen K, Alanen P, Jokela J, Söderling E. Dissolution of xylitol from a food supplement administered with a novel slow-release pacifier: preliminary results. Eur Arch Paediatr Dent 2007;8:123–125.

II Taipale T, Pienihäkkinen K, Salminen S, Jokela J, Söderling E. *Bifidobacterium animalis* subsp. *lactis* BB-12 administration in early childhood: a randomized clinical trial on effects on colonization of mutans streptococci and the probiotic. Caries Res 2012;46:69–77.

III Taipale T, Pienihäkkinen K, Alanen P, Jokela J, Söderling E. Administration of *Bifidobacterium animalis* subsp. *lactis* BB-12 in early childhood: A post-trial effect on caries occurrence at four years of age. Submitted 2012.

IV Taipale T, Pienihäkkinen K, Isolauri E, Larsen C, Brockmann E, Alanen P, Jokela J, Söderling E. *Bifidobacterium animalis* subsp. *lactis* BB-12 in reducing the risk of infections in infancy. Br J Nutr 2011;105:409–416.

1. INTRODUCTION

Microbial colonization of the gastrointestinal tract begins immediately after birth. During the first years of life, an adult-type pattern of complex indigenous microbiota is established. The microbiota is acquired via mother to child transmission, from other individuals, and from the environment, and it is further shaped by diet and hygiene (Salminen and Isolauri 2008, Adlerberth and Wold 2009). The developing intestinal and oral microbiota plays an important role in the health and wellbeing of the host. A shift in the compositional development of the microbiota may increase the risk of immunological and infectious diseases in infancy and later childhood (Collado et al. 2009).

The most common oral disease in children, early childhood caries, can also be considered to result from a shift in the balance of the resident microbiota. Increased environmental acidification in dental plaque leads to the initiation and progression of caries. Many acidogenic and aciduric bacteria, e.g. mutans streptococci (MS), *Veillonella*, *Actinomyces*, *Lactobacillus*, and *Bifidobacterium* are involved in the caries process in primary and permanent dentition (Aas et al. 2008, Belda-Ferre et al. 2012). Although the role of bacteria in the development of a caries lesion is crucial, dental caries can be considered a multifactorial, plaque-associated disease. Several factors at the tooth surface level, and at individual and population level may result in an imbalance in the equilibrium between biofilm and tooth (Fejerskov 2004).

Probiotic bacteria, mostly lactobacilli and bifidobacteria, are living microorganisms which, when administered in adequate amounts, confer a health benefit to the host (WHO/FAO 2002). In childhood, specific probiotic strains have been successfully used in the prevention and treatment of gastrointestinal diseases and allergies, and in the modulation of immune response (Floch et al. 2008, Salminen and Isolauri 2008). In the past last decade, several studies have investigated whether probiotic bacteria originally planned to promote general health could also be beneficial to oral health. Several studies have shown that short-term administration of probiotic lactobacilli can affect oral microbiota in adults (Stamatova and Meurman 2009). However, the role of bifidobacteria in the oral cavity is not completely understood. Bifidobacteria are highly aciduric and acidogenic and frequently found from deep caries lesions but not from the sound tooth surfaces (Aas et al. 2008). On the other hand, specific strains of probiotic bifidobacteria have been shown to reduce the salivary levels of MS in adolescents and in young adults (Caglar et al. 2005 and 2008b, Cildir et al. 2009, Singh et al. 2011). So far, no studies are available concerning the early administration effect of probiotic bifidobacteria on the colonization of erupting teeth and the future dental health of the child.

It has been suggested that exposure early in life to the probiotic organisms may have a long-term beneficial influence on the composition and development of the infant's microbial ecosystem, potentially leading to a reduced risk of diseases (Isolauri et al. 2002, Rautava 2007, Collado et al. 2009). Administration of probiotics to exclusively breastfed infants with a measuring cup or spoon is impractical and time-consuming. These factors have been overcome with the development of a new pacifier optimized for infant health. The aim of the present work was to test a food supplement containing xylitol and a probiotic organism, *Bifidobacterium animalis* subsp. *lactis* BB-12 (*B. lactis* BB-12), and to investigate its health effects, properties and safety when administered with a novel pacifier in early childhood.

2. REVIEW OF THE LITERATURE

2.1 Oral microbiota and dental caries in children

2.1.1 Development of the oral microbiota

During the birth process and rapidly thereafter, microbes from the surrounding environment start to colonize the gastrointestinal tract, including the mucosal surfaces of the oral cavity, until a dense and complex microbiota develops (Mackie et al. 1999, O'Hara and Shanahan 2006). Factors influencing the colonization pattern include genetics, mode of delivery, maternal microbiota, type of feeding, geographical location, and hygiene conditions around the child (Salminen and Isolauri 2008, Adlerberth and Wold 2009). The developing intestinal and oral microbiota plays an important role in the health and wellbeing of the host.

The first exposure to microorganisms in vaginally delivered infants occurs during passage through the birth canal, whereas infants born by caesarean section (C-section) acquire their first microbes from the skin of parents and health providers, and from medical equipment. The data concerning the acquisition of microorganisms from the birth canal to the oral cavity during the delivery are limited. Only *Staphylococcus epidermidis* present in the vaginal microbiota has been shown to persist on the oral surfaces of the children (Hegde and Munshi 1998). The mode of delivery has been shown to affect the diversity and type of acquired bacteria in the mouth. Higher numbers of taxa were reported among healthy, three-month-old infants delivered vaginally, compared with those delivered by C-section (Lif Holgerson et al. 2011). Moreover, C-section infants became colonized with *Streptococcus mutans* almost a year earlier than did vaginally delivered infants (Li et al. 2005). After the birth process, neonates are continuously exposed to new microbes from food, usually from breast milk. Breastfeeding affects the composition of the gut microbiota. However, the role of breast milk in the development of the oral microbiota is unclear. Breast milk has been shown to contain viable bifidobacteria and lactic acid bacteria (Martin et al. 2003, Gueimonde et al. 2007, Abrahamsson et al. 2009).

Adherence is a key initial event for bacteria to survive and persist in the oral cavity (Jenkinson and Lamont 1997, Kolenbrander et al. 2010). Most bacteria are rapidly swallowed or they show only transient colonization to the oral surfaces (Könönen 2000). In the oral cavity, the mucosal surfaces of lips, cheek, palate and tongue are highly selective for microorganisms during the first months of life. Only few species from the surrounding environment are able to adhere to the oral epithelial surfaces. Most oral

microbes are acquired from the mother, father, or siblings by vertical transmission via saliva (Berkowitz et al. 1981, Könönen 2000). Viridans streptococci, *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus salivarius* are the first and numerically dominant pioneer species of the human mouth, but also some anaerobic bacterial species can be recovered (Rotimi and Duerden 1981, Smith et al. 1993, Könönen 1999). The number and diversity of the oral microbiota increase during the first few months of life and form the basis for further colonization and coaggregation of bacteria. The eruption of teeth around the age of six months changes the oral environment significantly, creating a number of new attachment sites and niches in the oral cavity. Streptococci and Actinomyces are the first bacteria which adhere to the acquired salivary pellicle covering the enamel (Nyvad and Kilian 1987, Li et al. 2004, Diaz et al. 2006). Members of the genera *Fusobacterium*, *Neisseria, Prevotella, Veillonella*, *Lactobacillus*, and *Rothia* are also commonly isolated from the mouth during the first year of life (Kolenbrander et al. 2010). MS, i.e. *S. mutans* and *Streptococcus sobrinus,* have been proposed to colonize the mouth during a defined period called "the window of infectivity" at the age of between 19 and 31 months (Caufield et al. 1993). However, MS have been shown to occur even in predentate infants (Wan et al. 2001, Nakai et al. 2010, Plonka et al. 2012). It can be assumed that MS may colonize the oral cavity at any age in childhood depending on the presence and intensity of factors that favour their transmission and establishment. The colonization of MS has also been shown to increase with age (Köhler et al. 1984, Söderling et al. 2001).

Bifidobacteria are anaerobic bacteria that are commonly found in dental plaque and saliva (Beighton et al. 2008). Bifidobacteria have been isolated from the mouth of infants on the second day after birth (Rotimi and Duerden 1981). Relatively little is known about how oral bifidobacteria are acquired. As stated earlier, breast milk has been shown to contain high levels of bifidobacteria; the most widely present species are *Bifidobacterium longum*, *B. animalis*, and *Bifidobacterium bifidum* (Gueimonde et al. 2007). Thus, it is possible that breast milk is one source of oral bifidobacteria. Oral bifidobacteria could also be acquired later in life from probiotic food (Beighton et al. 2008). The predominant species found in the dentate mouth are *Bifidobacterium dentium* and *B. longum* (Munson et al. 2004, Beighton et al. 2008). *Bifidobacterium breve*, *Bifidobacterium subtile*, *Bifidobacterium adolescentis*, and *Bifidobacterium urinalis* have also been isolated from the oral cavity (Munson et al. 2004).

2.1.2 Dental caries – a plaque-mediated disease

Dental caries is defined as a localized chemical dissolution of a tooth surface resulting from metabolic events taking place in a dental plaque. Although the role of bacteria in the development of a caries lesion is crucial, dental caries can be considered a multifactorial,

plaque-associated disease. Several factors at the tooth surface level (e.g. composition of the dental plaque, salivary secretion, fluoride ion concentration) and at individual and population level (e.g. behaviour, education, knowledge, attitudes) may result in an imbalance in the equilibrium between biofilm and tooth (Fejerskov 2004).

Dental caries in the primary dentition is commonly referred to as early childhood caries (ECC). The disease of ECC is the presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in a child under the age of six (American Academy of Pediatric Dentistry; AAPD 2011). Despite the efforts and achievements in preventive measures in oral health, ECC is still a significant health problem even in developed countries. Children with ECC are at a higher risk of new caries lesions in primary and permanent dentitions, as well as acute oral infections (American Academy of Pediatric Dentistry; AAPD 2011). Moreover, the severe form of ECC is difficult to treat successfully because of the young age of the children, and therefore the children frequently require treatment under general anaesthesia resulting in increased treatment costs (Berkowitz 2003).

In the 1880s, the dentist W.D. Miller proposed in his epochal work that oral bacteria are major etiological agents in the development of caries (Miller 1889). During the past decades, several hypotheses have been proposed on the role of plaque bacteria in the aetiology of caries. The proposal that only a few bacterial species are actively involved in the caries process formed the basis of the specific plaque hypothesis, whereas the non-specific plaque hypothesis suggested that disease is the outcome of the overall activity of the total plaque microflora (Loesche 1986, Theilade 1986). The ecological plaque hypothesis proposed that the organisms associated with disease may also be present at sound sites, but levels are too low to be clinically relevant. According to the ecological plaque hypothesis, caries is the result of a shift in the balance of the resident microflora due to a response to a change in local environmental conditions (Marsh 1994 and 2004). An extended caries ecological hypothesis considers dental plaque a microbial ecosystem in which non-mutans bacteria are the key microorganisms responsible for maintaining dynamic stability on the tooth surface (Takahashi and Nyvad 2008) (Figure 1). The dynamic stability stage turns to the acidogenic stage when sugar is supplied frequently. Prolonged acidification increases the acidogenecity of non-mutans streptococci and a number of other aciduric bacteria. This process may shift the demineralization/ remineralization balance toward a net mineral loss, leading to the initiation of dental caries. Finally, under severe and prolonged acidic conditions, many bacteria, e.g. MS, *Veillonella*, *Propionibacterium*, *Actinomyces*, *Atopobium*, *Lactobacillus*, and *Bifidobacterium* are involved in the caries process (Aas et al. 2008, Belda-Ferre et al. 2012).

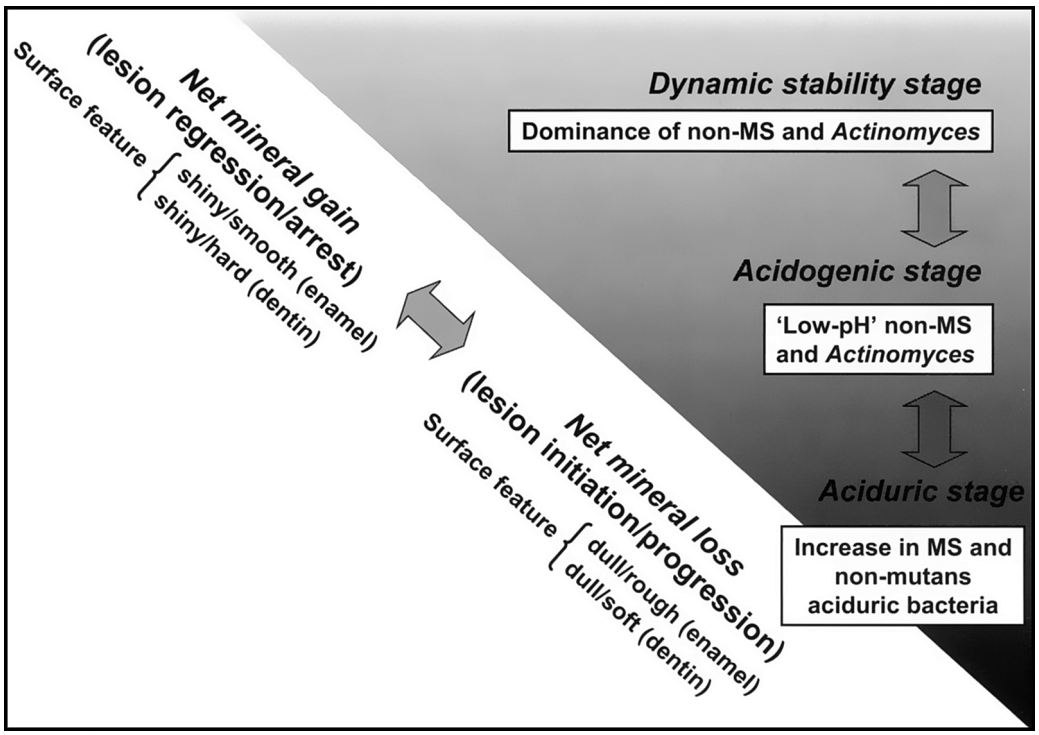

Figure 1. An extended caries ecological hypothesis according to Takahashi and Nyvad (2008; with permission).

