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Oral Administration of *Lactobacillus reuteri* During the First Year of Life Reduces Caries Prevalence in the Primary Dentition at 9 years of Age

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Short title Effect on Caries in Primary Teeth after Administration of *L. reuteri*

Declaration of Conflicts BioGaia AB, Lund, Sweden contributed with material and analyses of *L. reuteri* in saliva. T. Abrahamsson and M. Jenmalm have received honoraria from BioGaia AB for lectures. The other authors report no conflicts of interest.

Abstract

The aim of this study was to evaluate the effect, on oral health at age 9 years, of daily oral supplementation with the probiotic *Lactobacillus reuteri*, strain ATCC 55730 to the mothers during the last month of gestation and to the children through the first year of life. The study comprised a single-blind, placebo-controlled, multicenter trial involving 113 children: 60 in the probiotic and 53 in the placebo group. The subjects underwent clinical and radiographic examination of the primary dentition, carious lesions, plaque and gingivitis were recorded. Saliva and plaque were sampled, for determination of mutans streptococci (MS) and lactobacilli (LB) in saliva and plaque as well as salivary secretory IgA (SIgA). Forty-nine (82%) of the children in the probiotic group and 31 (58%) in the placebo group were caries free ($p < 0.01$). The prevalence of approximal caries lesions was lower in the probiotic group (0.67 ± 1.61 vs. 1.53 ± 2.64 ; $p < 0.05$) and there were fewer sites with gingivitis compared to the placebo group ($p < 0.05$). There were no significant differences between the groups with respect to frequency of tooth-brushing, plaque and dietary habits but to intake of fluoride supplements ($p < 0.05$). There were no intergroup differences with respect to *L. reuteri*, MS, LB or SIgA in saliva. Within the limitation of this study it seems that daily supplementation with *L. reuteri* from birth and during the first year of life is associated with reduced caries prevalence and gingivitis score in the primary dentition at 9 years of age.

There is increasing evidence that probiotic bacteria have an effect on the microbial balance in humans and consequently on health. During the last few decades, this positive effect has been documented with respect to several medical conditions, particularly disturbances of gastrointestinal function [for review, see Thomas et al., 2010]. Other studies have shown that perinatal probiotic supplementation may prevent the development of atopic eczema [Abrahamsson et al., 2007; Jenmalm & Duchén, 2013].

There are a number of reviews of the effect of probiotic bacteria on the oral microbiota and how this may affect dental caries and periodontal disease [Stamatova & Meurman, 2009; Bonifait L et al., 2009; Twetman & Keller, 2012; Twetman, 2012]. Several studies have shown positive effects of probiotic lactobacilli: reduction in the number of mutans streptococci, dental plaque and gingival inflammation [Nikawa et al., 2004; Krasse et al., 2006; Calgar et al., 2008; 2009; Marttinen et al., 2012]. To date, however, only five studies have been published with caries as the endpoint [Näse et al., 2002; Stecksén-Blicks et al., 2009; Petersson et al., 2011; Hasslöf et al., 2013; Taipale et al., 2013], three of which report lower caries increment after exposure to probiotic bacteria compared to control groups. In the study by Näse et al. [2002], preschool children were exposed to milk with *L. rhamnosus* five days a week over seven months at 1-6 years of age. A statistically significant lower caries increment was found in a subgroup of the children, 3-4 years of age, after exposure to probiotic bacteria. Furthermore, a 75% reduction in caries prevalence was observed in preschool children after consumption of milk supplemented with both *L. rhamnosus* and 2.5 ppm fluoride for almost 2 years [Stecksén-Blicks et al., 2009]. In a study in adults with established root caries, Petersson et al. [2011] found partial reversal of the lesions after daily intake, for 15 months, of milk supplemented with probiotic bacteria. In contrast, the study by Taipale et al. [2013] found no difference in caries prevalence in 4-year-old children who during the first year of life had been treated with probiotic bacteria, compared to those who received xylitol or sorbitol administered in the same way. Neither, Hasslöf et al. [2013] found any effect on caries at 9 years of age after supplementation of *L. paracasei* (LF19) in cereals for the first 4-13 months of life.

