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Citation for the published paper:

Hasslöf, P., West, C., Karlsson Videhult, F., Brandelius, C., Stecksén-Blicks, C. [Year unknown!]

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Caries Research

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Early intervention with probiotic *Lactobacillus paracasei* F19 has no long-term effect on caries experience

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Key words: dental caries, mutans streptococci, lactobacilli, probiotic intervention

Abstract

The aim of the study were to evaluate possible long-term effects of a cereal diet supplemented with *Lactobacillus paracasei* F19 (LF19) during weaning on caries experience, mutans streptococci (MS) and lactobacilli (LBC) in a group of 9-year old children. A secondary aim was to evaluate if the intervention resulted in the permanent integration of LF19 as part of the oral microbiota. The study followed up on a double-blind placebo-controlled randomised trial. Among 179 infants that were randomised to a daily diet that included cereals with or without LF19 from 4-13 months of age, 56 from the probiotic group and 62 from the placebo group participated in the follow-up at 9 years. Data were collected by oral clinical examination and questionnaires. MS and LBC levels were assessed with conventional cultivation; LF19 was detected by using randomly amplified polymerase chain reactions (RAPD-PCR). At the follow-up, neither decayed, missing and filled surfaces for primary teeth (dmfs) nor decayed, missing and filled surfaces for permanent teeth (DMFS) differed significantly between the probiotic and placebo groups ($p > 0.05$). MS and LBC levels were similar in both groups ($p > 0.05$). RAPD-PCR showed no evidence of oral colonisation with LF19 in the study group. It is concluded that an early intervention with LF19 did not affect the frequency of dental caries, MS or LBC. LF19 did not establish itself as a permanent facet of the oral microbiota in any of the subjects included in this study.

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Introduction

Dental caries is one of the most common diseases observed among children. It is a multifactorial disease with a variety of identified risk factors, including both genetic and lifestyle factors. The disease is characterised by a loss of hard tissue from the tooth due to an imbalance in the commensal microbiota in the dental biofilm. This equilibrium upset results from the overgrowth of acid-tolerating bacteria species, such as mutans streptococci (MS) and lactobacilli (LBC) [March 2003]. These species of bacteria produce acids as metabolic by-products, which in turn reduces the pH of the biofilm [Selwitz et al., 2007]. Modern molecular techniques have been used to demonstrate that MS are typically associated with early enamel demineralisation, while LBC are mainly found in deep caries lesions [Becker et al., 2002].

Probiotics are by definition 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [WHO/FAO, 2002]. The addition of probiotics to infant formulas and baby foods (e.g. gruel and cereals) is gaining in popularity. Notably, these products come into contact not only with the intestinal tract but also with the oral cavity. It has been suggested that the chronic use of probiotics to alter the gastrointestinal microbiota in young children may lead to undesirable side effects in the intestine and the oral cavity [Benno et al., 2010]. It is therefore necessary to conduct follow-up studies on the effects of probiotics on oral microbiota and caries development in infants. Intervention studies in children and young adults have shown that the ingestion of various strains of probiotic LBC can decrease oral microbiota levels of MS, although with somewhat differing results. This inconsistency may be due to the numerous strains investigated or differences in study design and/or study population [Näse et al., 2002; Cildir et al., 2009; Stecksén-Blicks et al., 2009; Lexner et al., 2010; Singh et al., 2011; Jindal et al., 2011; Cildir et al., 2012]. Certain naturally occurring LBC inhibit the growth of a patient's autologous MS *in vitro*. Among the LBC species, *Lactobacillus paracasei* exhibits maximal interference activity against *Streptococcus mutans*, particularly in caries-free subjects [Simark-Mattsson et al., 2007].

Though oral colonisation by probiotic LBC has been described as a transient phenomenon [Yli-Knuuttila et al., 2006; Caglar et al 2009; Saxelin et al., 2010; Stinkiewicz et al., 2010; Marttinen et al., 2011], it has also been questioned whether exposure early in life may facilitate a permanent incorporation into the oral microbiota [Meurman, 2005]. The few studies on probiotics that have examined caries as an outcome suggest that probiotics are beneficial rather than hazardous to dental health in children [Näse et al., 2002; Stecksén-Blicks et al., 2009] and adults [Petersson et al., 2011].