MS are the most extensively studied bacteria in early childhood caries (Loesche 1986, Parisotto et al. 2010). MS are frequently isolated from the caries lesions and they are highly acidogenic and aciduric. Furthermore, MS are able to produce water-insoluble glucan, which promotes bacterial adhesion to the tooth surface (Hamada and Slade 1980). Many previous reports have shown that children whose teeth are colonized earlier by MS show higher caries experience than those colonized later or not at all (Alaluusua and Renkonen 1983, Köhler et al. 1988). Over the past years much research has been targeted on the prevention of transmission of MS from mother to child. In clinical trials, inhibition of MS transmission has been achieved by chlorhexidine treatments (Tenovuo et al. 1992), by xylitol chewing gum consumed by mothers (Söderling et al. 2000, Thorild et al. 2003, Nakai et al. 2010), and by combined prevention programmes (Köhler et al. 1983). Recent long-term studies have shown that the prevention of early MS colonization significantly reduces caries prevalence even at the age of 10 and 15 years (Köhler and Andréen 2010, Laitala et al. 2012).

As indicated, many aciduric and acidogenic bacterial species are involved in the caries process. MS may persist on tooth surfaces without a caries lesion, and on occasion, disease can develop in the absence of these species (Takahashi and Nyvad 2008). Thus, MS play a role but are not necessarily the etiological agents of the disease (Beighton 2005). In early

childhood, however, the MS colonization seems to be a relevant and practical marker of increased caries risk (Thenisch et al. 2006, Meurman and Pienihäkkinen 2010).

Bifidobacteria are highly acidurid and acidogenic bacteria and usually connected to caries lesions in children (Mantzourani et al. 2009, Nakajo et al. 2010, Palmer et al. 2010). In a study carried out by Becker et al. (2002), the bacterial species found in ECC were compared to those found in caries-free children aged two to eight years. In that study, *Bifidobacterium* species were the most numerous bacteria identified in both cavitated and deep dentinal caries lesions, but they were not found from intact enamel or white spot lesions. Aas et al. (2008) compared the oral bacteria associated with dental caries in primary and permanent dentition. The study groups consisted of 15 children with caries and 14 caries-free controls from the age of two to 21 years. *Bifidobacterium* species were among the microbes that dominated deep-dentin lesions in primary dentition. Furthermore, in that study, bifidobacteria seemed to have a more dominating role in dentin caries lesions of primary teeth than in those of permanent teeth. The most frequently isolated *Bifidobacterium* species from active carious lesions has been *B. dentium*. Beighton et al. (2008) demonstrated that *B. dentium* was present at high levels in the saliva of adults, and their numbers were significantly correlated with the levels of MS. In summary, bifidobacteria belonging to the resident oral microbiota may play an important role in the progression of ECC. It may be hypothesized that a change in the local oral environment, when sugar is supplied frequently, increases the number and proportions of acidurid and acidogenic bacteria, including bifidobacteria, in the oral cavity.

2.2 Probiotic bifidobacteria and oral health

2.2.1 Probiotic activity in the oral cavity

The word probiotic meaning “for life” is currently used to name bacteria associated with beneficial effects on humans and animals. The idea of the positive role of selected bacteria dates back to 1900s decade when the Ukrainian bacteriologist Ilya Metchnikoff studying the flora of the human intestine suggested that usage of dairy bacteria fermented by lactic acid bacteria might have a positive effect on human health (Metchnikoff 1907). In the same decade, a French paediatrician Henry Tissier observed that children with diarrhea, contrary to healthy children, had in their stools a low number of Y-shaped “bifid” bacteria. He suggested that these bacteria, which were later called bifidobacteria, could be administered to patients with diarrhea to help restore a healthy gut flora (Tissier 1906). The currently used consensus statement defines probiotics as “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host” (WHO/FAO 2002).

The gastrointestinal tract has been the main field of probiotic research. However, in the past last decade several studies have investigated whether probiotic bacteria originally planned to promote gut health could also be beneficial to oral health. The mouth as a microbial habitat differs from the intestinal tract (The Human Microbiome Project Consortium 2012). Probiotic species with proven benefits in the gut might not necessarily be effective in the oral cavity. Recently, potential novel probiotics, e.g. strains of the genera *Propionibacterium, Streptococcus*, and *Weissella* have been studied and characterized beside the conventional *Lactobacillus* and *Bifidobacterium* strains for various oral health purposes, including caries, periodontal diseases, and halitosis (Meurman 2005, Teughels et al. 2008, Twetman and Stecksén-Blicks 2008, Stamatova and Meurman 2009, Haukioja 2010). In order to be an oral probiotic, a microorganism should survive in saliva, resist the defence factors of the saliva, adhere to oral surfaces, and colonize, at least temporarily, the surfaces of the oral cavity. Furthermore, probiotics should be safe for the host. It has been well documented that probiotic health effects are strain-specific, so each strain should be tested separately (Salminen et al. 2009). Data concerning the effect of specific combinations of probiotics in the prevention of oral diseases are limited. Most probiotics are commercially available in various dairy products, other foods, and food supplements, and thus the oral cavity is the first organ of the gastrointestinal tract where probiotics may exert their therapeutic or preventive effect. The current definition of probiotics points out the need for providing an adequate dose of probiotic bacteria in order to exert the beneficial effects. The consumption of 10^6–10^{10} viable cells of probiotic bacteria per day is recommended (Hatakka and Saxelin 2008). The optimal dose in the mouth is not clear, but the consumption of approximately 10^7–10^{10} probiotic bacteria per day, depending on the strain, have been used in clinical trials.

The oral cavity with specialized mucosal surfaces, teeth, saliva, and gingival crevicular fluid create a distinct and challenging environment for probiotic action. Potential mechanisms by which probiotic bacteria may exert their antagonistic effects against pathogens are partly unknown. Possible mechanisms of probiotic action are presented in Figure 2. Probiotic bacteria may interact with the oral epithelium by strengthening the epithelial barrier function or by modulating the innate and adaptive immune response (Stamatova and Meurman 2009). Lactobacilli and bifidobacteria have also shown to produce antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins, and short chain fatty acids against pathogens (Russell et al. 2011). Furthermore, probiotic bacteria may compete for nutrition and the same binding sites with pathogens, and hence, inhibit adhesion and aggregation of bacteria (Hatakka and Saxelin 2008).

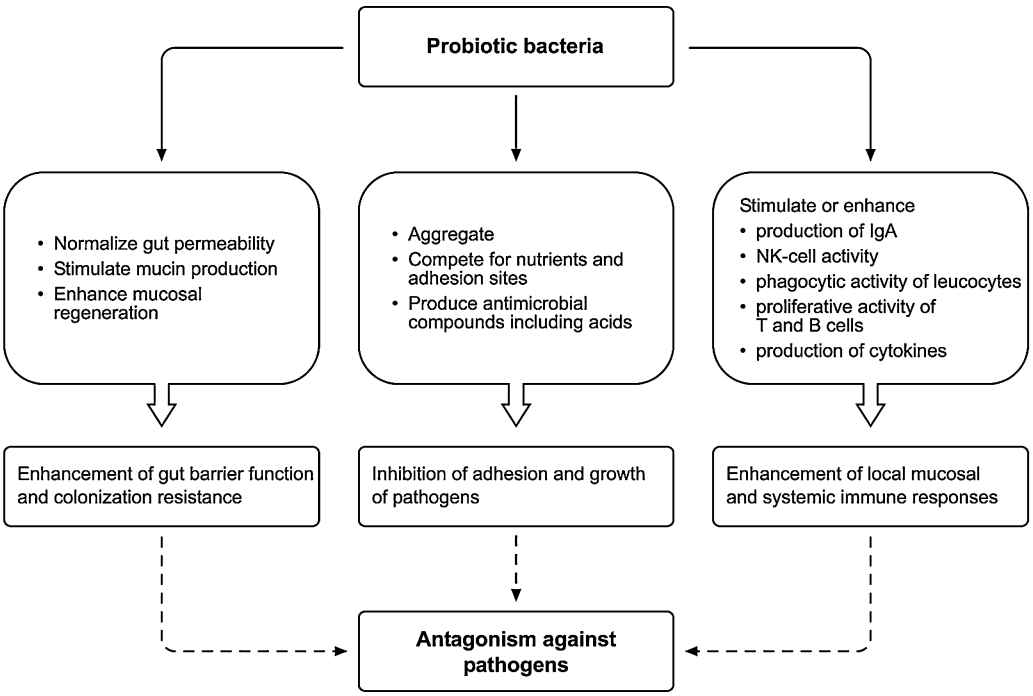

Figure 2. Possible mechanisms of probiotic action (from Söderling, 2012; with permission).

2.2.2 The effect of bifidobacteria on oral health

The genus *Bifidobacterium* belongs to the family Bifidobacteriaceae, which comprise seven genera according to the List of Prokaryotic names with Standing in Nomenclature (LPSN 2012); *Aeriscardovia*, *Alloscardovia*, *Bifidobacterium*, *Gardnerella*, *Metascardovia*, *Parascardovia*, and *Scardovia*. Bifidobacteria are Gram-positive, anaerobic, nonmotile, non-spore-forming, non-gas-producing, catalase-negative branched rods that occur singly, in chains, or in clumps when cultured *in vitro*. Bifidobacteria are found among the resident microbiota in the gastrointestinal tract and their metabolic activities may produce, e.g. conjugated linoleic acid, vitamins, short chain fatty acids, bacteriocins, and antibiotic-like substances, which have been shown to beneficially influence the human host (Russell et al. 2011). The genus *Bifidobacterium* consists of more than 30 distinct species, of which at least 11 species have been isolated in humans. The most common *Bifidobacterium* species in infants have been reported to be *B. bifidum*, *B. breve*, and *B. longum* (Roger et al. 2010). Strain-specific probiotic properties have been found in some bifidobacterial strains belonging to the species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum.*

In contrast to the extensive studies of bifidobacteria in the human gut microbiota and their beneficial health effects in that environment, little is known about the benefits that probiotic bifidobacteria might offer to oral health. Most studies concerning the effects

of probiotics on oral health have been conducted with lactobacilli (Teughels et al. 2008, Stamatova and Meurman 2009, Haukioja 2010). This section focuses on bifidobacteria even though some strains of lactobacilli of special interest are also presented.

Good attachment ability and persistence on the oral mucosa and teeth are important properties of probiotics from an oral health point of view. An *in vitro* study carried out by Haukioja et al. (2006), demonstrated that bifidobacteria could survive in saliva, and *B. lactis* BB-12 was even able to resist the activated peroxidase system, which is one of the defence factors in saliva. In that study, the authors also showed that the adherence of bifidobacteria to saliva-coated surfaces was weaker than that of the lactobacilli strains, but the presence of *Fusobacterium nucleatum*, which is regarded as a chain-microorganism in biofilm formation, enhanced the binding of bifidobacteria (Haukioja et al. 2006). In another *in vitro* study by the same group, *B. lactis* BB-12 was shown to modify the protein composition of the salivary pellicle and specifically prevent adhesion of other bacteria, such as *S. mutans* (Haukioja et al. 2008a).

So far, only one study has addressed the *in vivo* binding of bifidobacteria to the oral cavity. The persistence of a combination of *Lactobacillus rhamnosus* GG and LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS, and *B. lactis* BB-12 (DSM 15954) administered as capsules, yoghurt, or cheese was compared (Saxelin et al. 2010). In this randomized open-label trial, 36 adults consumed a probiotic mixture 10^{10} CFU/day. The daily dose of *B. lactis* BB-12 ranged from 1.2×10^6 to 1.4×10^{10} CFU/day depending on the vehicle. Unstimulated saliva samples were taken before, during, and after the two-week intervention period. During the intervention, only one participant in the yoghurt group and one in the cheese group carried the *B. lactis* BB-12. *B. lactis* BB-12 was recovered but only on the first day of follow-up, and from the same two participants who carried it during the intervention. In that study, *L. rhamnosus* GG was the only probiotic strain regularly recovered in saliva samples. Within ten days, *L. rhamnosus* GG was, however, eliminated from the saliva of approximately 80% of the subjects (Saxelin et al. 2010). These results are in line with earlier clearance studies conducted with lactobacilli. Probiotic lactobacilli colonize the mouth only transiently in adults so that after intervention the levels of bacteria in the oral cavity decrease rapidly (Yli-Knuuttila et al. 2006, Caglar et al. 2009). However, it has been suggested that exposure early in life to the probiotic organisms may facilitate a permanent installation and the effect of probiotics in the oral cavity (Twetman and Stecksén-Blicks 2008). Nothing is known about oral colonization when probiotic bifidobacteria are administered on a daily basis in early childhood.

Bifidobacteria are fermentative bacteria that produce major amounts of acetic and lactic acids. Lactose, galactose, sucrose, and especially human milk oligosaccharides are

fermented by a unique fructose-6-phosphate phosphoketolase pathway, known as "the bifid shunt". Acid production is one of the main functions of bifidobacteria in the intestine because it inhibits the growth of undesirable bacteria (Russell et al. 2011). On the other hand, from the oral health perspective, the production of organic acids from dietary sugars is elementary in the initiation and progression of caries. The acid production from sugars and sugar alcohols has been studied *in vitro* in four *Bifidobacterium* dairy and probiotic strains consisting of *B. lactis* BB-12, *B*. sp. 1100, *B. longum* 913, and *B.* sp. 420 (Haukioja et al. 2008b). In that study, all *Bifidobacterium* strains produced acids efficiently from glucose, and the decrease in pH was comparable to that caused by *S. mutans*. Only *B. lactis* BB-12 of the four tested *Bifidobacterium* strains caused a significant decrease in pH with lactose. However, none of the probiotic bifidobacteria caused a significant decrease in pH with sucrose and sugar alcohols (Haukioja et al. 2008b).

Most clinical studies on probiotics and oral health have been conducted in adults with strains of lactobacilli. These studies have focused on measuring changes in counts of MS. Intervention studies have shown that specific strains of lactobacilli, i.e. *Lactobacillus reuteri* SD2112, *L. reuteri* PTA 5289, *L. rhamnosus* GG, and *L. rhamnosus* 705 may reduce the counts of salivary MS (Näse et al. 2001, Ahola et al. 2002, Nikawa et al. 2004, Caglar et al. 2006, 2007, 2008a). However, contradictory results have also been reported. Interventions of *L. rhamnosus* LB 21, *L. rhamnosus* GG, or a combination of *L. reuteri* SD2112 and PTA 5289 had no effect on the MS levels of dental plaque (Stecksén-Blicks et al. 2009, Marttinen et al. 2012). Decreased counts of MS in saliva do not necessarily mean a decrease in the caries risk. The microbiota of unstimulated whole saliva resembles that of the tongue more than of dental plaque (Mager et al. 2003). However, MS originate almost exclusively from dental plaque. Decreasing MS without affecting the microbial homeostasis of dental plaque should improve the microbiological composition of the plaque and make it less virulent.