The somewhat contradictory results of these five studies indicate the need for further clinical studies with caries as the outcome. Moreover, there are no studies to date testing the hypothesis that probiotic supplementation from birth may affect the development of oral microbiota and prevent caries.

The aim of the present study was to compare caries prevalence in the primary dentition of 9-year old children whose mothers during the last month of gestation and the children had

received daily oral *L. reuteri* supplementation during the first year of life, against a true placebo group. The null hypothesis tested was that *L. reuteri* supplementation during the first year of life do not affect the caries prevalence in the primary teeth at 9 years of age.

Material and Methods

Study Population

In 2001-2003, 282 families with a history of allergic diseases and where the mothers were pregnant, were asked to participate in a prospective, randomised, double-blind, placebo-controlled study of the effect of oral *L. reuteri* supplementation on the development of allergic diseases [Abrahamsson et al., 2007]. The families were recruited at the Department of Paediatrics in the County Hospitals in Jönköping, Motala and Norrköping and at the University Hospital in Linköping, Sweden. 232 families were randomized into an active (probiotic) and a placebo (control) group. Randomization of the two groups was stratified for each of the four study centers. The mothers in the test group daily consumed five drops of oil containing living *L. reuteri* derived from breast milk (strain ATCC 55730) during the 4 weeks before the expected date of delivery and continued until the child was born. The drops consisted of freeze-dried *L. reuteri*, suspended in 3 parts refined coconut oil one part refined peanut oil, containing cryo-protective components. The total bacterial content of the five drops was approximately 10^8 colony-forming units. From birth, the infant was daily given five drops orally throughout the first year of life (thus 365 days). The mothers and children in the placebo group were treated in the same way. The placebo drops consisted of the same two oils without any bacteria and could not be differentiated from the active product by smell, taste or visual appearance. All products were manufactured by BioGaia AB (Lund, Sweden). The drops were easy to administer and compliance was high: 188 of the 232 infants (81%) completed the first year of the study; the drop outs were evenly distributed in the two groups [Abrahamsson et al., 2007]. The parents were blinded until the clinical follow-up at two years of age. The study is registered at ClinicalTrials.gov (NCT01285830). At 7 years of age, a medical and interview follow-up focusing on allergy development was performed [Abrahamsson et al., 2013]. Some of the socioeconomic data in the present study originate from this follow up.

At 9 years of age, these 188 children were invited to an oral examination, including radiographs, saliva sampling and a semi-structured interview. Of the 188 invited, 113 agreed to participate in the present study, *i.e.* 49% of the originally enrolled 232 families and 60% of the 188 invited families. The main reason for attrition was that families had moved from the district. The probiotic group finally comprised 60 subjects and the placebo 53. The number of girls/boys was 28/32 in the probiotic and 30/23 in the placebo group. The mean ages (\pm SD) were 9.1 (\pm 0.3) in the probiotic and 9.2 (\pm 0.4) in the placebo group.

Clinical Examination

The 113 children were examined by one of the authors (S.C.) at the respective public dental clinics at which the children received their regular dental care. The examination was performed with a mirror and probe, under optimal light conditions. The examiner was not aware which group the child belonged to. In order to avoid interference with erupting and exfoliated teeth, only primary molars and canines were examined, for caries and restorations (a maximum of 12 teeth). Clinical visible initial caries (d_i) was defined as “a demineralised surface with a chalky appearance” and manifest caries (d_m) as “the minimal level that could be verified as a cavity by gentle probing” [Koch, 1967]. Ten children (in all 486 tooth surfaces) were examined twice at an interval of 14 days: there was complete agreement between the examinations (Cohen’s kappa value = 1).