The aims of this study are to: i) assess the frequency of caries at 3, 6, and 9 years of age in a group of children who had eaten cereals supplemented with *L. paracasei* ssp. *paracasei* strain F19 (LF19) or the same cereals without any probiotic (placebo) from 4–13 months of age; ii) identify whether LF19 established a niche in the oral microbiota and whether MS and LBC were less prevalent in 9-year-olds who had ingested a probiotic-laden diet. The null hypothesis was that there were no differences between the probiotic and placebo groups in the frequency of caries or colonisation by LF19, MS, and/or LBC.

Material and methods

Study design

The study is a follow-up of a double-blind, randomised controlled trial described previously in detail [West et al., 2008]. The follow-up study was approved by the Regional Ethical Review Board in Umeå (§08-214M). In the baseline study, the effect of a diet that included LF19 during weaning was studied with regard to allergies and immunological development. The study population comprised 179 infants, aged 4 months at the beginning of the intervention period. All were full-term babies (gestational age 37–42 weeks) with birth weight >2500 g who were delivered vaginally. Infants were randomised to a daily diet of cereals supplemented with LF19 or the same cereals without LF19 (Semper AB, Stockholm, Sweden); the infants adhered to this diet from 4–13 months of age. The recommended dose was at least 1 serving a day, which translated to the ingestion of 1×10^8 colony forming units (CFU) of strain LF19 (Fig. 1). Compliance with the protocol was evaluated

through questionnaires completed by the parents and evaluating stool samples for the presence of LF19 using randomly amplified polymerase chain reactions (RAPD-PCR) [West et al., 2008].

ClinicalTrialsIdentifier NCT00894816.

Study population

Of the 179 infants that were randomised to receive study cereals with or without LF19, 171 (84 in the probiotic group, 87 in the placebo group) completed the intervention phase. The baseline study was conducted during the period from August 2000–November 2003. From April 2009–June 2011, 118 infants from the original study population (56 probiotic, 62 placebo) participated in the follow-up study described here (Fig. 2). The main outcomes in the follow-up were dental caries and immunological development as well as the prevalence of allergic disease, obesity (which will be reported separately).

General health and eating habits

With the assistance of their parents, all participants were asked to complete a questionnaire regarding general health, eating habits (including the consumption of soft drinks, sweets and products containing probiotic LBC), social background (parents' education level), parents' smoking habits, and dental hygiene.

Caries experience

Two certified dentists (PH and CB) performed the oral examinations. The presence of dentine caries was defined according to the WHO criteria [1987]; enamel caries was defined as proposed by Koch et al. [1967]. Bitewing radiographs were taken when approximal surfaces were unavailable for visual inspection. Decayed, missing, and filled surfaces were calculated for primary and permanent teeth (dmfs/DMFS). Molars, canines and incisors extracted due to caries were registered as 3 and 2 missing surfaces, respectively. Total caries experience was calculated as the sum of the dmfs and DMFS values. Data on dmfs and DMFS values were also collected from Public Dental Health Service records collected at the child's first visit at 3 years of age (n = 114) and at 6 years of age (n = 118). Dental records for 4 of the participants were not available, as these

infants resided in other parts of Sweden from 2–6 years of age.

Saliva sampling

Saliva samples were obtained in the morning. The participants were not allowed to brush their teeth or eat or drink anything from the time they woke up until after the sampling had been completed. The participants were instructed to chew on a piece of paraffin for 3 minutes and to collect the resulting saliva in a test tube. The secretion collected during the first minute was discarded. The rest of the saliva sample was immediately transported to the lab and divided into 2 aliquots: 1 was frozen at -80°C and 1 was analysed immediately (see below).