Little information is available about the relationship between probiotic bifidobacteria and the oral microbiota. Four studies have demonstrated that specific probiotic bifidobacteria might reduce the counts of salivary MS. Caglar et al. (2005, 2008b) were the first to report the effect of bifidobacteria-derived probiotics on caries-associated bacteria. The aim of their first study was to examine whether short-term consumption of yoghurt containing *B. lactis* DN-173 010 (7×10^7 CFU/g) would affect the salivary levels of MS and lactobacilli in 21 young adults aged 21–24 years. A double-blind, randomized crossover study consisted of a run-in period (one week), an intervention period (two weeks), a washout period (four weeks), and the second intervention period (two weeks). The subjects consumed 200 g yoghurt per day with or without probiotic bacteria. A statistically significant reduction of salivary MS levels was observed following the

consumption of the test yoghurt in contrast to the control yoghurt. No significant alteration in the lactobacilli counts was measured (Caglar et al. 2005).

A similar effect with a comparable study design was reported when Caglar et al. (2008b) studied the effect of probiotic ice cream containing *B. lactis* BB-12 (1×10^7 CFU/g) in young adults. In this study, 24 healthy adults (mean age 20 years) ingested 53 g probiotic ice cream or a control ice cream once a day for 20 days. Salivary levels of MS decreased significantly after the consumption of the probiotic. The salivary levels of lactobacilli were unaltered (Caglar et al. 2008b).

In the study carried out by Cildir et al. (2009), 24 healthy children (12–16 years) undergoing orthodontic treatment received 200 g control yoghurt or yoghurt supplemented with *B. lactis* DN-173010 (2×10^8 CFU/g) once a day. The study design was a double-blind, randomized, crossover study, with two separate two-week intervention periods. A statistically significant reduction in salivary MS level was recorded after probiotic yoghurt consumption, while no changes were found after the control product intake. No significant alteration in the lactobacilli counts was observed (Cildir et al. 2009).

Recently, the effect of probiotic ice cream containing *B. lactis* BB-12 (ATCC27536) and *Lactobacillus acidophilus* La-5 was studied in 40 caries-free 12–14-year-old children (Singh et al. 2011). The probiotic ice cream weighing 54 g contained 1×10^6 CFU/g of each probiotic strain. Two separate intervention periods lasted for two weeks. The salivary levels of MS decreased significantly after consumption of probiotic ice cream. However, a decline in high MS counts was also evident after intake of control ice cream. The levels of lactobacilli were unaltered.

No studies have been reported on the effect of probiotic bifidobacteria on the occurrence of caries. However, two studies have investigated the effect of administration of probiotic lactobacilli on caries occurrence. In a Finnish study, 594 one to six-year-old children attending a day-care centre received *L. rhamnosus* GG-containing (10^6 CFU/mL) milk five days a week for seven months. The milk use reduced dental caries significantly in the three to four-year-old children (Näse et al. 2001). In a recent Swedish study, 248 one to five-year-old preschool children received for lunch 150 mL either standard control milk or test milk supplemented with both fluoride (2.5 mg/L) and *L. rhamnosus* LB21 (10^7 CFU/mL) for 21 months (Stecksén-Blicks et al. 2009). Caries occurrence figures were significantly lower in the test group, but as the authors themselves state, it was impossible to differentiate the role of the probiotic organism and fluoride in the result. These findings suggest that specific probiotic lactobacilli may promote dental health.

Probiotic bacteria originally planned for gut health may be beneficial rather than hazardous to dental health. New probiotics are emerging and research groups have identified resident bacteria in the oral microbiota of healthy subjects as potential probiotics. However, the number of studies concerning probiotic bifidobacteria and oral health is limited. It is not known which bifidobacteria could have possible probiotic properties in the oral cavity. It is clear that bifidobacteria are aciduric and acidogenic, and at least some bifidobacteria used in various probiotic products may transiently colonize the oral cavity during the time they are in active use. Thus, more clinical studies are needed in order to understand the effects of probiotic bifidobacteria on oral health.

2.3 Clinical effects of probiotic bifidobacteria in children

The effect of probiotic bacteria in the prevention and treatment of infectious diseases and allergies in children has been studied extensively in recent years. The most widely investigated species belong to the genera *Lactobacillus* and *Bifidobacterium*. In childhood, particularly *L. rhamnosus* GG seems to be effective in the treatment of infectious diarrhea, in the prevention of antibiotic-associated diarrhea, and in the modulation of immune response (Floch et al. 2008). Moreover, *L. rhamnosus* GG has been successfully used in a combination with *B. lactis* BB-12 in the prevention and treatment of atopic eczema associated with cow's milk allergy (Isolauri and Salminen 2008).

2.3.1 *B. lactis* BB-12 and general health

Members of the genus *Bifidobacterium* are used globally as probiotics in many food products such as yoghurt, milk, infant formula, cheese, and dietary supplements. The most thoroughly studied *Bifidobacterium* strain is *B. lactis* BB-12 (Masco et al. 2004, Garrigues et al. 2010). The efficacy of *B. lactis* BB-12 in children is most comprehensively documented in the prevention and treatment of diarrheal and respiratory infections, which are reviewed in Section 2.3.2. *B. lactis* BB-12 has also been used in preterm infants in the establishment of a healthy microbiota. Supplementation with *B. lactis* BB-12 increased the number of bifidobacteria, while reducing the numbers of enterobacteria and clostridia (Mohan et al. 2006). In neonates, the use of a probiotic mixture containing *B. lactis* BB-12 has been shown to reduce both the incidence and severity of necrotizing enterocolitis (Bin-Nun et al. 2005). Furthermore, *B. lactis* BB-12 alone or in combination with *L. rhamnosus* GG has been successfully used in the prevention and treatment of allergic disorders, such as atopic eczema (Isolauri et al. 2000).

Bifidobacteria have been considered to be important bacteria in the development of the immune system in early childhood. Bifidobacteria are the predominant species in

breast-fed infants, whereas formula-fed infants harbour a more complex gut microbiota (Harmsen et al. 2000, Turroni et al. 2012). Moreover, breast-fed infants generally harbour a more diverse *Bifidobacterium* population compared to formula-fed infants (Roger et al. 2010). Breast milk contains secretory immunoglobulin A (IgA), which plays a central role in local immunity and has a significant function in creating a barrier against infections by pathogenic bacteria or viruses. *B. lactis* BB-12 has been observed to increase the faecal levels of total IgA during intake of the formula in healthy, weaned infants (Fukushima et al. 1998). In addition, *B. lactis* BB-12 supplementation in combination with *L. rhamnosus* GG has been shown to increase protective cow's milk-specific IgA responses at the time of introduction of cow's milk to the infant's diet (Rautava et al. 2006).

An important property of probiotic bifidobacteria is their ability to survive passage through the gastrointestinal tract. Faecal recovery during the oral administration of a probiotic strain is a standard method of showing survival in the gastrointestinal tract (Saxelin et al. 2010). *B. lactis* BB-12 has been shown to survive in the intestinal passage in a dose-dependent manner (Larsen et al. 2006). Faecal recovery does not necessarily mean that bacteria are able to adhere to the intestinal mucus. *B. breve*, *B. bifidum*, and *B. longum* are considered to be indigenous or mucosa-adherent bifidobacteria that can be cultured from intestinal as well as faecal samples in early infancy (Turroni et al. 2009). Studies in infants have demonstrated that *B. lactis* BB-12 is able to persist in the gastrointestinal tract only transiently so that, after intervention, the levels of bacteria decrease rapidly (Fukushima et al. 1998). However, little is known about the colonization of intestinal gut mucosa when probiotic bifidobacteria are administered on a daily basis in early childhood.

2.3.2 Prevention and treatment of common infectious diseases

2.3.2.1 Diarrhea

A recent consensus statement on the use of probiotics recommends that specific probiotic strains, including *Saccharomyces boulardii*, *L. rhamnosus* GG, and *L. reuteri* SD2112, are effective in the treatment of childhood infectious diarrhea (Floch et al. 2008). A few studies have also examined the role of bifidobacteria in the prevention and treatment of acute infectious diarrhea (Table 1) and antibiotic-associated diarrhea in infancy. The rationale for using probiotic bacteria in diarrhea is based on the assumption that they balance and modify the gut microbiota and act against intestinal pathogens (Szajewska and Mrukowicz 2001, Hatakka and Saxelin 2008).

Most clinical studies have examined the preventive effect of bifidobacteria on the occurrence of community-acquired diarrhea among healthy children in day-care centres.

Only one study has been conducted among hospitalized children (Saavedra et al. 1994). In that study, a statistically significant decrease in the incidence of diarrheal episodes was observed in the probiotic group compared to the control group (7% vs. 31%). Furthermore, the shedding of rotavirus was significantly lower in those receiving the probiotic-supplemented formula.

Community-based intervention studies among healthy children have demonstrated that specific probiotic bifidobacteria or a probiotic mixture can reduce the incidence and duration of diarrheal episodes. In the study by Weizman et al. (2005), *B. lactis* BB-12-supplemented infant formula significantly reduced the number of days and number of episodes with diarrhea in healthy infants aged 4–10 months. A probiotic mixture containing bifidobacteria significantly reduced gastrointestinal diseases in preschool children (Lin et al. 2009). Contradictory results have also been reported. Two studies conducted in a French population could not show any beneficial effect of an infant formula containing bifidobacteria on diarrheal episodes (Chouraqui et al. 2004, Thibault et al. 2004). Also in a Finnish study, an infant formula supplemented with probiotics did not reduce the incidence of early or recurrent gastrointestinal infections during the first year of life (Rautava et al. 2009).

Probiotic bacteria have also been shown to reduce the risk of antibiotic-associated diarrhea in children. A previous meta-analysis of six randomized, placebo-controlled clinical trials stated that treatment with probiotics compared with placebo reduced the risk of diarrhea in children treated with antibiotics from 28.5% to 12.0% (Szajewska et al. 2006). In a study carried out in Brazil, the combination of *B. lactis* and *Streptococcus thermophilus* significantly reduced the incidence of antibiotic-associated diarrhea in infants, 6 to 36 months of age (Corrêa et al. 2005).

In summary, these intervention studies demonstrated that specific probiotic bifidobacteria might be beneficial in the treatment of infectious diarrheal diseases in children, especially in hospitalized, high-risk pediatric patients. However, community-based studies in healthy infants provide only moderate or weak evidence on the efficacy of bifidobacteria in infectious diarrhea. The data concerning the effect of bifidobacteria against antibiotic-associated diarrhea are only limited.

2.3.2.2 Respiratory infections

Increasing evidence suggests that specific probiotic bacteria may also reduce infections outside the gastrointestinal tract. Recent studies have demonstrated that probiotic supplementation in infancy may be more beneficial in viral infections than in bacterial infections (Guandalini 2011). In many cases, respiratory infections are viral in origin,

and the mucosal surfaces of the respiratory tract may be an appropriate area for probiotic immunostimulation (Hatakka and Saxelin 2008). To date, seven randomized, placebo-controlled studies have been published concerning the effect of probiotic bifidobacteria on the prevention and treatment of respiratory infections in infants or preschool children (Table 2). These studies have been community-based studies among healthy children, conducted all over the world.

Administration of *B. lactis* Bi-07 in combination with *L. acidophilus* NCFM significantly reduced the incidence and duration of fever, coughing, and rhinorrhea in Chinese children (Leyer et al. 2009). A similar effect on respiratory illnesses was reported in India, where milk containing *B. lactis* HN019 reduced the incidence of pneumonia and acute lower respiratory infections in children (Sazawal et al. 2010). Two studies in the Finnish population showed promising results concerning the effect of bifidobacteria against recurrent respiratory infections (Hatakka et al. 2007, Rautava et al. 2009).

In contrast, three studies were unable to find any beneficial effect of probiotic bifidobacteria on respiratory infections. Administration of *B. lactis* BB-12 did not reduce the occurrence or duration of acute respiratory illnesses in young children in Israel (Weizman et al. 2005). In that study, significantly fewer febrile episodes were, however, observed in the probiotic group. No positive effect of bifidobacteria on respiratory illnesses were found in Taiwanese children when three commercial multiple probiotic products were compared in the treatment and prevention of pediatric infectious diseases (Lin et al. 2009). Furthermore, in the USA, a probiotic-containing yoghurt-based drink supplemented with *B. lactis* BB-12 did not reduce self-reported illnesses in children, including the presence of respiratory infection (Merenstein et al. 2010).

To sum up, there are clinical data suggesting that some probiotic bifidobacteria may reduce the occurrence, recurrence, and duration of respiratory infections in healthy children. However, most studies have been conducted with different genera of probiotics; hence it is difficult to separate the effect of bifidobacteria and lactobacilli in the result. In addition, geographical location and environmental conditions may affect the outcome. The mechanisms by which probiotic bifidobacteria can exert their protective effect in the respiratory tract are partly unknown. Specific probiotic strains have been shown to modulate innate and adaptive immune responses (Russell et al. 2011). Probiotic bifidobacteria may also suppress the growth of the pathogens locally (Hatakka and Saxelin 2008).

Table 1. Randomised, double-blind, placebo-controlled studies on the effect of bifidobacteria on acute diarrheal infections in children.

Intervention	Duration, vehicle	Subjects	Main result	Authors
B. lactis BB-12 (1.9×10^8 CFU/g) and *S. thermophilus* TH-4 (1.4×10^7 CFU/g)	2.5 months IF	Hospitalized infants, aged 5–24 months (n=55)	Incidence of diarrhea ↓ Duration of diarrhea ↔ Rotavirus shedding ↓	Saavedra et al. 1994
B. breve and *S. thermophilus* (dose not known)	5 months IF	Pediatric centres, aged 4–6 months (n=971)	Incidence of diarrhea ↔ Duration of diarrhea ↔ Need for dehydration ↓	Thibault et al. 2004
B. lactis BB-12 (10^8 CFU/day)	4.5 months IF	Residential care setting, aged <8 months (n=90)	Incidence of diarrhea ↔ Duration of diarrhea (↓)	Chouraqui et al. 2004
B. lactis BB-12 or *L. reuteri* (55730) (1.2×10^9 CFU/day)	3 months IF	Day-care centres, aged 4–10 months (n=201)	Incidence of diarrhea BB-12 (↓), *L. reuteri* ↓ Duration of diarrhea ↓	Weizman et al. 2005
L. rhamnosus GG and *B. lactis* BB-12 (10^{10} CFU/day)	12 months IF	Community-based,aged 0–12 months (n=81)	Incidence of diarrhea ↔	Rautava et al. 2009
L. casei rhamnosus or *L. rhamnosus* T cell-1 or multiple probiotics	7 months capsules	Preschool, 4–5 years (n=1062)	Incidence of diarrhea in multiple probiotic group ↓	Lin et al. 2009
B. lactis HN019 (1.9×10^7 CFU/g) and prebiotic olicosaccharides	12 months milk	Community-based, aged 1–4 years (n=624)	Incidence of diarrhea ↔	Sazawal et al. 2010

↓=decreased risk ($p<0.05$), (↓)=marginally decreased risk ($0.05<p<0.20$), ↔=no effect, IF=infant formula

Table 2. Randomized, double-blind, placebo-controlled studies on the effect of bifidobacteria on the prevention and treatment of respiratory illnesses in children.

