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Intestinal microflora of Estonian and Swedish infants

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The intestinal microflora of 1-y-old healthy Estonian ($n = 27$) and Swedish infants ($n = 29$) was studied by quantitative culture of faecal samples. The major differences were high counts of lactobacilli and eubacteria in the former and increased numbers of clostridia in the latter babies. Bifidobacteria and anaerobic cocci prevailed equally in both groups, while eubacteria and enterococci were the major microorganisms in many Estonian infants and bacteroides and clostridia in many Swedish infants. The microflora of the Estonian infants was in many aspects similar to the flora prevailing in infants of western Europe in the 1960s. The results suggest a shift in the intestinal microflora among infants in western industrialized countries.
□ *Geographic differences, infants, intestinal microflora*

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The composition of the intestinal microflora, the regulatory mechanisms and the significance for the host have been the object of extensive studies for many years. There are geographic variations in the human gastrointestinal microflora, as recently summarized by Salminen and coauthors (1). For example, in western Europe the intestinal flora comprises more anaerobic bacteria (bifidobacteria, bacteroides) and significantly fewer streptococci, enterococci, lactobacilli and yeasts than the flora in Uganda, Japan and southern India (2, 3). These differences may be associated with the high consumption of meat and fat by many Europeans and a more vegetarian diet in other parts of the world. Recently, it was shown that a change to an uncooked extreme vegan diet, known as living food, and subsequent return to a conventional mixed Western diet, both induced major changes in the faecal microflora (4, 5).

Several other factors may also explain the differences in the intestinal flora between populations, starting from infancy. In a comparative study of neonates it was found that Pakistani infants were colonized with aerobic Gram-negative bacteria at a significantly earlier age and with more species of enterobacteria than Swedish babies (6). Similarly, extensive colonization with enterobacteria was found in Estonian but not in Finnish neonates (7, 8). It is possible that major improvements in general living standards in western Europe over the past few decades and highly effective antiseptic and aseptic cleaning procedures at delivery (9–11) have induced changes in the maternal vaginal microflora. Furthermore, an increased use of antibiotics may have altered the normal microflora of infants in many countries (12, 13).

There is increasing evidence that both the prevalence and severity of atopic diseases are increasing in industrialized countries with a market economy (14, 15). The intestinal flora exerts a regulatory effect on the maturation of the immune system (16). It is not known, however, whether differences in the composition of the intestinal microflora during infancy could influence the postnatal maturation of the immune system. This possibility has been addressed in prospective studies of new-born infants in Estonia with a low prevalence and in Sweden with a high prevalence of childhood allergy (17). As a part of these studies the faecal microflora was compared in healthy 1-y-old Estonian and Swedish infants.

Material and methods

Study group

The study group comprised 27 Estonian (12M, 15F; group 1) and 29 Swedish (15M, 14F; group 2) healthy 12-month-old infants. The collection of specimens was carried out in Tartu, Estonia, from October to December 1995 and in Linköping, Sweden, from March to December 1995.

The duration of exclusive or partial breastfeeding, the number of atopic infants, defined by a positive skin prick test and eczema or asthma (17, 18), and the number of infants treated with antibiotics according to the retrospective questionnaire were similar in the two groups. Unfortunately, as the study was cross-sectional no accurate information was available on the use of antibiotics in individual children (Table 1).

Approximately 1 g voided stool was collected into sterile plastic containers by the parents. In Estonia, the samples were kept in a domestic refrigerator at 4°C for no more than 2 h before delivery to hospital, where they were frozen at −20°C. The Swedish families all had a deep freezer at home and the samples were frozen at home within 1 h,

Table 1. Clinical data of Estonian ($n = 27$) and Swedish ($n = 29$) infants.

Clincal data	Estonian	Sweden
Number of infants fed exclusively by breastmilk	23	18[a]
Duration (months)		
Median	3	4
Range	0–12	0–8
Number of infants fed by any breastmilk	26	25
Duration (months)		
Median	5	7.5
Range	0–12	1–12
Antibiotic treatment (no.)		
One period	5	4
Two or more periods	4	8
Atopic disease (no.)	7	7

[a]Data on the breastfeeding of four Swedish children are omitted, as their microflora data did not differ from the others.