Microbial analysis

The saliva samples were vortex-mixed and diluted 10-fold, 5 times, then plated on selective media. After serial dilutions, selective mitis salivarius-bacitracin (MSB) agar plates [Gold et al., 1973] were used for MS cultivation. The plates were incubated in aerobic conditions at 37°C for 72 hours. For LBC cultivation, Difco™ Rogosa SL-agar (Becton Dickinson and Company Sparks, USA) and MRS-agar (de Man, Rogosa, Sharpe, Oxid, Hampshire, England) were used. HCl or HAc respectively were added, and the pH was adjusted to 5.4. The total viable counts of bacteria (TVC) were estimated on blood agar oral plates (BAO). MRS, Rogosa SL, and BAO plates were incubated in an anaerobic atmosphere for 72 h at 37°C. The number of CFU was estimated using a stereo-microscope. Thereafter, the MRS and Rogosa SL plates were transported to Arla, Stockholm and upon arrival they were kept in cold storage (+4 °C) until reading. Typical colonies on the plates were streaked on MRS plates, pH 5.4, and incubated anaerobically at 37°C for 72 hours and checked by microscopy. DNA was extracted from all characteristic colonies and screened by randomly amplified polymerase chain reaction (RAPD-PCR) with the primer LBC-19 (5'-AGT AGC CAC-3'). Isolates positive in the screening were confirmed with a second primer OPA-02 (5'-TGC CGA GCT G) [Björneholm et al., 2002]. The detection limit for the RAPD-PCR was < 10 CFU/g saliva. To secure an unbiased identification of the

isolates, positive and negative controls were randomly included in the identification series of lactobacilli.

Statistical analysis

The data were analysed using PASW statistics software (version 18.0, Chicago, IL, USA). The numbers of CFUs of MS, LBC, and TVC in saliva were logarithmically transformed to improve normality. For group comparisons the non-parametric Mann-Whitney U-test was used for continuous data and Pearson's chi-squared test for categorical data. Linear regression was used to test the association between selected variables and dental caries. A p-value of < 0.05 was considered as statistically significant.

Results

There were no differences between the probiotic and the placebo group in background factors, such as parents' educational level, duration of breastfeeding, smoking history; reported history of chronic disease; consumptions of sweets, soft drinks, milk, and oral hygiene habits (Table 1).

Caries experience at first dental examination and 6 years of age

Data collected from the dental charts obtained when participants were 3 years of age showed no child with caries in the probiotic group while 2 children in the placebo group had caries. The mean dmfs was 0.2 ± 1.0 in the placebo group. The records from examinations performed at 6 years of age showed caries experience in 20 % of the probiotic group and 26 % of the placebo group. The mean total caries experience (dmfs+DMFS) were 0.6 ± 1.7 and 0.7 ± 2.4 , respectively. (Fig.3). The median values was zero in both groups at both examinations. There were no statistically significant differences in caries experience between the two groups at any timepoint ($p > 0.05$).

Clinical measurement of dmfs and DMFS values

At 9 years of age, 41% of the probiotic group and 37% of the placebo group had caries. The median values were zero in both groups and the mean caries experience (dmfs+DMFS) were 1.4 ± 2.8 and 1.3 ± 2.2 , respectively (Fig. 3). The mean dmfs-values were 1.1 ± 2.8 and

0.9 ± 1.9 in the probiotic and placebo group, respectively and the mean DMFS values were 0.3 ± 0.7 and 0.4 ± 1.0 , respectively. The two groups were similar with respect to every aspect of caries experience examined ($p > 0.05$).

Colonisation by LBC and MS

The bacterial counts are displayed in Figure 4. LBC colonies were detected in 84% of subjects in the probiotic group and 77% in the placebo group. RAPD-PCR showed no evidence of oral LF19 colonisation in the study group. In each group, 76% had detectable levels of MS. There was no statistically significant difference between the groups in the number of MS (CFU/ml) in saliva ($p > 0.05$). The MS ratio of TVC was 0.2% in the probiotic group and 0.1% in the placebo group ($p > 0.05$). There was a statistically significant correlation between MS (CFU/ml) and total caries experience at 9 years of age in the placebo group ($R = 0.363$, $p < 0.01$) but not in the probiotic group ($R = 0.208$, $p > 0.05$).