Intervention	Duration, vehicle	Subjects	Main result	Authors
B. lactis BB-12 or *L. reuteri* (55730) (1.2×10^9 CFU/day)	3 months infant formula	4–10 months (n=201)	Occurrence of ARI ↔ Duration of ARI ↔ Days with fever ↓ Episodes of fever ↓	Weizman et al. 2005
L. rhamnosus GG and LC705 and *B. breve* 99 and *Propionibacterium freudenreichii* ($8–9 \times 10^9$ CFU/capsule)	5 months capsules	10 months – 6 years (n=309)	Occurrence of AOM ↔ Occurrence of ARI ↔ Occurrence of recurrent ARI (↓)	Hatakka et al. 2007
L. rhamnosus GG and *B. lactis* BB-12 (10^{10} CFU/day)	12 months infant formula	0–12 months (n=81)	Incidence of AOM ↓ Recurrence of ARI ↓	Rautava et al. 2009
L. casei rhamnosus or *L. rhamnosus* T cell-1 or multiple probiotics	7 months capsules	4–5 years (n=1062)	Occurrence of ARI ↔ in multiple probiotics group	Lin et al. 2009
L. acidophilus NCFM or *L. acidophilus* NCFM and *B. lactis* Bi-07 (10^{10} CFU/day)	6 months milk	3–5 years (n=326)	Symptoms of ARI ↓ Duration of ARI ↓	Leyer et al. 2009
B. lactis HN019 (1.9×10^7 CFU/day) and prebiotic olicosaccharides	12 months milk	1–4 years (n=624)	Incidence of ARI ↓ Incidence of pneumonia ↓ Days with fever ↓	Sazawal et al. 2010
B. lactis BB-12 (10^{10} CFU/day)	90 days yoghurt	1–3 years (n=182)	Symptoms of ARI ↔ Absence from daycare ↔	Merenstein et al. 2010

↓=decreased risk ($p<0.05$), (↓)=marginally decreased risk ($0.05<p<0.20$), ↔=no effect, ARI=acute respiratory infection, AOM=acute otitis media

2.4 Safety of bifidobacteria

The growing market and increasing use of products containing live probiotic bacteria have raised the question of their possible health risks. In theory, live bacteria cannot be considered totally safe. Probiotic bacteria may be responsible for four types of side effects consisting of infection, deleterious metabolic activity, excessive immune stimulation, and gene transfer (Marteau and Seksik 2004). Bifidobacteria are considered to be very safe since no cases of a probiotic bifidobacteria having caused an infection have been reported. Also indigenous bifidobacteria, which belong to the resident microbiota of the gastrointestinal tract, very rarely cause infections (Saarela et al. 2000). In addition, probiotic bifidobacteria have not been shown to cause any harmful enzymatic activity or unwanted immunomodulation (Saarela et al. 2007). However, the tetracycline resistance gene tet(W) has been found in *B. lactis* (Gueimonde et al. 2010).

In many countries a wide range of *Bifidobacterium* species are recommended to be given to infants. In early childhood, the gut microbiota and associated immune system have not fully developed, and the infant does not receive the probiotics in the context of a stable and established microbiota. Several long-term intervention studies concerning the safety and tolerance of consumption of bifidobacteria have been performed in children, and even in preterm infants (Chouraqui et al. 2004, Saavedra et al. 2004, Bin-Nun et al. 2005, Mohan et al. 2006, Weizman ans Alsheikh 2006, Dekker et al. 2009, Allen et al. 2010). These studies have shown that the administration of specific strains of bifidobacteria, i.e. *B. bifidum* CUL20, *B. longum* BL999, *B. lactis* BB-12, *B. lactis* HN019, and *B. lactis* CUL34 is safe and well tolerated. These *Bifidobacterium* strains have also the qualified presumption of safety (QPS) status of the European Food Safety Authority, and they are generally recognized as safe (GRAS) by the FDA in the US. (FDA 2002, EFSA 2009).

From an oral health perspective, safety issues connected with the use of bifidobacteria are difficult to predict. Adhesion of bacteria to the surfaces of the oral cavity is a key factor in the selection of oral probiotic strain but it could also be a risk factor in the progression of caries (Teughels et al. 2008). On the one hand, bifidobacteria are highly aciduric and acidogenic bacteria and frequently found in deep caries lesions but not from sound tooth surfaces (Becker et al. 2002, Aas et al. 2008, Mantzourani et al. 2009, Nakajo et al. 2010). Bifidobacteria have also been isolated from infected root canals (Chávez de Paz et al. 2004). Thus, resident oral bifidobacteria are able to colonize the carious dentin if the local environmental conditions, i.e. poor oral hygiene and frequent intake of cariogenic foods, favour the growth of bifidobacteria. On the other hand, specific strains of probiotic bifidobacteria have been shown to reduce the salivary levels of mutans streptococci in children (Caglar et al. 2005, 2008b, Cildir et al. 2009, Singh et al. 2011). All the subjects in those four studies were caries-free, but the possibility remains that if the children had dental decay the bifidobacteria might have colonized the lesions. So far, no studies are available concerning the administration effect of probiotic bifidobacteria on the occurrence of caries in early childhood.

3. AIMS OF THE STUDY

The aim of the study was to test a food supplement containing xylitol and a probiotic organism, *B. lactis* BB-12, and to investigate its health effects, properties and safety when used in a novel pacifier in early childhood. The specific objectives were:

1. To monitor the pattern of xylitol release and salivary xylitol concentrations during sucking of a slow-release pacifier **(I)**. The hypothesis was that salivary xylitol concentrations inhibitory to mutans streptococci (MS) would be reached during sucking, and that the presence of *B. lactis* BB-12 in the tablet would not affect the pattern of xylitol release.

2. To characterize the effect of early administration of *B. lactis* BB-12 and xylitol on oral colonization by MS and the probiotic in children **(II)**. Sorbitol was used as a control since it is considered inert from a dental point of view. We hypothesized that *B. lactis* BB-12 might reduce the colonization by MS, and that *B. lactis* BB-12 would show poor colonization in the mouths of the children.

3. To evaluate the effect of early administration of *B. lactis* BB-12, xylitol, and the sorbitol control on caries occurrence **(III)**. The hypothesis was that *B. lactis* BB-12 would not increase the caries risk of the children by age four.

4. To identify markers of dental caries in early childhood in a Finnish population with good dental health **(III)**. We hypothesized that the early colonization of MS and visible plaque in the dentition would associate with the occurrence of caries in young children.

5. To investigate the safety and efficacy of *B. lactis* BB-12 in reducing the risk of acute infectious diseases in infants **(IV)**. We hypothesized that *B. lactis* BB-12 could reduce the risk of acute infectious diseases in infancy.

6. To study the feasibility of the novel administration method. The hypothesis was that the novel method would be practical and acceptable by the parents.

4. MATERIALS AND METHODS

4.1 Subjects and study designs

The characterization of the study designs, subjects and interventions are shown in Table 3. The clinical trial profile for studies II, III, and IV is presented in Figure 3.

Study I was performed in adults in order to obtain optimal cooperation and to be able to standardize the saliva collection. Studies II, III, and IV were based on a randomized, double-blind, placebo-controlled, intervention study conducted in newborn infants. Studies II and III consisted of three, and study IV, of two parallel groups. A part of the parents participating in the clinical trial evaluated the feasibility of the novel administration method with a structured questionnaire (Taipale et al. IADR/CED abstract 0502, 2007).

In study I, 10 women, belonging to the personnel of the Institute of Dentistry in the University of Turku, volunteered for the study in 2006. These test subjects did not take part in the clinical trial. The inclusion criteria for the study were that subjects were healthy and showed salivary flow rates exceeding 1 mL/min. In studies II, III, and IV, the families were recruited for the clinical trial from Muurame and Korpilahti, in Central Finland, between September 2004 and February 2007. During the recruitment period a total of 479 pregnant mothers received an information leaflet at well-baby clinics. After delivery, 163 families were willing to participate in the clinical trial. The inclusion criteria for the trial were: (1) the child was healthy, (2) the parents were willing to use the novel slow-release pacifier and the tablet, and (3) the child started to receive the tablet before the age of two months. In cases where the child did not start using the pacifier before the age of two months but the parents were motivated to remain in the trial, they were offered the possibility of delivering the crushed tablet to the child using a spoon. In the feasibility study, the families filled in a structured questionnaire on the novel administration method after the child had used the pacifier for one month.

In studies II and III, the required sample size was based on earlier studies examining MS transmission from mother to child (Berkowitz et al. 1981, Söderling et al. 2000, Gripp and Schlagenhauf 2002). In study IV, sample size was calculated on the basis of a previous study examining the incidence of acute otitis media and respiratory infections during the first year of life (Vesa et al. 2001). Estimated sample sizes were increased by 25–30% in order to compensate for the proportion of dropouts.

Table 3. Characterization of the study design and subjects.

Study	Design Subjects	Study groups	Number starting/ completing study (%)	Follow-up
I	Pilot Adults	1. BB-12 + XYL 2. XYL	10/10 (100%)	–
II	RDBPC Children	1. BB-12 + XYL 2. XYL 3. SOR	106/96 (91%)	2 years
III	RDBPC Children	1. BB-12 + XYL 2. XYL 3. SOR	106/94 (89%)	4 years
IV	RDBPC Children	1. BB-12 + XYL 2. XYL	75/69 (92%)	8 months

RDBPC=randomized, double-blind, placebo-controlled; BB-12=*Bifidobacterium animalis* subsp. *lactis* BB-12; XYL=xylitol; SOR=sorbitol

4.2 Ethical aspects

The Ethics Committee of the Hospital District of Southwest Finland approved the clinical studies. Written informed consent was obtained from adult subjects (I), or from the infants parents (II, III, and IV). All data were treated confidentially. The probiotic strain used has well-documented safety properties in infant studies and in food products. The bulk agents of the test tablets, xylitol and sorbitol, are considered to be safe for dental health because they cannot be utilized by the microorganisms in dental plaque. No laxative effects were expected to occur with small daily doses (200–600 mg) of sugar alcohols. Most Finnish children use a pacifier. Pacifier use has been thought to shorten the duration of exclusive breastfeeding. In our clinical trial, the families were not pressured to use a pacifier, and breastfeeding was vigorously supported.

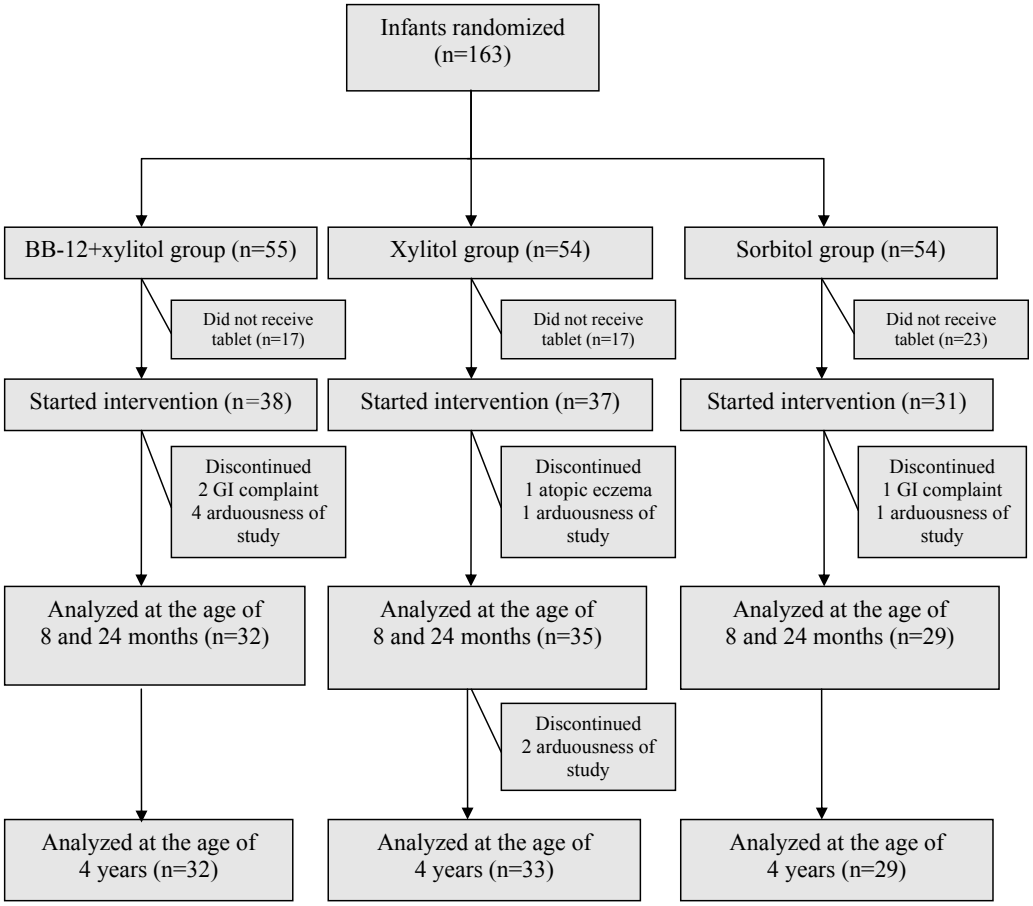

Figure 3. Clinical trial profile. GI=Gastrointestinal.

4.3 Test tablets and their administration

In studies II, III, and IV, test tablets were administered from the age of 1–2 months with a novel slow-release pacifier (Alanen and Söderling, U.S. Patent 6.203.566, 2000) or dissolved in water with a spoon. The pacifier contained a pouch in which the tablets were inserted (Figure 4). The children received the tablets twice a day via a small pacifier (volume 120 μL) until 6–8 months of age, thereafter via a larger pacifier (volume 250 μL) until the age of two years. In study I, only the larger pacifier was used in adults. The pacifiers were manufactured by Plastone, Konnevesi, Finland and donated to the study.

Figure 4. The novel slow-release pacifier with a pouch.