Radiographic Examination

Two bitewing radiographs were taken; one on each side. Initial approximal caries ($d_{i;a}$) was defined as “a caries lesion in the enamel that has not reached the dentinoenamel junction or a lesion that has reached or penetrated the dentinoenamel junction, but does not appear to extend into the dentine” and manifest caries ($d_{m;a}$) as “a caries lesion that clearly extends into the dentine” [Alm et al., 2007]. The radiographs were analysed by two of the authors (S.C. and M.S.). In the event of disagreement, the findings were discussed until consensus was reached.

Saliva and Plaque Sampling

The participants were instructed not to eat or drink for two hours preceding the sampling. Paraffin-stimulated whole saliva was collected (\approx 3 ml) in a sterile test tube. A pooled plaque sample was collected from 4 approximal sites (in the maxillary and mandibular right molar

regions), for determination of the level of mutans streptococci (MS) and lactobacilli (LB) in relation to the total number of cultivable flora, determined on blood agar. The following analyses were carried out:

1. Analysis of *L. reuteri* was performed by BioGaia AB (Lund, Sweden). The saliva was diluted 10 fold by adding 8 ml of 0.15 M NaCl and 1 ml glycerol, mixed and frozen at -80°C until analysis. The samples were then plated on de Man Rogosa Sharpe (MRS) agar plates, containing 50 mg/L of vancomycin and 20 g/L of sodium acetate (Acumedia, Ljusne, Sweden) The MRS plates were incubated for 48-72 h at 37°C under anaerobic conditions. Reuterin positive colonies were randomly selected from the replicated MRS media and frozen in a freezing medium, containing 4.7 mM K₂HPO₄, 1.3mM KH₂PO₄, 2.0 mM sodium citrate, 2.1 mM Mg-SO₄, 15% glycerol (Merk, Lund, Sweden) and stored at -80° C until further identity using PCR analysis as described by Sinkiewicz et al. 2010]. The microbiological analysis was conducted with the detection limit of $\geq 1.0 \times 10^2$ cfu/ml.

2. MS and LB in saliva and plaque were analysed at the Institute of Odontology, Sahlgrenska Academy, University of Gothenburg, Sweden. The samples were dispersed on a Whirlimixer for 30 s (saliva sample) and 10 s (plaque sample) and serially diluted in 0.05 M phosphate buffer (pH 7.3). 25- μ l portions were plated in duplicate on mitis salivarius with bacitracin (MSB) agar for growth of MS and on Rogosa Selective Lactobacillus (SL) agar for growth of LB. The MSB agar plates were incubated anaerobically in candle jars at 37°C for 2 days and the SL agar plates aerobically at 37°C for 3 days. The number of colony-forming units (CFU) of MS was counted on the MSB agar and identified by their characteristic colony morphology. All CFU in SL agar were considered to be LB. The detection limit for both MSB and SL agars was 100 CFU. Total number of microorganisms in plaque was estimated on blood agar plates (BAP; containing 5% defibrinated blood), which were incubated at 37°C in 95% H₂ and 5% CO₂ for seven days. Number of MS and LB in plaque was then expressed as percentage (%) of the total number of CFU on BPA.

3. SIgA was analysed at the Division of Inflammation Medicine, Linköping University, Sweden, as described previously by Böttcher et al. [2002]. Briefly, an anti-human secretory component antibody (Dakopatts AB, Täby, Sweden) was used for coating, alkaline phosphatase-conjugated goat-anti-human-IgA (Sigma Immunochemicals, Stockholm, Sweden) for detection and human IgA (Sigma Immunochemicals) as a standard.

Gingival Bleeding and Plaque index

Four sites on each primary tooth were registered as “bleeding” or “no bleeding” in response to gentle probing [Löe & Silness, 1967]. After the same teeth had been dried with compressed air, the presence of plaque was registered on the same tooth surfaces as “visible” or “no visible” plaque [Silness & Löe, 1964]. The two indices were calculated by adding the surfaces with gingival bleeding or plaque, divided by the number of examined surfaces and expressed as a percentage.