None of the differences was statisticially significant (Mann–Whitney *U*-test).

delivered to the hospital within 1 month and then transported to Estonia in dry ice for analysis. All samples were kept at –20°C for not more than 3 months.

Bacteriological analysis

Weighed samples of faeces were serially diluted (10^{-2} to 10^{-9}) under a stream of CO_2 in prereduced phosphate buffer (pH 7.2). The bacteria were quantitated by serial dilutions on 10 different freshly prepared media, i.e. Fastidious Anaerobes Agar (FAA, LabM) for total anaerobes; Columbia CNA agar (BBL) for Gram-positive anaerobes; Schaedler agar (BBL) with vancomycin and nalidixic acid supplement (Oxoid) for Gram-negative anaerobes; Cefoxitin–Cycloserine–Fructose Agar (CCFA, Oxoid) supplemented with egg yolk and sodium taurocholate for *Clostridium difficile*; yeast-extract agar for total aerobes; yeast-extract agar with 6.5% of sodium chloride for staphylococci; Endo agar for coliforms; Leeds Acinetobacter Medium (LAM) with vancomycin at 10 mg l^{-1}, cefsulodin at 15 mg l^{-1} and cephradine at 50 mg l^{-1} for acinetobacteria (19); MRS agar (Amersham) for microaerophiles such as lactobacilli and streptococci; and Sabouraud medium supplemented with penicillin (50 000 U l^{-1}) and streptomycin (40 000 U l^{-1}) for yeasts and fungi. The total counts of clostridia were estimated on FAA after ethanol treatment (20).

The yeast-extract agar, salt-yeast-extract agar, Endo medium, LAM and Sabouraud medium were incubated for 24–48 h aerobically at 37°C and inspected after 24 and 48 h. MRS medium was incubated in a microaerophilic atmosphere (GampyPak Plus, BBL) for 72 h, whereas FAA, Columbia CNA, Schaedler agar and CCFA medium were incubated for up to 4–5 d in an anaerobic container with 85% N_2, 10% CO_2 and 5% H_2 (BBL).

The colony counts of different dilutions were recorded and all colonies of different morphology from the highest dilutions with growth were Gram stained and subjected to microscopy. The microbial counts were given in log colony-forming units per gram faeces (CFU g^{-1}). The detection limit of microorganisms was 3 log CFU g^{-1}.

The microorganisms were identified mostly on the genus level. For identification of enterobacteria on a species level, standard methods were used [estimation of oxidase, indole, growth/fermentation pattern on Kliegler iron agar (BBL), Simmons citrate agar, acetate and sodium malonate agar]. Coagulase test was employed for differentiation of *Staphylococcus aureus* and coagulase negative staphylococci (CNS). Streptococci and enterococci were identified by catalase production and fermentation of esculine, and lactobacilli according to their cell morphology and negative catalase test. The Gram-negative coccobacteria were identified as acinetobacteria by expressing a particular fermentation/growth pattern on Kliegler agar, negative oxidase and motility tests (21). The identification of anaerobes was performed according to their growth on selective media, colonies and cells morphology (22, 23). *Clostridium difficile* was identified according to its ability to grow on CCFA, colonial and cellular morphology, Gram staining, typical smell and absence of growth in aerobic and microaerophilic conditions (21).

Statistical methods

The total count (log $_{10}$ CFU g^{-1}) of microorganisms and the counts of various genera and species were calculated for each child. In addition, the relative amounts of the particular microbes were expressed as a proportion of the total count (%), employing the statistical program "Bioquant" for personal computer, which gives output data for every microorganism as the absolute count (log CFU g^{-1}) and their percentage in the total count with its normal values, elaborated by investigating healthy 5–12-month-old infants. The microorganisms were considered potentially predominant if they made up more than 10% of total population, e.g. a difference more than 1 log from the subordinate microbes. In healthy infants bifidobacteria, eubacteria, anaerobic cocci or bacteroides as single or combined populations usually prevail (7). If another microorganism comprised more than 10% of the total population, it was named the "major microorganism".

Since the counts of microorganisms were skewed, the Mann–Whitney *U*-test was used to compare the prevalence and the counts of bacteria in faecal samples of Estonian and Swedish infants.