In all studies, the probiotic tablet contained 5×10^9 CFU of *B. lactis* BB-12 (DSM 15 954; Chr. Hansen A/S, Hoersholm, Denmark) in addition to bulk agent xylitol. In study I, the adult test subjects received, in random order, the probiotic tablet containing 300 mg of xylitol and the reference xylitol (300 mg) tablet (Danisco Limited, Kotka, Finland). In studies II and III, the infants were randomized into three groups receiving probiotic bacteria in addition to xylitol (100 mg or 300 mg), xylitol (100 mg or 300 mg), or sorbitol control (100 mg or 300 mg) according to the size of the pacifier. Since study IV investigated only children's infectious diseases during the first eight months of life, the sorbitol control group was not used as a control group in it. Thus, in study IV, only the probiotic group and the xylitol control group were compared.

All tablets were manufactured by Oy Karl Fazer Ab (Vantaa, Finland) and packed in white plastic bottles with colour codes. Freshly made tablets were delivered to the families biannually and stored in a refrigerator. The families received tablets and pacifiers until the child turned two. If the families wanted to use a regular pacifier as well, they were offered the pouch-free version of the slow-release pacifier (Alanen and Varrela, US patent 5.922.010, 1999).

4.4 Data collection

In the clinical trial (II, III, and IV), the infants and the parents visited the municipal health care centres at the age of one month, eight months, two years, and four years. At these appointments, a trained professional, a dental nurse or a dental hygienist, interviewed the guardian using structured questionnaires validated in earlier studies (Kilpi et al. 2002).

At the one-month visit, the parents were interviewed for background information on the birth and the family, as well as on breastfeeding and possible complementary feeding. The guardians reported diseases in parents and siblings, parents' smoking habits, and special diets. At this visit, the parents also received detailed instructions on the use of study diaries concerning the nutrition of the child, the health of the child, and the use of medications.

At the eight-month visit (IV), the parents were interviewed for information on breastfeeding status, use of the tablet and the pacifier, use of complementary foods in the infants diet, dairy products, and products containing probiotics. All infections and other health problems during the first eight months of the children's life were recorded in special diaries by the parents. The occurrence of respiratory infections, gastrointestinal disorders, doctor-diagnosed acute otitis media, and antibiotic treatments were recorded in detail. The symptoms of respiratory infections included runny nose, nasal congestion, cough, and shortness of breath. Respiratory infection was diagnosed when the child had at least two infectious symptoms during one day or one symptom during two consecutive days. Parents also reported respiratory infections with complications (bronchitis, pneumonia, and sinusitis) diagnosed by a doctor (municipal or private) unrelated to the study. The AOM occurrences included all doctor-diagnosed acute ear infections. GI infections included every episode with watery diarrhea or vomiting. Fever episodes included elevated body temperatures (37˚C) lasting for at least one day. Parents also recorded any occurrence of atopic diseases or allergic sensitisations. All adverse effects were recorded in detail.

At the two-year visit (II), similar interviews concerning breastfeeding, use of test tablets and children's diet, were performed. In addition, the oral hygiene habits and the use of fluoride and xylitol were recorded in detail.

At the age of four years, the parents were interviewed about the children's oral health-related variables in detail (III). For the sake of this visit, the parents also kept a three-day food diary on the child. In addition, a separate questionnaire was filled in concerning the consumption of sweet snacks and sweet drinks.

One dental nurse and one dental hygienist carried out the data collection in Korpilahti and Muurame health care centre. The research nurses had a long history and experience

of children's health education and caries prevention, and they were additionally trained for the purposes of the present trial. The training consisted of study meetings bimonthly (20 hours) with the dentist (TT) and five rehearsal sessions (10 hours) in the University of Turku with the supervisors (ES and KP).

4.5 Analytical methods

4.5.1 Oral microbiological sample collections

Oral microbial samples were collected from the children at the age of eight months and two years for *B. lactis* BB-12 determination (qPCR) and plate culturing of mutans streptococci (MSB, TYCSB), lactobacilli (Rogosa), total facultatives (blood agar), and yeasts (Sabouraud) (II). At the age of four years, children's MS levels were determined using the Dentocult® SM Strip mutans test (Orion Diagnostica, Espoo, Finland) (III). At the beginning of the clinical trial, MS levels of the mothers were also determined using the Strip test (II). For all studies, the dental biofilm samples were collected from proximal spaces of the maxillar and mandibular incisors and molars in a standardized manner, using microbrushes (Quick-Stick, Dentsolv AB, Huddinge, Sweden) (II) or dental floss (Plackers®, Lidingö, Sweden) (III). The oral mucosal samples were collected from buccal sulcus of the mandible using cotton swabs (II). The ends of the microbrushes/swabs were cut with sterile scissors into transport tubes containing 1 mL tryptic soy broth (Difco, Detroit, Michigan, USA) with 10% glycerol v/v. The tubes were stored at –20˚C for a maximum of two months, transported on dry ice to the Institute of Dentistry, Turku, and stored there for a maximum of three months at –70˚C before microbiological analysis. The strips were analyzed in Korpilahti and Muurame.

4.5.1.1 Determination of mutans streptococci, lactobacilli and yeasts (II)

The plate-culturing of microbes was performed as follows. After 10-fold serial dilutions, the samples were plated on Mitis salivarius agars (Difco, Detroit, MI, USA) containing sucrose and bacitracin (MSB; Gold et al. 1973), TYC/LAB 35 agars (Lab M Ltd, Lancashire, UK) containing sucrose and bacitracin (TYCSB; Van Palenstein Helderman et al. 1983), Rogosa agars (Difco), and blood agars (Orion Diagnostica, Espoo, Finland), as well as Sabouraud and Nickerson agars (Difco). The MS grown on the MSB agar were incubated for 3 days in a 7% CO_2 atmosphere, and the TYCSB agars anaerobically for 3 days at +37˚C. The numbers of MS were identified on the basis of colony morphology and counted by means of a stereomicroscope. The identification of *S. mutans* was based on consistent findings of "rough" colony morphology on the MSB plate, positive fermentation with sorbitol, mannitol, raffinose, and melibiose, and negative dextran

agglutination. Identification of *S. sobrinus* was based on "smooth" colonies on the MSB plate, positive fermentation with mannitol but negative with raffinose, and melibiose, and positive dextran agglutination. Aciduric microbiota including lactobacilli were grown on Rogosa agars for 3 days and total facultatives on blood agars anaerobically for 3–4 days at +37˚C. Yeasts were grown on both Sabouraud and Nickerson agars aerobically for 2–3 days and the identification of yeast colonies was made on the basis of colony morphology and colour. All oral microbial samples contained counts of total facultative bacteria of approximately log CFU 6 or more. Thus, microbial sample collection can be considered to have been successful.

4.5.1.2 Chairside-assay of mutans streptococci (III)

The strips of the Dentocult® SM Strip mutans test were incubated in their vials at 35–37˚C for 48– 72 hours. Trained research nurses in Korpilahti and Muurame evaluated the dried strips with the naked eye according to the manufacturer's classification chart. The strip was considered positive if typical round and spherical MS colonies were detected. The positive strips were further categorized into three classes: 1 ($<10^5$ CFU/mL), 2 (10^5–10^6 CFU/mL), and 3 ($>10^6$ CFU/mL). The strip was considered negative if no MS colonies were detected: 0 ($<10^4$ CFU/mL). All strips were double-checked by the dentist (TT) together with the research nurses.

4.5.2 Determination of *B. lactis* BB-12 from oral samples (II)

In study II, *B. lactis* BB-12 was detected from the oral microbial samples using quantitative and qualitative PCR analyses. Total DNA was extracted from the samples using the QiaAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Quantitative PCRs (qPCR) were conducted as described by Collado et al. (2008). The specific primers were used to detect the *B. animalis* group (Ventura and Zink 2002). Real-time PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, Calif., USA). Spiking samples with known amounts of *B. lactis* BB-12 showed that the extraction efficiency and detection limit was 10^4 CFU/g. Because of the low numbers of *B. lactis* BB-12 found in the samples, the results were confirmed with conventional qualitative PCR analyses, described in detail by Rinne et al. (2005). Amplification of the DNA was performed using the Veriti 96-well Thermal Cycler (Applied Biosystems). Amplified products were subjected to gel electrophoresis in 1% agarose gel, and visualized by ethidium bromide staining. *B. lactis* BB-12 was used as a positive control. The determinations were performed in the Functional Foods Forum, University of Turku, Finland.

4.5.3 Determination of *B. lactis* BB-12 in faeces (IV)

In study IV, faecal samples were collected from the children at the age of eight months. The faecal samples were stored at the health care centres at –20˚C for up to two months and then delivered on dry ice to the University of Turku. In the University of Turku, the samples were stored at –70˚C before transportation on dry ice to Denmark. *B. lactis* BB-12 DNA was quantified by a *B. animalis* subsp. *lactis*-specific quantitative PCR assay based on partial bifidobacterial 23S ribosomal DNA sequences. Total DNA was extracted from 200 mg of faeces (QiaAmp DNA Stool Mini Kit), mixed with 1mL ASL buffer and 0.1 mm zirconia beads, and treated for 5 minutes in a Mini-Beadbeater (BioSpec, Bartlesville, OK, USA) according to the instructions of the manufacturer. Extraction efficiency and detection limit were determined to be 5% and 10^5 CFU/g. Faecal analyses were performed in the laboratory of Chr. Hansen A/S Hoersholm, Denmark.

4.5.4 Determination of xylitol concentrations of saliva (I)

In study I, the xylitol concentrations of saliva were determined during sucking the pacifier and test tablets. The pacifier was taken from the mouth at 2.5 minutes intervals and a small saliva sample was spat through a funnel into a test tube on ice. The sample collections were repeated at 2.5 minutes intervals until most of the tablet had dissolved. The xylitol concentrations of the saliva samples were assessed using the kit by Boehringer (R-Biopharm AG, Darmstadt, Germany). All assays were completed in triplicate. Analyses were performed in the Institute of Dentistry, University of Turku, Finland.

4.6 Clinical examination

In study II, the oral health status of the children was registered at the age of two years according to the schedule of the Korpilahti-Muurame public health care centre prevention programme. The examination was carried out, in a dental chair with operating light using a mouth mirror and air syringe, the caretaker holding the child. Dental caries was recorded using idmfs index (i=incipient carious lesion, d=a dentinal or pulpal carious lesion, m=missing due to caries, f=filled, and s=tooth surface). The presence of visible plaque accumulation was also registered. One dental nurse and one dental hygienist carried out the clinical examinations at the two-year visit in Korpilahti and Muurame.

In study III, the clinical examination at the age of four years consisted of a dental examination and assessment of oral hygiene status. One dentist (TT) examined all children in Korpilahti and Muurame. The examination was performed in a dental chair with operating light using a mouth mirror, air syringe, WHO periodontal probe, and fibre-optic transillumination (FOTI). Radiographs were not included in the examinations.

Before dental assessment, the presence of visible plaque accumulation was registered on a 0–2 scale (0=no plaque, 1=some visible plaque, 2=plenty of visible plaque). Visual and tactile detection of dental caries followed the International Caries Detection and Assessment System (ICDAS) described in detail by Ismail et al. (2007). The clinical status of each tooth surface was classified on an ordinal scale (from 0 to 6 depending on the severity of the lesion) and marked on an ICDAS recording sheet. The activity of the carious lesion was determined to be active or inactive. For the trial, dmf indices were also recorded. Before the clinical trial, the examiner trained for two days using the ICDAS interactive e-learning package (www.icdas.org) and literature related to ICDAS. After non-clinical training, five children aged four to eight years and presenting carious lesions were recruited for the assessment of inter-examiner reliability conducted by a senior examiner (KP). Immediately after these examinations all findings were compared and discussed. Due to the length of the data collection phase, the ICDAS e-learning programme and the ICDAS quiz were repeated every six months. Furthermore, intra-examiner reliability was assessed by recalling five children for caries examination.

4.7 Outcome measures

The primary and secondary outcome measures are presented in Table 4. In studies II, III, and IV the pre-specified outcome measures were defined prior to starting the clinical trial.

Table 4. The primary and secondary outcome measures of the study.

Study	Primary outcome	Secondary outcome
I	Xylitol concentration in saliva	Pattern of xylitol release
II	MS colonization at the age of 2 years	*B. lactis* BB-12 presence in the oral samples at the age of 2 years
III	Caries occurrence at the age of 4 years	MS colonization at the age of 4 years
IV	Cumulative incidence of ARI and AOM at the age of 8 months	*B. lactis* BB-12 presence in the faecal samples at the age of 8 months

ARI=acute respiratory infection, AOM=acute otitis media

4.8 Statistical methods

Statistical analyses were performed using IBM SPSS Statistics versions 12.0–19.0 (IBM Inc., New York, USA). In the clinical trial (II, III, and IV), only those children who completed the study protocol were included in the analyses. In all studies, probability values of <0.05 were considered statistically significant.

In study I, the time effect, differences between salivary collection periods, and the effect of BB-12 on the xylitol concentration were tested with the Wilcoxon signed ranks test for repeated measurements. In studies II and III, the baseline characteristics and the differences between the groups were analyzed for statistical significance using the Pearson's chi-square for categorized/dichotomized variables and interval level variables using analysis of variance (ANOVA).

In study IV, baseline characteristics and the differences between the groups were tested using the Student's *t* test and the chi-square test. Differences between the intervention groups in the occurrence of reported illnesses (respiratory infections, AOM, fever, and GI infections) and the use of antibiotics were analyzed using the chi-square test, for original frequency as well as for dichotomized data (at least one). The risk ratio and its 95% CI were calculated to measure the group differences in relation to the cumulative incidence of the studied diseases.

5. RESULTS

5.1 Dissolution of xylitol from the food supplement (I)

In the pilot study with 10 adults, dissolution of the xylitol and xylitol-BB-12 tablet from the pacifier pouch was tested. *B. lactis* BB-12 did not affect the solubility of the food supplement. Both tablets dissolved, exhibiting salivary xylitol concentrations with no clear concentration peaks (p=0.139) (Figure 5). No statistically significant differences in the individual values at any collection time in relation to xylitol concentrations were detected (p=0.285). All subjects showed xylitol concentrations exceeding 10 mg/mL, that is 1% at least at one examination point. Both tablets dissolved in a similar way within 7.5–15 minutes.