Discussion

Devine and Marsh hypothesised that it could be easier to alter the caries-associated microbiota at the time of colonisation compared to later in life when the microbiota is firmly established [2009]. This study showed no long-term effect of LF19 on the oral microbiota, as measured by 4 parameters: MS-TVC ratio and MS, LBC and LF19. To our knowledge, this is the first study describing possible long-term effects on caries-associated microorganisms and dental caries in a group of children who received controlled probiotic lactobacilli supplementation from 4–13 months of age.

The results of the study confirmed the null hypothesis: early intervention with LF19 had no effect on caries at 3, 6, or 9 years of age. Similarly, the number of MS, the MS-TVC ratio, the number of LBC, and the LF19 colonisation were not different from those of an untreated placebo group. Interestingly, MS was associated with dental caries in the placebo group but not in the probiotic group at the follow-up examination, 8 years after the intervention phase.

The dropout rate for the baseline study was low and compliance with the study protocol during the intervention was considered as excellent, based on the confirmed presence of LF19 in stool in a majority of the LF19 infants [West et al., 2008]. Of 171 infants that completed the baseline study, 118 participated in the follow-up study conducted 8 years after the intervention phase. Lower socioeconomic status is associated with a higher rate of caries among pre-schoolers [Stecksén-Blicks et al., 2008]. Notably, the education level of the parents who participated in the present study was high. The incidence of caries was low in both groups of children, which limited the statistical power of the main outcome measure analysis.

The infant acquires oral bacteria from the mother during early childhood [Li et al., 1995]. A recent study showed that health related streptococci and LBC were more common in children delivered vaginally as compared to those delivered by caesarean section [Nelun Barfod et al., 2011]. Vaginally born children are exposed to maternal commensal bacteria at birth and colonised later in life with cariogenic bacteria [Li et al., 2005]. It has also been shown that breastfeeding promotes colonisation with LBC more effectively than formula feeding [Holgersson et al., 2012]. In the present study, all included children were delivered vaginally and the majority were breastfed [West et al., 2008]. The rationale for introducing a probiotic LBC during weaning was to maintain the presence of LBC in the gut and oral cavity. We hypothesised that early exposure to probiotic LBC could prevent or delay MS colonisation and accordingly prevent dental caries. Substantial epidemiologic evidence links MS to caries [van Houte et al., 1994] but it has been shown that the development of caries may involve more complex communities of bacterial species. This idea, known as the ‘ecological plaque hypothesis’, maintains that increased acidity favours the proliferation of certain bacterial species (e.g. MS) [March 2003].

In vitro studies have demonstrated that various probiotic LBC can survive in human saliva [Haukioja et al., 2006]. *L. paracasei* LF19 has a low acid production from dietary

sugars [Hedberg et al., 2008], can inhibit growth of MS [Hasslöf et al., 2010; Keller et al., 2011] while the ability to co-aggregate with MS is lower than for *L. plantarum* 299v, *L. plantarum* 931, *L. rhamnosus* GG, *L. reuteri* PTA 5289 and *L. acidophilus* 117 [Keller et al., 2011]. Earlier clinical studies on a possible installation of probiotic LBC in the oral cavity have been performed in subjects with a mature microbiota. These studies showed that different strains of probiotic LBC act as transient colonisers in the oral cavity [Yli-Knuuttila et al., 2006; Caglar et al. 2009; Saxelin et al., 2010; Stinkiewicz et al., 2010; Marttinen et al., 2011]. One recent study in infants showed that early exposure (from 2–24 months) to *Bifidobacterium animalis* subsp *lactis* BB-12 did not result in permanent colonisation by probiotic bacteria [Taipale et al., 2012]. Our finding supports the view that probiotic LBC are transient colonisers, even when administered early in life. The environmental conditions in the current study may not have been optimal for permanent colonisation by LF19 in the oral cavity and the short contact time of the extrinsically administered probiotics to the oral cavity may have been a limiting factor. Notably, adherence is a key factor for colonisation; *in vitro* experiments have shown that *L. paracasei* F19 adheres poorly to saliva-coated surfaces [Haukioja et al., 2006]. The DSMZ16671 *L. paracasei* strain co-aggregates with MS but not with other oral commensals and does not bind to hydroxyapatite [Lang et al., 2010] while heat-killed bacteria from the same strain inhibited caries development in rats [Tanzer et al., 2010]. The best way to use this knowledge to elucidate microbiota establishment in the infant remains unclear.

