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Clinical microbiology

Long-term monitoring of the human intestinal microbiota from the 2nd week to 13 years of age

Akihito Endo [a, *, 1], Anna Pärtty [b], Marko Kalliomäki [b], Erika Isolauri [b], Seppo Salminen [a]

[a] Functional Foods Forum, University of Turku, Itäinen Pitkäkatu 4A, Turku 20014, Finland
[b] Department of Pediatrics, Turku University Hospital, University of Turku, Turku, Finland

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ABSTRACT

Microbial contact begins prior to birth and continues rapidly thereafter. Few long term follow-up studies have been reported and we therefore characterized the development of intestinal microbiota of ten subjects from the 2nd week of life to 13 years of age. PCR-denaturing gradient gel electrophoresis combined with several bacterial group-specific primer sets demonstrated the colonization steps of defined bacterial groups in the microbiota. *Bifidobacterium* species were seen throughout the test period in all subjects. *Bacteroides fragilis* group and *Blautia coccoides–Eubacterium rectale* group species were not detected in several subjects during the first 6 months of life but were commonly seen after 12 months of life. *Streptococcus* group appeared during early life but was not seen in several subjects at the age of 13 years. Although a few species were linked with the increasing age, major bacterial species in the groups did not change dramatically. Rather considerable changes were found in the relative abundances of each bacterial species. Clustering analysis of total bacterial flora indicated that the microbiota changed considerably between 6 months and 12 months of life, and, at the age of 12 months, the intestinal microbiota was already converted toward a profile characteristic of an adult microbiota. Probiotic supplementation in the beginning of life did not have major impacts on later microbiota development.

1. Introduction

The adult human intestinal tract is colonized by a large and complex microbiota, the largest collection of active cells in the body. This microbiota plays various important roles in host systems, including food digestion, immunological homeostasis, protection against colonization by pathogens, host fat storage and brain development and behavior [1–5].

Traditionally, the infant prior to birth was thought to be sterile, the initial contact with bacteria occurring during birth. However, recent studies have reported that microbial contact begins prior to birth and continues thereafter in an accelerated manner [6,7]. From birth, the intestinal microbiota develops rapidly in a succession of bacterial strains. Numerous host and environmental factors are associated with this process [8–10], and importantly, the development of the intestinal microbiota has been connected to that of a number of health problems such as gastroenteritis, irritable bowel syndrome, and even overweight [9,11]. Despite the reported significance of microbiota development, composition and activity, very few studies have monitored it over a longer term, perhaps due to difficulties in organizing a controlled follow-up. Long-term monitoring of the microbiota in early life would thus be essential in characterizing the relationships between microbiota development and any health consequences.

In the present study, we monitored the development of the intestinal microbiota in ten subjects from the 2nd week of life to 13 years of age. PCR-denaturing gradient gel electrophoresis (PCR-DGGE), in combination with DNA primer sets specific for the *Bifidobacterium*, *Bacteroides fragilis*, *Blautia coccoides–Eubacterium rectale*, *Lactobacillus*, *Streptococcus* groups and total bacteria, was used for analysis of the intestinal microbiota.

2. Methods

2.1. Ethics statement

The study was approved by the committees on ethical practice in Turku University Hospital and the Health Office of Turku. Written

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

* Corresponding author. Tel.: +358 2 333 6894.
E-mail addresses: akihito.endo@utu.fi, a3endou@bioindustry.nodai.ac.jp (A. Endo).
[1] Present address: Department of Food and Cosmetic Science, Faculty of Bioindustry, Tokyo University of Agriculture. Yasaka 196, Abashiri, Hokkaido 099-2493, Japan.

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informed consent was obtained from the parents and children, when the children were of receptive age.

2.2. Subjects

The subjects participating in this study were enrolled for an ongoing randomized, double-blind, placebo-controlled study involving perinatal *Lactobacillus rhamnosus* GG (ATCC 53103) intervention (http://www.clinicaltrials.gov/ct/gui/show/NCT167700), as described in detail elsewhere [12,13]. In brief, the mothers of children-to-be were recruited in the antenatal clinics of the city of Turku (population 170,000). The recruiting criterion for the intervention study was that at least one family member had atopic dermatitis, allergic rhinitis or asthma. The mothers were randomized in a double-blind, placebo-controlled manner to receive 1×10^{10} colony-forming units of *L. rhamnosus* GG (ATCC 53103) or placebo (microcrystalline cellulose) capsules once a day for 4 weeks before expected delivery. After delivery, the capsule contents were given either to the child, mixed with water, or continuously to the mother, if breast-feeding, for 6 months. Fecal samples were collected at the age of 2 weeks, 3 months, 6 months, 12 months, 24 months and 13 years. The inclusion criterion for the monitoring study was that subjects had available fecal samples from at least 2-time points at 2 weeks, 3 months or 6 months, 1 time-point at 12 months or 24 months and at 13 years. In total, ten subjects (six with perinatal probiotic administration and four with placebo administration) fulfilled the criterion and were enrolled for the present study (Tables 1 and 2).