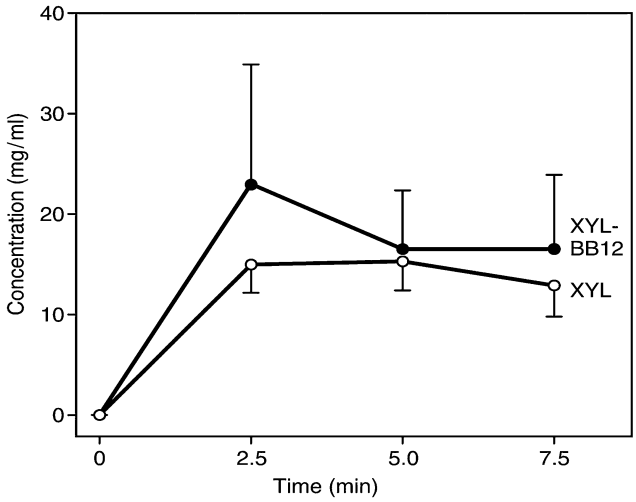

Figure 5. Xylitol concentrations (mg/mL saliva) of whole saliva samples collected during sucking of the slow-release pacifier. Number of subjects 10; curves show means±SE. (Taipale et al. 2007).

5.2 Feasibility of the novel administration method

In the substudy, the families participating in the clinical trial were interviewed for their opinions of the practicality and acceptability of the pacifier use (Taipale et al. 2007). Forty-seven families fulfilled the inclusion criteria. The hands-on instructions preceding the use of the pacifier were considered to be adequate by 96% (45/47) of the families. Of the families, 85% (40/47) reported that the insertion of the tablet into the pouch of the pacifier was easy (Figure 6). The recommended frequency of administration, twice a day, was considered acceptable by 85% (40/47) of the families (Figure 7). About half of

the families reported that they would discontinue their participation, if the administration frequency were raised to three times a day.

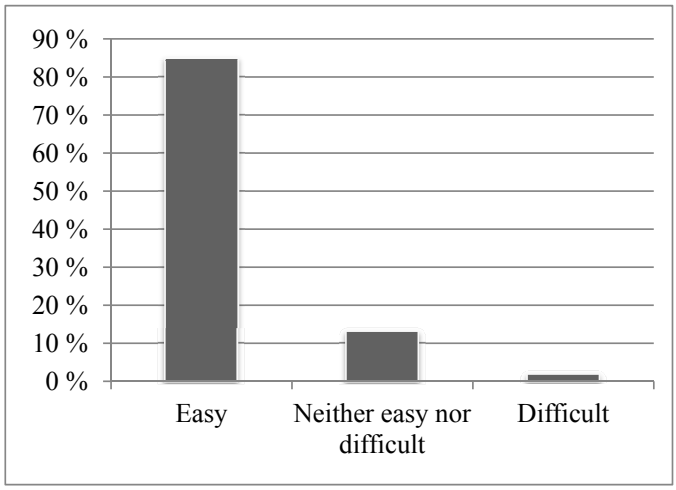

Figure 6. Proportions of reported answers to question: "How easy was the insertion of the food supplement tablet into the pouch of the pacifier?" Easy (85%), neither easy nor difficult (13%), difficult (2%).

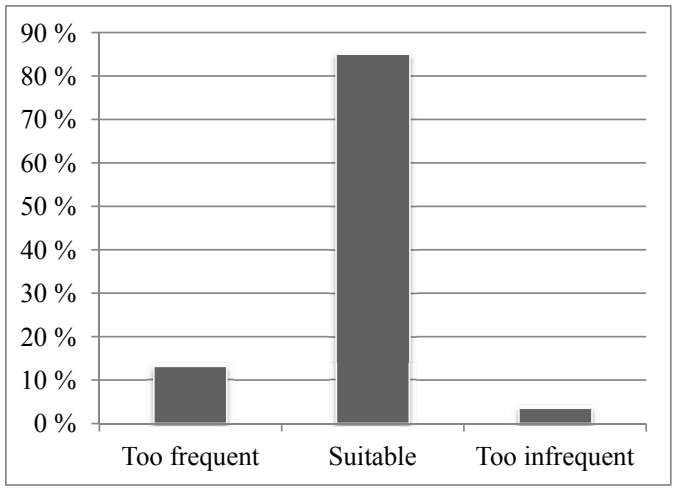

Figure 7. Proportions of reported answers to question: "How acceptable was the recommended frequency of tablet administration, two times per day?" Too frequent (13%), suitable (85%), too infrequent (2%).

5.3 Clinical pacifier trial: baseline characteristics (II–IV)

The baseline characteristics of the children participating in the clinical trial (II, III, and IV) are presented in Table 5. No significant differences were seen in the background

variables of the families between the intervention groups. In addition to the variables listed in Table 5, there were no differences between the groups in terms of the family health history, educational background of the parents, number of parents following special diets, or the proportion of children attending day-care centres. The use of antibiotics for treatment of infectious diseases in the children and the diet histories of the children in the three groups were both similar.

Table 5. Baseline characteristics of the families by group. Number and proportion (%) of subjects in relation to categorized variables and mean and standard deviation (SD) in relation to continuous variables.

	BB-12 (n=32)	XYLITOL (n=35)	SORBITOL (n=29)	p value
Boys, n (%)	16 (50)	20 (57)	13 (46)	0.838
Gestational age, weeks, mean (SD)	40 (1.1)	39.7 (1.2)	39.9 (1.0)	0.676[a]
Caesarian section, n (%)	11 (32)	7 (20)	5 (17)	0.324
Age of mothers, years, mean (SD)	31.4 (5.5)	30.5 (5.3)	32.3 (4.9)	0.389[a]
Primipara, n (%)	13 (38)	19 (54)	9 (31)	0.532
Exclusive breastfeeding, months, mean (SD)	3.4 (1.7)	3.8 (1.9)	3.9 (1.4)	0.386
Total breastfeeding, months, mean (SD)	9.4 (6.0)	9.6 (6.3)	9.3 (3.7)	0.397
Siblings, mean (SD)	1.1 (1.5)	1.1 (1.2)	1.3 (1.1)	0.877[a]
Maternal smoking, n (%)	4 (11)	7 (21)	3 (10)	0.427
Paternal smoking, n (%)	9 (26)	10 (29)	5 (17)	0.481
Maternal MS ≥105 CFU/mL, n (%)	31 (94)	32 (91)	28 (97)	0.571

[a] Statistical analysis with ANOVA.

5.4 Clinical effects of *B. lactis* BB-12 in the oral cavity (II, III)

Of those infants who fulfilled all inclusion criteria and started the intervention, 84% (32/38) in the BB-12 group, 89% (33/37) in the xylitol group, and 94% (29/31) in the sorbitol group completed the four-year follow-up. According to the questionnaires at the age of eight months and two years, 81% (26/32) of the children in the BB-12 group, 85% (28/33) in the xylitol group, and 83% (24/29) in the sorbitol group were reported to have received the test tablets twice a day, and the rest of the participants once a day. Mean duration of tablet delivery was 14.9±6.7 months and did not differ between the groups (p=0.780). At the beginning of the trial, 19% (20/106) of the infants started receiving the crushed test tablets with a spoon. Furthermore, many parents continued the delivery of tablets with a spoon after the child stopped using a pacifier.

5.4.1 Recovery of *B. lactis* BB-12 in oral samples (II)

In the clinical trial (II), the recovery of *B. lactis* BB-12 in dental plaque and oral mucosa samples was analyzed. Of the children, 22% (7/32) received the *B. lactis* BB-12 tablet until two years, 66% (21/32) until 8–19 months, and 12% (4/32) until less than eight months of age. During the intervention period in the eight-month samples, *B. lactis* BB-12 was recovered in 9% (3/32) of the children in the BB-12 group. At the end of the intervention period, none of the children in the BB-12 group harboured *B. lactis* BB-12 in their oral samples.

5.4.2 *B. lactis* BB-12 administration and MS colonization (II, III)

MS levels of the children were analyzed from the oral samples at the age of eight months, two years, and four years. MS colonization percentages at the age of two and four years are presented by study groups in Figure 8. At the age of eight months, only one child showed detectable counts of MS in dental plaque. At the age of two years (II), 6% (2/32) of the children in the BB-12 group, 31% (11/35) in the xylitol group, and 10% (3/29) in the sorbitol group harboured MS. The colonization percentage at the age of two years was significantly higher in the xylitol group compared to the BB-12 and the sorbitol control groups (p=0.012). No significant differences between BB-12 and sorbitol groups in terms of MS colonization were detected (p=0.56). In study II, the culturing results of the microbial samples were similar on MSB and TYCSB agar. At the age of four years (III), the MS colonization percentages (MS $\geq 10^5$ CFU), 56% (18/32) in the BB-12 group, 70% (23/33) in the xylitol group, and 46% (13/29) in the sorbitol group, did not differ between study groups (p=0.180).

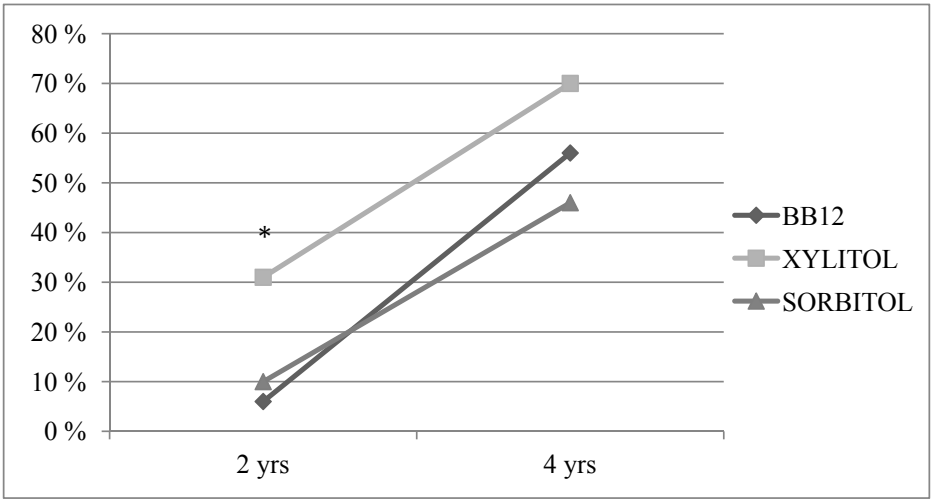

Figure 8. MS-colonization percentages of the children at the age of two and four years. The MS levels at the age of two years were determined using plate-culturing techniques (MSB, TYCSB) and at the age of four years using the Dentocult® SM Strip mutans test. * p<0.05.

5.4.3 *B. lactis* BB-12 administration and counts of lactobacilli and yeasts (II)

Very few children harboured mucosal yeasts (>log CFU 3) at the age of eight months or two years. Mucosal yeasts were found only in 6% (2/29) of the two-year-old children in the sorbitol group. Mucosal lactobacilli (aciduric flora including lactobacilli log CFU >4) were found in 17–35% of the eight-month-old and in 10–31% of the two-year-old children. No statistically significant differences were detected between the groups in the proportion of children harbouring mucosal or plaque lactobacilli either at eight months or at two years of age. The MS, lactobacilli and total facultatives showed no significant decrease in viability resulting from their storage and transportation. However, an approximate a loss of 1 log CFU in the viability of *Candida albicans* ATCC 28366 was found in the storage and transportation procedures described above.

5.4.4 *B. lactis* BB-12 administration and caries occurrence (III)

The occurrence of combined enamel and dentinal caries lesions (ICDAS lesion code 2–6) at the age of four years in the study groups is shown in Figure 9. The occurrence of obvious dentinal caries lesions (ICDAS lesion code 4–6) is presented in Figure 10.

Of the children, 70% (65/93) had no visually detectable caries lesion (BB-12 group 69% (22/32), xylitol group 64% (21/33), sorbitol group 79% (22/28); p=0.441). No differences were detected between the study groups in the occurrence of enamel caries (ICDAS lesion code 2–3; p=0.268), obvious dentinal caries (ICDAS lesion code 4–6; p=0.201), or combined enamel and dentinal caries (ICDAS lesion code 2–6; p=0.441). All detected caries lesions were recorded to be active (in a state of progression). Only one child in the xylitol group had fillings in his primary dentition. No sealants or extracted/missed teeth due to caries were recorded.

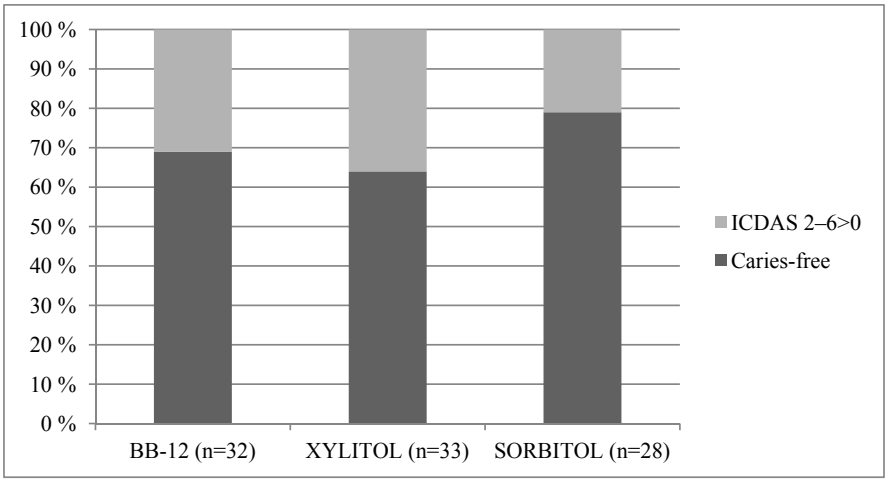

Figure 9. The percentages of caries-free subjects versus those with ICDAS 2–6 caries lesions at the age of four years by group.

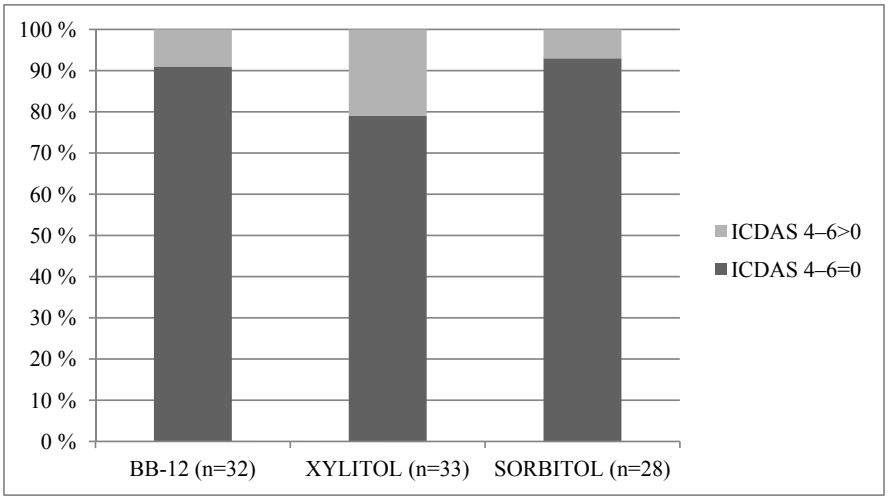

Figure 10. The percentages of subjects with obvious dentinal caries lesions at the age of four years by group.