The methods that are most commonly used for prevention of dental caries (e.g. dietary advice and fluoride exposure) are ineffective in certain segments of the paediatric population. The use of probiotics represents a potential addition to regular prevention. Most studies on probiotics and oral health have measured changes in MS counts [Stamatova et al., 2009], but a few have used dental caries as the endpoint measure. One report suggested that *L. rhamnosus* LGG could reduce the incidence of caries in young children [Näse et al., 2002]. A study on *L. rhamnosus* LB21

suggested similar effects, although a confounding effect from the fluoride added to the test product cannot be excluded [Stecksén-Blicks et al., 2009]. Although the current study found no such effect of probiotic consumption on dental caries more studies are needed to evaluate the possible effects of this approach.

In conclusion, the consumption of a diet that included LF19 during weaning had no effect on dental caries at 3, 6, or 9 years of age and no effect on MS or LBC at 9 years of age. The presence of LF19 was not detected in saliva samples from any of the study participants.

Acknowledgements

Financial support was provided through a regional agreement between Umeå University and Västerbotten County Council (TUA, ALF, FoU); Arla Foods AB, Sweden; the European Union's Seventh Frame Work Programme under grant agreement n° 222720; the Swedish Society for Medical Research; and the Ekhaga and Oskar Foundations. The authors are most grateful to all of the children who participated as well as their parents. Inger Sjöström, Elisabeth Granström and Maria Hedberg are gratefully acknowledged for their skilful laboratory assistance.

Authors' contributions

PH, CEW, and CSB designed the study. PH and CB performed the clinical examinations and executed the saliva samplings. PH performed the laboratory work. FKV analysed the dietary data. PH and CSB analysed the data and drafted the manuscript. CEW and CSB revised the manuscript critically.

Declaration of interest

Dr. Christina West has received funding and a speaker's honorarium from Arla Foods. The authors declare no personal or financial conflict of interest.

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Table 1. Characteristics of the children at the 9-year follow-up.

	Probiotic	Placebo
Gender boys/girls	22/34	30/32
Number of siblings,	1.4 ± 0.6	1.6 ± 0.6
Healthy (%)	91	89
Mother with high school/university education (%)	98/68	100/69
Father with high school/university education (%)	98/45	97/52
Breastfeeding (months)	8.0 ± 3.9	7.9 ± 4.2
Exclusive breastfeeding (months)	3.7 ± 1.3	3.8 ± 1.2
One or both parents with a history of smoking (%)	11	12
Soft drinks per week, mean±SD	1.3 ± 1.1	1.4 ± 1.0
Sweets per week, mean±SD	1.6 ± 2.7	1.3 ± 0.5
Milk intakes per day, mean±SD	2.4 ± 1.3	2.5 ± 1.1
Toothbrushing < once per day (%)	100	95
Fluoride toothpaste (%)	96	100

Fig 1. Overview of the study protocol.

Fig 2. Participant flow chart. **A.** Randomised to the probiotic or placebo group. **B.** Completed the intervention phase. **C.** Participated in the follow-up study.

*Delivered by caesarean section. **Withdrawal of consent by parents. ***Had moved from the region/did not consent.

Fig 3. Total caries experience (mean dmfs + DMFS) in the probiotic /placebo group at 3, 6, and 9 years of age. Black lines denote standard deviation

Fig 4. Mutans streptococci, Lactobacilli and Total viable counts (mean log¹⁰ CFU/ml) in the probiotic and placebo groups. Black lines denote standard deviation.