Clinical microbiology

The impact of perinatal probiotic intervention on gut microbiota: Double-blind placebo-controlled trials in Finland and Germany

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ABSTRACT

Specific probiotic combinations during early feeding, via the mother or incorporated in early formula-feeding, mold the intestinal microbiota composition in infants. The objective was to analyze the impact of probiotic administration to mother or infant on gut microbiota composition in 6-month-old Finnish and German infants. In Finland probiotics were given to mothers ($n = 79$) for 2 months prior to and 2 months after delivery. In Germany probiotics were started in infants ($n = 81$) at weaning, at the latest at 1 month of age, and continued for 4 months. A breast-fed group of 6-month-old infants (22 from Finland, 8 from Germany) were compared. Gut microbiota were analyzed by FCM-FISH and qPCR methods. In breast-fed infants a trend toward higher counts of bifidobacteria was detected in Finland ($p = 0.097$) as against Germany, where a more diverse microbiota was reflected in higher *Akkermansia* ($p = 0.003$), *Clostridium histolyticum* ($p = 0.035$) and *Bacteroides–Prevotella* ($p = 0.027$) levels and a higher percentage of *Akkermansia* ($p = 0.004$). Finnish LPR + BL999 intervention group (*Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999) had higher percentages of fecal *Lactobacillus–Enterococcus* (9.0% vs. 6.1% placebo, $p = 0.003$) and lower bifidobacteria levels (10.03 log cells/g vs. 10.68 log cells/g placebo, $p = 0.018$). Probiotic treatment had different impacts on gut microbiota composition in Finnish and German infants due to differences in mode of feeding and the early commensal microbiota. Probiotic treatment had different impacts on gut microbiota composition in Finnish and German infants due to differences in mode of feeding and the basic commensal microbiota.

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1. Introduction

Intestinal colonization of a newborn starts at birth and continues during infancy. After weaning, the gut microbiota becomes more diverse, and continues to develop toward an adult microbiota [1]. Microbiota development depends on the first inoculum, the mother's microbiota, mode of delivery and the environment, including feeding practices and use of antimicrobials [2,3]. Bifidobacteria constitute up to 60–70% of the total microbiota of healthy breast-fed infants [2,4,5], with *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium breve* as the most predominant species in different geographic areas [6–10]. The gut microbiota of formula-fed infants, again, may be more diverse, harboring more *Bacteroides*, *Clostridium* and *Enterobacteriaceae*

[3,6]. Deviations in microbiota composition such as low numbers or aberrant species of bifidobacteria have been associated with a higher risk of allergic and infectious diseases [11,12] and even obesity [13,14].

A probiotic is defined as a "live microorganism which when administered in adequate amounts confers a health benefit on the host" [15]. Administration of probiotics perinatally and during the first months of life may have a long-term beneficial influence on the composition and development of the infant's bifidobacteria, potentially leading to a reduced risk of atopic disease [16,14]. The limited number of intervention studies thus far have yielded different outcomes [17,18]. Reasons for this may be related to differences in target populations or probiotic strains used in preparation and in dosing together with the mode of administration. We hypothesized here that the diet of the infant, the probiotic preparation, but also the pre-existing microbiota and the environment, may influence the probiotic potential to modify the gut microbiota composition.

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We therefore investigated the intestinal microbiota composition and structure in Finnish and German infants who received specific probiotic combinations during early feeding either via the mother or incorporated in early formula-feeding. Breast-fed infants were also included to detect differences in gut microbiota composition between two breast-fed cohorts in different countries.

2. Methods

2.1. Study design and subjects

The study was conducted as a randomized, double-blind, placebo-controlled clinical trial with 3 groups in each country.

In Finland the subjects were informed about the study by leaflets distributed during their first visit to maternal welfare clinics in the city of Turku and neighboring areas in South-West Finland. Interested recipients contacted the research nurse, who gave further information on the study and scheduled their first visit to the study clinic in Turku University Central Hospital. Finnish clinical trial is registered at ClinicalTrials.gov identifier: NCT00167700.

The Finnish study cohort comprised 79 infants from an ongoing allergy prevention study. Power analysis was made for the main study, where the primary outcome was prevalence of atopic eczema at 2 years of age. The probiotic product and placebo were given to the mothers for 2 months before delivery and for 2 months after delivery during breastfeeding. The test product was provided by Nestle and it was based on product Pro Natal but the prebiotic ingredients such as inulin and oligofructose had been removed and specific probiotic added. The placebo was Pro Natal matrix without prebiotics and probiotics. Probiotics or placebo were used for a maximum of 4 months. Twenty-eight mothers received a probiotic product consisting of Lactobacillus rhamnosus LPR (CGMCC 1.3724) with B. longum strain BL999 (ATCC: BAA-999), (LPR + BL999), 29 mothers received Lactobacillus paracasei ST11 (CNCM 1-2116) with B. longum BL999 (ST11 + BL999), and 22 placebo. The dose to the mother was 10^9 CFU/day of each probiotic strain provided in one sachet of 7 g per day (powder form), diluted in a glass of water. The mothers in Finland were allocated to study groups according to a computer-generated, blocked randomization list and were chosen on the criterion that infants were breast-fed exclusively until 4 months of age and partially/exclusively breast-fed until 6 months.