5.4.5 Oral health-related background variables (II, III)

The results of clinical examinations and interviews concerning the oral health-related background variables at the age of two and four years are presented in Table 6 (II) and in Table 7 (III).

Table 6. The oral health-related background variables at the age of two by group.

	BB-12 (n=32)	XYLITOL (n=35)	SORBITOL (n=29)	p value
Number of teeth, mean (SD)	16.7 (1.6)	17.0 (2.0)	17.0 (1.5)	0.685[a]
Brushing started, months, mean (SD)	8.5 (1.8)	7.6 (2.3)	7.8 (2.2)	0.156[a]
Teeth brushed, times/day, mean (SD)	1.3 (0.5)	1.2 (0.4)	1.2 (0.4)	0.876[a]
dmf, n	0	0	0	
Use of xylitol, times/day, mean (SD)	1.4 (1.6)	1.5[b] (1.3)	1.1 (1.2)	0.771[a]
Use of probiotics started, months, mean (SD)	11.1 (1.4)	11.9[b] (2.9)	10.9 (2.3)	0.220[a]
No visible plaque, n (%)	16 (50)	20 (57)	14 (48)	0.746

[a] Statistical analysis with ANOVA. [b] n=32.

Table 7. The oral health-related background variables at the age of four by group.

	BB-12 (n=32)	XYLITOL (n=33)	SORBITOL (n=29)	p value
Teeth brushed twice a day, n (%)	16 (50)	18 (55)	8 (28)	0.176
Use of fluoride toothpaste, n (%)	32 (100)	32 (97)	28 (97)	0.586
Use of fluoride tablets, n (%)	0 (0)	0 (0)	1 (3)	0.322
Fluoride level of drinking water >1mg/L, n (%)	1 (3)	0 (0)	0 (0)	0.303
Xylitol use ≥3 times/day, n (%)	8 (25)	17 (53)	8 (28)	0.035
Sweet snacks daily, n (%)	14 (45)	20 (63)	11 (38)	0.140
Sweet drinks daily, n (%)	5 (16)	7 (22)	3 (10)	0.476
No visible plaque (whole dentition), n (%)	13 (41)	15 (46)	16 (54)	0.582

Since no differences were detected in oral health-related background variables in study II, the three intervention groups were combined. The MS counts of the mothers were associated with the MS detected in the dentition of the children at the age of two years (p=0.041). No association was detected between the MS colonization and the educational background of the families, or the mode of delivery. However, of the children whose mothers smoked, 38.5% (37/96) were colonized with MS, while the corresponding figure was 13.3% (13/96) for children of non-smoking mothers (p=0.039).

In study III, the use of commercial xylitol products at least three times per day was highest in the xylitol group (p=0.035). No other significant differences were seen between intervention groups in the clinical trial. The association of background variables and occurrence of caries at the age of four years was analyzed with the combined study groups. The occurrence of caries (ICDAS lesion code 2–6) was associated with daily use of sweet drinks (p=0.028), observed visible plaque in the whole dentition (p=0.002), observed visible plaque in the buccal surfaces of the maxillary incisors (p=0.003), and MS detected in dental plaque at the age of two (p=0.001) and four (p=0.002). The occurrence of caries was not associated with daily use of sugar snacks (p=0.766), tooth-brushing frequency (once vs. twice a day; p=0.209), use of xylitol (≥3 times/day vs. <3 times/day; p=0.530), the educational background of the mothers (academic vs. non-academic; p=0.099), or total breastfeeding (≥12 months vs. <12 months; p=0.717). Five children who had not been colonized with MS at age four showed enamel caries lesions (ICDAS lesion code 2–3). However, none of these MS-negative children had obvious dentinal caries lesions (ICDAS lesion code 4–6). In addition, all the children who had obvious dentinal carious lesions were colonized with MS at age four.

5.5 The effect of *B. lactis* BB-12 on infections in early childhood (IV)

In study IV, the BB-12 group and the xylitol control group were compared. The follow-up was completed by 92% (69/75) of the infants. Cumulative incidence of self-reported infections and antibiotic use are presented in Figure 11. Delivery of *B. lactis* BB-12 significantly reduced the occurrence of respiratory infections during the first eight months of life (p=0.014). Of the children, 65% (22/34) in the BB-12 group and 94% (33/35) in the control group experienced one or more episodes of respiratory infections. No significant differences between the groups were observed in reported gastrointestinal infections (p=0.605), acute otitis media (p=0.455), fever episodes (p=0.676), or antibiotic treatments (p=0.535).

As for non-infectious diseases, parents reported colic symptoms in 6% (2/34) of the infants in the BB-12 group and 11% (4/35) of the infants in the control group. Atopic eczema was detected in 26% (9/34) of the infants receiving the BB-12 tablet, and 34% (12/35) receiving the control tablet. Dairy milk allergy was diagnosed in three children, two in the probiotic group and one in the control group.

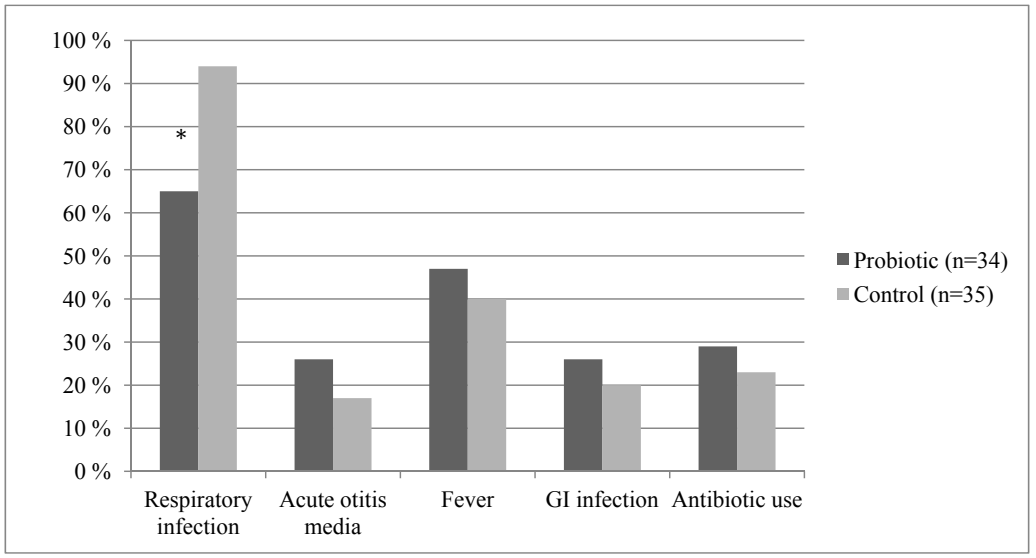

Figure 11. Cumulative incidence of self-reported infections (at least one episode) and the use of antibiotics during the first eight months of life. * p<0.05.

5.6 Recovery of *B. lactis* BB-12 in faecal samples (IV)

Faecal samples were available from all eight-month-old children in the BB-12 and xylitol control groups (IV). *B. lactis* BB-12 was recovered in the faeces of 62% (21/34) of the infants receiving the BB-12 tablet and 17% (6/35) of the infants receiving the control

tablet. All BB-12-positive and negative children in the BB-12 group were reported to have received the tablet either via the pacifier or with a spoon until the eight-month examination.

5.7 Adverse effects (II–IV)

No serious adverse effects were detected during the clinical trial. Two infants receiving *B. lactis* BB-12 and one receiving sorbitol withdrew from the clinical trial as a result of gastrointestinal complaints. One infant in the xylitol group was diagnosed with atopic eczema, and his physician recommended the family to discontinue the intervention. Gastrointestinal complaints could be explained by osmotic diarrhea when sugar alcohols were ingested. However, since the daily xylitol and sorbitol dose at the time of withdrawal was only 200 mg, this is unlikely.

6. DISCUSSION

The present study introduced a novel delivery method when xylitol and probiotic organisms were administered with the slow-release pacifier. The novel method was found to be practical and acceptable. The administration of *B. lactis* BB-12 significantly reduced respiratory infections in early childhood. Optimally, a probiotic strain with proven benefits for general health could also be used to benefit dental health. *B. lactis* BB-12 showed poor colonization in the oral cavity and did not reduce the levels of MS or the occurrence of caries. Importantly, however, no negative effects on dental health were observed, which is an important finding since bifidobacteria are also considered to be caries-associated microorganisms.

6.1 Administration of xylitol and probiotics with the pacifier

In study I, the novel delivery method was tested in adults for the sake of standardization of sample collection. The *B. lactis* BB-12-xylitol tablet and the control xylitol tablet dissolved from the pouch of the pacifier both slowly and completely, with no clear concentration peaks during 7–15 minutes of sucking. Thus, the probiotic tablet could be delivered in a safe and controlled way with the new pacifier. The concentration peak and elevated salivary xylitol concentrations have been suggested to play a role in the clinical effect of xylitol (Mäkinen et al. 1995, Tapiainen et al. 2002). With the slow-release pacifier no concentration peak could be observed, but on the other hand, elevated salivary xylitol concentrations were detected for longer periods as compared with other xylitol products (Lif Holgerson et al. 2006).

Another preliminary study with the pacifier was conducted in the beginning of the clinical trial. A part of the families participating in the trial was interviewed in order to examine the feasibility of the novel delivery method (Taipale et al. 2007). We wanted to examine how practical and time-consuming the novel method was when included in daily routines. Eighty-five percent of the families reported that the insertion of the probiotic tablet into the pouch of the pacifier was easy and that the recommended frequency of administration, twice a day, was considered acceptable. However, about half of the families reported that they would discontinue their participation, if the delivery frequency were raised to three times a day. Thus, the frequency of two daily administrations should not be exceeded.

The most common non-nutritive habit of infants and young children is pacifier sucking. Non-nutritive sucking habits seem to be of importance to children from a psychological point of view. Dentists and orthodontists recommend the use of pacifiers to prevent digit sucking, which is a significant cause of malocclusions. Pacifier use has been thought to

shorten the duration of exclusive breastfeeding (Chaves et al. 2007) and also to increase the risk of morbidity, especially AOM (North et al. 1999, Niemelä et al. 2000). These notions could not be confirmed in the present clinical trial. No association between the occurrence of AOM and pacifier usage (sucking time per day, duration of usage) could be detected. Previous systematic reviews are also in line with our findings concerning an adverse relationship between pacifier use and the duration or exclusivity of breastfeeding (O'Connor et al. 2009, Jaafar et al. 2011). In our clinical trial, breastfeeding was strongly supported and the families were not pressured to use the pacifier.

6.2 *B. lactis* BB-12 and oral health

We investigated whether *B. lactis* BB-12 could adhere to or at least temporarily colonize the oral surfaces when administered on a regular basis in early childhood and at the time of tooth eruption (II). Our findings suggest that *B. lactis* BB-12 shows only transient retention in the oral mucosa and the dental surfaces of the children. It has been suggested that exposure early in life to the probiotic organisms may facilitate its permanent installation in the oral cavity (Twetman and Stecksén-Blicks 2008). This was not confirmed here with *B. lactis* BB-12. No other reports are available concerning the oral persistence of this probiotic strain when administered in infancy. Our result is in agreement with the study of Saxelin et al. (2010) who tested the same strain in adults. They reported that only one participant carried *B. lactis* BB-12 in saliva samples during and after a two-week administration of *B. lactis* BB-12 in yoghurt or cheese. Also other probiotics like *L. reuteri* and *L. rhamnosus* GG appear to show rapid clearance from the mouth in adults (Yli-Knuuttila et al. 2006, Caglar et al. 2009, Saxelin et al. 2010). However, probiotics have been suggested to exert beneficial effects on oral health without permanently colonizing the site (Teughels et al. 2008).

B. lactis BB-12 was also recovered from a few children in the xylitol and sorbitol group. The assay method that we used is *B. animalis* group-specific. Breast milk has been shown to contain high levels of bifidobacteria including subspecies of *B. animalis* (Gueimonde et al. 2007). Thus, it is possible that breast milk was one source of the recovered bifidobacteria. More likely, the reason for the oral recovery of *B. lactis* BB-12 in the xylitol and sorbitol groups was maternal consumption of dairy probiotic products containing undefined mixtures of bifidobacteria.

One aim of the clinical trial was to characterize the effect of early administration of *B. lactis* BB-12 on oral colonization by MS. We hypothesized that the administration of *B. lactis* BB-12 might reduce MS colonization. At the age of two years (II), all three study groups showed lower colonization percentages than expected. The lowest MS colonization percentage was found in the BB-12 group. However, the administration of

B. lactis BB-12 was not necessarily connected with the low MS colonization in the BB-12 group since the colonization percentage was also low in the sorbitol control group. In the clinical trial, the intervention started in early childhood, and lasted on average 14 months. No other long-term studies are available on the effect of bifidobacteria on the oral microbiota. Earlier short-term intervention studies, lasting 20–28 days, have demonstrated that consumption of ice cream or yoghurt containing *B. lactis* BB-12 or *B. lactis* DN-173010 can reduce MS levels in adolescents (12–16 yrs) and in young adults (Caglar et al. 2005 and 2008b, Cildir et al. 2009, Singh et al. 2011). Contrary to the studies above, we determined the MS from dental plaque and oral mucosa, not from saliva. With the exception of MS, the microbiota of whole saliva resembles that of the tongue more than that of plaque (Mager et al. 2003). In addition, strain-specific differences in probiotic effects and the use of combinations of probiotics, as in the study by Singh et al. (2011), may influence the results. The mechanism of possible probiotic action in the oral cavity is not fully understood. Obviously, in adults, one single strain is not able to induce a permanent microbiological shift in a complex and fully matured oral ecosystem (Teughels et al. 2008). However, in early childhood, the modification of microbiota may be possible. In theory, probiotic bacteria can produce antimicrobial compounds against MS (Meurman et al. 1995, Spinler et al. 2008, Simark-Mattsson et al. 2009), and compete for the same adhesion sites, nutrients, and growth factors (Haukioja et al. 2006 and 2008a). In addition, the possible influence on local and systemic immune response cannot be excluded (Fukushima et al. 1998, Hatakka and Saxelin 2008).