Interview

At the time of the clinical examination, a parental semi-structured interview was conducted, including questions about tooth-brushing, use of fluorides (mouthrinses and/or lozenges) and dietary habits, including regularly exposure of commercial products containing probiotic bacteria (e.g. yoghurt and juice) during the last 3 months. The frequency of daily “caries-risk products” was analyzed with a frequency index as described by Wendt & Birkhed [1995]. Data concerning background factors and potential confounders during infancy (Abrahamsson et al, 2007) and at seven years of age (Abrahamsson et al., 2013) are presented in Table 1.

Statistical Analyses

Before the study, a power analysis was performed based on 8-year-old children in the same geographical area and with a known caries prevalence. The caries prevalence in the sample was 1.9 decayed/filled tooth surfaces with a standard deviation of 3.6 and with an assumption to detect a reduced caries prevalence by half with a significance of 5% and power of 80%. The necessary number of individuals in each group was estimated to around 50. The data were analysed using the Statistical Package for the Social Sciences software program SPSS ver. 19.0 (SPSS Inc., Chicago, IL, USA). Chi-squared tests were used to test the association with categorical dependent variables. Mann-Whitney *U*-tests were used for continuous dependent variables. The results for some continuous variables are presented as mean \pm standard deviation (SD), range, and 95% confidence interval (CI). The level of significance was set at $p < 0.05$. For all comparisons, the *p*-values are presented, even if not significant.

Ethical Considerations

The present odontological study and the original medical study [Abrahamsson et al., 2007] were approved by the Regional Ethics Committee for Human Research at Linköping

University, Sweden (M122-31 and M171-07, respectively). Written informed consent was given by the parents or guardians before the dental examination.

Results

Dental Caries

Caries prevalence at 9 years of age is presented in Table 2. The mean number \pm SD of primary teeth was 10.9 ± 2.5 in the probiotic group and 10.7 ± 3.3 in the placebo group. In the probiotic group, 49 (82%) of the children were caries free in the primary dentition, compared with 31 (58%) of the children in the placebo group (Chi square test, $p = 0.008$) and the null hypothesis was rejected. The mean number \pm SD of initial and manifest approximal caries lesions was 0.67 ± 1.61 in the probiotic group and 1.53 ± 2.64 in the placebo group ($p < 0.05$). There were no statistically significant differences between girls and boys with respect to caries prevalence.

Microbiological Factors

No statistically significant intergroup differences were found with respect to MS or LB in saliva or in plaque. The mean concentration $\text{mg/L} \pm \text{SD}$ of salivary SIgA was 132 ± 71 in the probiotic group and 111 ± 59 in the placebo group ($p = 0.08$; Table 2). *L. reuteri* was detected in only 4 children (2 in each group).

Gingival Bleeding and Plaque Index

The frequency of sites with gingival bleeding was lower in the probiotic group than in the placebo group ($p = 0.05$). No statistically significant difference was found for Plaque Index (Table 3).

Dietary Habits, Oral Hygiene and Socioeconomic factors

Dietary habits including consumption of sugary drinks and other products containing sugar were similar in the two groups as well as intake of commercial products containing probiotics. Fluoride supplements were more frequently used in the placebo group (Table 3). At 7 years of age, no differences were found between the groups concerning family size, day-care, parental smoking and breastfeeding at 6 months of age (Table 1).