2.3. DNA extraction from fecal samples and reference strains

DNA extraction from fecal samples was performed using the ZR Fecal DNA MiniPrep™ (ZYMO Research Corp. CA). A number of bacterial strains, including *Bifidobacterium* spp., *Bacteroides* spp., *Parabacteroides distasonis*, *Blautia* spp., *Clostridium nexile*, *Eubacterium hallii*, *Ruminococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Weissella confusa* and *Enterococcus* spp. were used as references for DGGE analysis. These strains were cultured in GAM broth (Nissui Co., Tokyo, Japan) supplemented with 0.5% glucose at 37 °C overnight under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in an anaerobic work station (Concept 400 anaerobic chamber, Tuskin Technology, Leeds, UK), except that *Lactobacillus* spp., *Leuconostoc* spp., *W. confusa* and *Enterococcus* spp. were cultured in MRS broth (Oxoid Ltd., Hampshire, England). DNA extraction from the reference strains was performed by a method previously described [14].

Table 1
Clinical Characteristics of the subjects.

Subject	Gender	Delivery	Maternal and neonatal probiotics/placebo	Exclusive and total breast-feeding terms (month)	Number of antibiotics during the first 6 month of life
1	Male	Vaginal	Placebo	0.5, 2[a]	3
2	Male	Caesarian	Probiotics	0.5, 2	0
3	Female	Vaginal	Probiotics	3, 7	1
4	Female	Vaginal	Probiotics	1, 3.5	1
5	Female	Vaginal	Placebo	4, 5	0
6	Male	Vaginal	Probiotics	3.5, 11.5	0
7	Female	Vaginal	Probiotics	6, 7	1
8	Female	Vaginal	Placebo	4.5, 11	0
9	Male	Vaginal	Placebo	1.5, 3.5	0
10	Male	Vaginal	Probiotics	4.5, ND[b]	2

[a] exclusive/total breastfeeding term.
[b] no data available.

Table 2
Time points used for analysis.

Subject	2 weeks	3 months	6 months	2 months	24 months	24 months
1	A[a]	A	NA	A	A	A
2	A	A	NA	A	A	A
3	A	NA	A	A	A	A
4	A	NA	A	NA	A	A
5	A	NA	A	A	A	A
6	NA	A	A	NA	A	A
7	NA	A	A	A	A	A
8	NA	A	A	A	NA	NA
9	NA	A	A	A	A	A
10	NA	A	A	A	A	A

[a] A, available.

2.4. PCR-DGGE and data analysis

The PCR primers used in the present study are shown in Table 3. PCR amplification of DNA from *Bifidobacterium* group and *Lactobacillus* group was conducted as described by Endo et al. [15], that of total bacteria and the *B. fragilis* group as described by Vanhoutte et al. [16], that of the *B. coccoides–E. rectale* group as described by Maukonen et al. [17], and of *Streptococcus* as described by Endo et al. [14]. Amplification was confirmed by agarose gel electrophoresis. The detection limits for the PCRs ranged from 10^5 to 10^6 CFU/g of feces.

DGGE analysis of each PCR product was conducted with a DCode System (Bio-Rad Laboratories, CA), as previously described [14]. DNA bands were identified by migration comparison of DNA bands of the reference strains. The DNA bands which were predominantly found but which could not be identified by migration comparison were identified by sequencing as described elsewhere [14]. The determined sequences were compared with known sequences at GenBank by BLAST analyses. The nucleotide accession numbers for the sequences determined are AB780427-AB780439.

DGGE images were imported into the BIONUMERICS software ver. 6.6 (Applied Maths, Belgium) for normalization and band detection. Band search and band matching using a band tolerance of 1% were performed as implemented in the BioNumerics. The relative abundance of each species in each bacterial group was determined according to the software instructions. Further, the dendrogram was constructed from the DGGE images for total bacteria using Pearson correlation by the unweighted pair-group method using the arithmetic averages (UPGMA) clustering method.

3. Results

3.1. Total bacterial flora

All samples generated PCR amplicons in the universal bacterial primer set and these were subjected to DGGE analysis. Clustering analysis of the DGGE profile produced two main clusters. The first was composed mainly of the samples at 12 months, 24 months and 13 years (childhood cluster; Fig. 1). The second cluster contained the samples from 2 weeks, 3 months and 6 months (infant cluster, Fig. 1). Samples taken at 12 and 24 months from subject no. 1, who had three courses of antibiotics during the first 6 months of life, were also included in the latter cluster (Fig. 1).

3.2. Bifidobacterium microbiota

All the samples produced PCR amplicons by *Bifidobacterium*-specific primer set and the amplicons were subjected to DGGE analysis. *Bifidobacterium longum* was dominant bifidobacterium in