The administration of *B. lactis* BB-12 in early childhood did not increase occurrence of caries at the age of four years (III). This result was in synch with study II where *B. lactis* BB-12 did not affect the counts of MS or lactobacilli during the first two years of life. Thus, *B. lactis* BB-12 may be considered a safe probiotic strain from the oral health point of view. The present study was the first to examine the administration effect of a probiotic bifidobacteria on the occurrence of caries. In earlier studies, the strains of lactobacilli, *L. rhamnosus* GG and *L. rhamnosus* LB21 appeared to reduce caries risk in preschool children (Näse et al. 2001, Stecksén-Blicks et al. 2009). Our "no-effect" result with *B. lactis* BB-12 may be explained by the fact that probiotic effects are strain-specific. Lactobacilli have been shown to adhere better to saliva-coated surfaces than bifidobacteria *in vitro* (Haukioja et al. 2006). Good attachment ability to the oral cavity may enhance a probiotic strain to act locally through the biofilm by competing with the caries-associated bacteria and, systemically, by modifying the immune system (Stamatova and Meurman 2009).

B. lactis BB-12 was selected for the study because it has a long history of safe use in infant studies, it has the qualified presumption of safety status of the European Food Safety Authority, and it is generally recognized as safe by the FDA in the US (FDA 2002,

Saavedra et al. 2004, EFSA 2009). Moreover, at the time of the trial, in Finland, the availability of *B. lactis* BB-12-containing products was limited, and the strain was only found in a small number of products containing undefined mixtures of bifidobacteria and in tablets sold in some pharmacies for preventing travellers' diarrhea in adults. In addition, although *B. lactis* BB-12 has been commercially available for more than 25 years and it is one of the most thoroughly studied probiotic strains, only a few studies have examined its effect on oral health.

6.3 Oral health-related aspects

At the age of two years, the highest MS-colonization percentage was detected in the xylitol group (II). This result indicates that the xylitol doses of up to 600 mg per day were not high enough to interfere with the colonization of MS. Also in earlier clinical studies, low-dose (1–1.5 g/day) xylitol interventions have not been able to reduce MS levels or prevent caries in young children (Oscarson et al. 2006, Meurman and Pienihäkkinen 2010). According to the literature, a daily dose of at least 5 g of xylitol with a daily consumption frequency of at least three is needed to reduce counts of MS both in children and adults (Mäkinen et al. 1989, Milgrom et al. 2009, Seki et al. 2011). In theory, however, even low xylitol doses could inhibit MS. *In vitro* studies have shown that low xylitol concentration, from 0.1% to 1%, can inhibit the growth of MS (Söderling et al. 2008). In study I, we found that xylitol released from the food supplement resulted in 0.5–1% xylitol concentrations in saliva. This result was found in adults, thus children with smaller mouths were expected to exhibit even higher xylitol concentrations in dental plaque. In order to examine the effect of the administration of low xylitol doses on MS colonization, we added a sorbitol control group to the study. Sorbitol is generally considered to be an inert polyol and is commonly used as a control in clinical xylitol studies (Mäkinen et al. 1995, Milgrom et al. 2009).

The effect of xylitol on risk markers of caries was also evaluated at the age of four years in the combined study groups (III). According to the interviews of the parents, most children used commercial products only 1–2 times per day, usually in the day-care centre. One third of the children used xylitol products at least three times per day. Thus, the dosages of xylitol were only 0.8–1.8 g per day, depending on the different products. This dosage of xylitol was not associated with reduced level of MS or visible plaque in the dentition at the age of four. In addition, no significant association between the daily use of xylitol and the occurrence of caries was found. This result is also in accordance with earlier studies by Oscarson et al. (2006) and by Meurman and Pienihäkkinen (2010).

Surprisingly, in all study groups, the levels of MS were rather low at the age of two years considering the MS counts of the mothers (II). According to earlier MS transmission

studies, half and even more than half of the children whose mothers show salivary MS counts of log CFU 5 or more should be colonized by MS at the age of two years (Berkowitz et al. 1981, Gripp and Schlagenhauf 2002). Also in the mother-child study carried out in Finland, the MS-colonization percentage at the age of two years was 49% in the fluoride control group (Söderling et al. 2000). Thus, in our clinical trial, nearly half of the children in the three groups were expected to be MS-positive at the age of two years. Participating families were highly motivated to improve their children's oral and general health. In addition, the high level of knowledge concerning MS transmission in the research communities may explain the low MS colonization percentages. Our public health-care centre has a well-organized oral health recall-system where the parents and the children regularly visit a dental hygienist or a dental nurse. During the study recruitment period, parents received information from leaflets and from newspaper articles published to promote the trial. In addition, the transmission of MS via saliva was carefully explained to the families at the first study appointment, which took place when the child was one month old.

When the children in the three groups were pooled at the age of two years maternal smoking showed a significant association with the MS colonization of the child (II). A recent Finnish study has shown that the early MS colonization in young children is strongly associated with the socioeconomic status of the family (Meurman and Pienihäkkinen 2010). That study was conducted in a city, a very different kind of community than ours. Our clinical trial revealed no associations between the educational background of the families and the MS colonization in the children. The impact of the mode of delivery earlier reported to influence the early colonization of MS (Li et al. 2005) was not observed in our clinical trial.

One aim of the clinical trial was to identify risk markers of caries in early childhood in a Finnish population with good dental health. For this purpose, the study groups were combined at the age of four years (III). Interestingly, the frequency of tooth brushing was not associated with the occurrence of caries. For caries prevention the quality of the tooth cleaning may be more important than its daily frequency (Bellini et al. 1981). However, the amount of visible plaque was strongly associated with the occurrence of caries. This result is in accordance with an earlier study by Alaluusua and Malmivirta (1994), which followed 92 children for one and a half years, from the age of 19 months to 36 months. Their results suggested that the best of the studied indicators for the risk of caries in early childhood was visible plaque on the labial surfaces of the maxillary incisors. In addition to plaque, MS colonization of the teeth at the ages of two and four proved to have a significant association with the occurrence of caries. A recent systematic review showed that the presence of MS, both in plaque and saliva in caries-free two- to five-year-old children is associated with a considerable increase in caries risk (Thenisch et al. 2006).

However, in our clinical trial, five children who were MS-negative at age four showed enamel caries lesions (ICDAS lesion code 2–3). None of these five children had obvious dentinal caries lesions (ICDAS lesion code 4–6). This finding is in line with the present plaque hypothesis where MS play an important role but are not necessarily in a causative relationship with caries (Takahashi and Nyvad 2011). We also found the association between the daily use of sweet drinks and the occurrence of caries. According to a survey of the National Public Health Institute of Finland, the intake of sucrose among one- to six-year-old children is higher than recommended, and the main sucrose source is sweet drinks (Publications of the NPHI 2008). A recent Australian study was in line with these results, stating that increased intake of sweetened drinks with added sucrose is a matter of great concern to the health profession worldwide (Lee and Brearley Messer 2011).

6.4 *B. lactis* BB-12 in reducing the risk of early infections

In developed countries, infants experience three to six respiratory tract infections during the first year of life, and 40% of them suffer from at least one episode of acute otitis media (Vesa et al. 2001). We found a significant reduction in the incidence of respiratory infections during the first eight months of life with probiotic supplementation. No other health-related differences between study groups were found in healthy breastfed infants. In Finland, 51% of the infants, and in the present hospital district area, 66% are exclusively breastfed at three months (Hasunen and Ryynänen 2005). With respect to the duration of exclusive and total breastfeeding, the mothers in the present trial represented typical Finnish mothers. Thus, *B. lactis* BB-12 may add to the protection provided by human breast milk. The duration of breastfeeding may indeed explain the low incidence of infectious diseases and also the insignificant differences in infectious diseases, especially AOM, between the groups, contributing also to the low number of antibiotic prescriptions in each. Beyond providing antibodies and non-specific antimicrobial molecules, breastfeeding influences the composition of indigenous intestinal microbiota by promoting the growth of bifidobacteria (Harmsen et al. 2000).

Only two studies have investigated the effect of bifidobacteria on infections during the first year of the infant's life. In those studies, all infants were weaned from breastfeeding before the probiotic supplementation started. Weizman et al. (2005) did not find any effect of *B. lactis* BB-12 on respiratory illnesses during a 12-week follow-up in infants aged 4–10 months. In that study, significantly fewer febrile episodes were, however, observed in the BB-12 group. In the study by Rautava et al. (2009), the combination of *B. lactis* BB-12 and *Lactobacillus* GG reduced the risk of early AOM and recurrent respiratory infections in children requiring formula before the age of two months. In that study, the intervention started before the age of two months and the follow-up period was 12 months, longer than in Weizman's study. In addition, the combination of probiotics may

have influenced the results. It is obvious that starting age and duration of intervention may be crucial in the possible beneficial effects of probiotic supplementation in early childhood. In early infancy, the maturing immune system might be more amenable to probiotic modification (Salminen and Isolauri 2008). Likewise, the diet of the infant and the food matrix contribute to the outcome (Isolauri et al. 2008). Hence, generalisation of our results to other infant populations may require further clinical demonstrations.

Faecal recovery of DNA of the ingested probiotic strain was used as the measure of successful delivery and intestinal passage of the probiotic (IV). *B. lactis* BB-12 has been shown to survive intestinal passage in a dose-dependent manner (Larsen et al. 2006), and to reside only transiently in faecal samples (Fukushima et al. 1998, Isolauri et al. 2002). In the present study, a relatively high variation in recovery rates from individual to individual was observed. Most children in the BB-12 group showed a positive recovery result. The negative results can be explained by the fact that the detection limit of the faecal recovery of *B. lactis* BB-12 was rather high. The possible reason for the faecal recovery of *B. lactis* BB-12 in the control group could be maternal consumption of dairy probiotic products containing undefined mixtures of bifidobacteria, or breastfeeding. The assay method we used is subspecies-specific but not strain-specific. Maternal breast milk and intestinal bifidobacteria, including species belonging to the *B. animalis* group, guide the compositional development of the *Bifidobacterium* microbiota in infants (Gueimonde et al. 2007).

6.5 Methodological considerations

This kind of clinical trial is optimally performed in a small community, such as Muurame or Korpilahti, in order to avoid interruption due to study subjects moving to another area, as well as to ensure a uniform socio-economic background. In practice, all families in the present area use the public health care services of the well-baby clinics irrespective of their socio-economic background. The present clinical trial was conducted according to the guidelines of the CONSORT (Consolidated Standards of Reporting Trials) statement to improve the quality of reporting, and to yield unbiased results (Moher et al. 2010). The study setup, e.g. randomization, blinding, and training was designed to be ideal for the trial. The recruitment of the families can be considered to have been successful. We recruited for as long as it was possible, in practice 2.5 years. Between September 2004 and February 2007 all pregnant mothers (n=479) received a leaflet regarding the trial at well-baby clinics. Almost 40% of the families wanted to take part in the trial even though the clinical trial programme was demanding. The four-year follow-up period required several visits to the dental clinic and compliance at home when using test tablets. It is well known that many parents do not want to take part in any study which is conducted in newborn infants. Moreover, those families who started the intervention were highly

motivated and remained in the trial. The dropout rates were unlikely to have caused bias in the trial.

The interviews were based on the structured questionnaires, which had been validated in earlier studies (Kilpi et al. 2002). One dental nurse and one dental hygienist performed all the interviews. The structured interview guided the discussion and ensured the similarity of the study appointments. At these appointments the study diaries were checked and supplemented if necessary. The questionnaires were also used to estimate the compliance of the families. The daily frequency of tablet administration was calculated based on the frequency of usage reported in the eight-month and two-year questionnaires, and the duration of tablet delivery from the two-year questionnaire. According to the questionnaires, over 80% of the children in all study groups were reported to have received the test tablets twice a day, and the rest of the participants once a day. In addition, faecal recovery of *B. lactis* BB-12 at the age of eight months could also be considered as a measure of compliance. Most children in the BB-12 group showed a positive recovery result (IV).

The diagnoses of infections and other illnesses during the first eight months of life were mainly based on the subjective evaluation of symptoms by the parents (IV). The infants are unable to communicate their own symptoms, and thus, it is possible that the prevalence of infections may have been over- or underestimated. Parents also reported respiratory infections with complications, i.e. AOM, bronchitis, pneumonia, and sinusitis, diagnosed by a doctor (municipal or private) unrelated to the study. The accuracy of these diagnoses was impossible to standardize. However, it can be assumed that reporting of these infections occurred equally in all study groups.

Experienced professionals performed the sample collections and the detection of bacteria with well-known equipment according to the manufacturer's instructions. The oral samples were easily and quickly collected from children in a standardized manner with microbrushes and cotton swabs. The chair-side test correlated well with plate culturing of MS. The storage and transportation procedure of bacteria was tested before the trial.

We followed the ICDAS to describe the severity of dental caries lesions. In the ICDAS examination protocol, a tooth surface is first viewed wet and after that it is dried for five seconds to detect the first visual changes in enamel; lesion code 1. The prolonged air-drying was not possible for all children at age four. Thus, we used only lesion codes 2 and 3 to present enamel caries, and lesion codes 4, 5, and 6 for obvious dentinal caries. The ICDAS was chosen because it includes the early state of initial lesions, and it has been shown to be reproducible and accurate (Jablonski-Momeni et al. 2008). One dentist (TT) performed all caries examinations, which increased the reliability of the diagnosis.

7. CONCLUSIONS

On the basis of the results presented in this thesis, the following conclusions can be drawn:

1. The salivary xylitol concentrations inhibitory to mutans streptococci (MS) can be reached during sucking of the food supplement containing xylitol and *B. lactis* BB-12 administered with the novel pacifier. The presence of *B. lactis* BB-12 in the tablet does not affect the pattern of xylitol release.

2. The early administration of *B. lactis* BB-12 does not result in permanent oral colonization of this probiotic and does not significantly affect the colonization of MS in children.

3. The early administration of *B. lactis* BB-12 does not seem to increase the occurrence of dental caries in children aged four. Thus, the early administration of *B. lactis* BB-12 should be safe with regard to the future dental health of the child.

4. The early colonization of MS and the visible plaque in the dentition are strongly associated with the occurrence of caries in young children. Low xylitol doses, up to 600 mg per day, are not high enough to interfere with the colonization of MS *in vivo*.

5. Controlled administration of *B. lactis* BB-12 in early childhood may reduce respiratory infections in breastfed children.

6. The food supplement tablet containing *B. lactis* BB-12 and xylitol can be delivered in a safe and controlled way with the novel pacifier. The present new method is practical and acceptable by the parents.

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Teemu Taipale

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