The German study population comprised 81 infants receiving early hypoallergenic formula with or without probiotics as part of an allergy prevention study. All children had a familial atopic background, proven by the presence of specific IgE in at least one parent. Recruitment commenced before 1 July 2005.

Twenty-four infants received partially hydrolyzed formula supplemented with L. rhamnosus LPR (CGMCC 1.3724) and B. longum BL999 (ATCC: BAA-999) (LPR + BL999), 25 received partially hydrolyzed formula with B. longum BL999 (ATCC: BAA-999), and 32 received partially hydrolyzed formula. The control and formulas were based on Beba-HA (=Nan-HA), the partially hydrolyzed 100% whey formula produced by Nestle for the study. The probiotic and placebo formulas were administered to the infants when they went onto formula, at the latest at 1 month of age. The intervention lasted for 4 months. The dose of the probiotic was at least 10^9 CFU/day of each strain provided in metallic tins, each containing 400 g of study formula.

In addition, in Germany 8 breast-fed 6-month-old infants who were not randomized into the study were included.

For the present study all subjects were randomly chosen and identified by a statistician. The randomization codes were available to the statistician only in order to maintain blindness for the ongoing main studies.

This study was conducted according to the guidlines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Committee on Ethical Practice of Turku University Hospital in Finland and in the Marien-Hospital Wesel in Germany. Written informed consent was obtained from all participants.

From both Finish and German study groups, fecal samples were collected when the infants reached the age of 6 months. These were stored frozen and transported to the laboratory in the Functional Foods Forum, University of Turku in Turku, Finland, and stored at $-70\ ^\circ$C until analyzed.

2.2. Sampling preparation and DNA extraction

Fecal samples were taken for analysis at 6 months of age. The samples (0.5 g) were weighed, diluted 1:10 (w/v) in phosphate buffer PBS (pH 7.4) and homogenized by thorough agitation in a vortex. Aliquots of these dilutions were used for DNA extraction. DNA from both feces and from pure cultures of the different bacterial strains used as reference were extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.3. Real-time polymerase chain reaction (qPCR) analysis

Quantitative real-time PCR was used to characterize the fecal microbiota using group- and species-specific primers as previously described. These oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany).

Briefly, PCR amplification and detection were performed with an ABI PRISM 7300-real-time PCR system (Applied Biosystems, Foster City, California). Each reaction mixture of 25 μl was composed of Power SYBR Green PCR Master Mix (Applied Biosystems), 1 μl of each of the specific primers at a concentration of 0.2 mol/L, and 1 μl of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the non-targeted PCR product.

The bacterial concentration in each sample was calculated by comparing the C_t values obtained from standard curves. A standard curve was made from serial dilutions of DNA isolated from each pure culture of the different reference strains. A linear relationship was observed between cell numbers and C_t values ($r^2 = 0.99-0.97$).

The following reference strains were used to construct the corresponding standard curves: B. longum (DSM 20219) (this strain was also used as the standard strain for quantification of the Bifidobacterium genus), Bifidobacterium catenulatum (JCM 7130), Bifidobacterium bifidum (DSM 20456), Bifidobacterium lactis (DSM 220606), B. infantis (DSM 20090), Bifidobacterium adolescentis (DSM 20083), B. breve (DSM 20213), Akkermansia muciniphila (ATTC BAA-835), Staphylococcus aureus (DSM 20231), Clostridium coccoides (DSM 935^T), Clostridium leptum (DSM 753^T), Clostridium difficile (DSM 1296^T) and Clostridium perfringens (DSM 756). The primer sequences of the reference strains and the annealing temperatures of the primers have been published elsewhere [7,13,19,20].

2.4. Flow cytometry − fluorescent in situ hybridization (FCM-FISH) analysis

Homogenized fecal samples were fixed overnight in 4% paraformaldehyde and stored in PBS-ethanol at $-20\ ^\circ$C until analyzed. Fluorescent in situ hybridization (FISH) with flow cytometer was performed as previously described [13,21]. In brief, samples were hybridized at specific temperatures in hybridization buffer with specific probes at a concentration of 5 ng/μl. After overnight