Discussion

The most important results in the present study are that 9-year old children who had received perinatal *L.reuteri* supplementation from birth and during their first year of life were more often caries free and had a lower prevalence of approximal caries than those in the placebo group. Our findings are in accordance with those of Näse et al. [2002], Stecksén-Blicks et al. [2009] and Petersson et al. [2011] but not with Taipale et al. [2013] and Hasslöf et al. [2013]. Direct comparison of the studies is difficult because of lack of uniformity in design: the probiotics were administered in different ways, with different strains of bacteria and in different combinations. However, most of the clinical studies with caries as the end point support the finding of the present study that the probiotic bacteria supplement has a certain caries-reducing effect.

The present study did not disclose any effect of exposure to probiotic bacteria on the quantities of MS and LB cultured on agar plates. In contrast, most earlier studies [Näse et al., 2002; Ahola et al., 2002; Nikawa et al., 2004; Calgar et al., 2005; 2008; 2009; Marttinen et al., 2012], report inhibited growth of MS. However, in accordance with the present results, other studies have found no effect of probiotic supplementation on the cariogenic microbiota [Mont alto et al., 2004; Stecksén-Blicks et al., 2009; Peterson et al., 2011, Hasslöf et al. [2013]. These conflicting findings might be explained by the different intervals between administration of probiotics and the oral microbiota analyses. In the present study, the probiotics were administered around 8 years before the saliva sampling and oral microbiota analyses. Previous studies demonstrated that probiotic bacteria are not permanently established, but only temporary [Calgar et al., 2008; Yli-Knuutila et al., 2006]. It should be noted that culturing cariogenic microorganisms on selective agar plates reveals only the number of CFU and provides no information about their virulence. One interesting finding was that the SIgA levels tended to be higher in the children receiving probiotics than in the placebo group ($p = 0.08$). This might have inhibited the activity of MS and caries development [Bratthall et al., 1997], but needs to be further investigated.

The probiotic group had a statistically significantly lower frequency of gingival inflammation than the control group. While an effect on gingival inflammation has been

found earlier [Krasse et al., 2006], it is difficult to compare the finding in our study, as such a long-lasting effect after administration of probiotics has not previously been reported. In a study by Abrahamsson et al. [2009], the authors concluded that *L. reuteri* may be detected in breast milk after supplementation to the mother. At start of the original study this possibility was discussed and was therefore the rationale for giving the mothers the probiotics to have an optimal colonization of *L. reuteri* in the children. Furthermore, in a recent study by Holgerson et al. [2013], presence of lactobacilli in breast milk inhibited the growth of MS, which may affect caries development in children. With today's knowledge of the positive effects of administration of probiotics on both general and oral health, future studies may evaluate higher total dose of probiotic bacteria than in the present study.

Although dietary and oral care habits were similar in the two groups and that the placebo group showed a higher intake of fluoride supplement, children in the probiotic group had lower caries prevalence. If the group-awareness after 2 years of age influenced the use of probiotics or general care is difficult to assess. However, not until the age of 9 years the question of any effect on oral health was raised. Thus no changes in oral health behavior could be expected in the two groups. One limitation of the present study is that socio-economic factors, which are recognized as influencing caries development, were not analyzed at 9 years of age. However, the participating children were randomized into the probiotic and placebo groups at birth, reducing the risk for bias. The double-blind placebo-controlled design contributed to the strength of the study. Data from the interview at 7 years of age revealed no difference concerning socioeconomic factors between the two groups.

Compliance with treatment was high, and during the children's first year of life there was no commercial product with *L. reuteri* on the market in Sweden that might have interfered with the test bacteria [Abrahamsson et al., 2007].

The uniqueness of this study is that the supplements had been given from birth, before the establishment of the oral microbiota (the so called "open window effect") and continued longitudinally during the first year of life. At 9 years of age, no differences in microbiological values or salivary factors could be detected. Despite this, the difference between the two groups with respect to caries prevalence in the primary dentition was significant. This is noteworthy, given that the primary teeth examined were unerupted at the time of the intake of probiotic bacteria. Further studies are warranted, to study the effect of early oral

supplementation with probiotics, preferably from birth, and to analyse further the effects of probiotic supplementation.

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