The mean values and 95% confidence intervals were used for comparison of the relative amounts of microorganisms in the children of the two groups. The prevalence of the predominating microorganisms in Estonian and Swedish infants was compared by the χ^2 test.

Results

Quantitative comparisons

The Estonian children harboured lactobacilli ($p < 0.01$) more frequently and the counts were higher than in the

Table 2. Composition of the faecal microflora of 1-y-old Estonian and Swedish infants, the prevalence of various species and the counts (log CFU g^{-1}).

	Estonian			Swedish		
	%	Median	Range	%	Median	Range
Aerobes	100	8.0	5.3–11.3	100	7.1	5.3–9.7
CONS	37	0	0–6.8	48	0	0–8.6
S. aureus	48	0	0–6.8	55	4.3	0–8.3
Enterococci	85	7.0	0–9.6	90	6.3	0–9.3
Coliforms	93	5.0	0–11.3	83	4.6	0–9.3
Streptococci	15	0	0–9.3	21	0	0–9.3
Lactobacilli	63[b]	4.2	0–10.3[c]	38[b]	0	0–8.6[c]
Candida	44	0	0–8.8	45	0	0–6.8
Anaerobes	100	8.0	5.3–10.6	100	8.5	5.8–11.3
Peptostreptococci	70	6.0	0–9.0	59	5.3	0–9.6
Bifidobacteria	70	7.0	0–9.3	72	7.3	0–11.2
Eubacteria	37[c]	0	0–10.6[c]	0[c]	0	0[c]
Bacteroides	74	7.0	0–9.8	90	7.0	0–10.3
Clostridia	74	4.0	0–8.3[a]	69	5.8	0–10.0[a]
C. difficile	4[c]	0	0–4.0[b]	34[c]	0	0–7.3[b]

0 = detection limit values < 3.0 log CFU g^{-1}.
[a]$p < 0.05$; [b]$p < 0.01$; [b]$p < 0.001$ (Mann–Whitney *U*-test).

Swedish babies ($p < 0.001$, Table 2). Furthermore, eubacteria were detected in 37% of Estonian samples but in none of the Swedish children ($p < 0.001$). In contrast, clostridia counts were higher in the Swedish infants ($p < 0.05$) and, in particular, *C. difficile* was more common than in the Estonian infants ($p < 0.001$).

The Estonian and Swedish infants were similarly colonized with staphylococci (CNS and *S. aureus*), streptococci, enterococci, coliforms, anaerobic cocci, bifidobacteria, bacteroides and yeasts (Table 2). Acinetobacteria were not found in any of the faecal samples, in either Estonian or Swedish infants.

Escherichia coli was isolated from 11 Estonian and 12 Swedish infants and *Klebsiella pneumoniae* from three and four infants, respectively. The corresponding figures for *Citrobacter freundii* were one and two infants, respectively, and *Enterobacter aerogenes* from two infants in both groups. Gram-negative nonfermenters, bacilli, sarcina, corynebacteria and fungi were isolated in occasional cases at a similar frequency (data not shown).

Relative amounts of microorganisms

The relative amounts of anaerobic (Fig. 1) and aerobic (Fig. 2) microorganisms differed in the Estonian and the Swedish infants. Thus, the mean proportion of enterococci ($p < 0.01$) was higher in Estonian children, while the proportions of streptococci ($p < 0.05$) and *C. difficile* ($p < 0.001$) were higher in the Swedish infants.

Eubacteria were part of the predominating flora in six of the Estonian but none of the Swedish children ($p < 0.01$). This was also true for enterococci (14 vs 4 infants, $p < 0.05$, Fig. 2), while bacteroides and clostridia were more commonly the major microorganisms in the Swedish babies (6 vs 14, $p < 0.05$, and 1 vs 6, $p < 0.01$, respectively, Fig. 1). Bifidobacteria and anaerobic cocci were equally often part of the predominating flora in both groups of infants (13 vs 16 and 7 vs 9 infants, respectively, Fig. 1).

Discussion

Several parameters were chosen to compare the intestinal microflora of Estonian and Swedish infants. In addition to the usual analysis of the prevalence and absolute counts of species and genera of microbes, the proportion of the total microbial count was estimated. This approach allowed a comparison to be made of the predominance pattern of faecal microorganisms in individual infants.

The major differences in the composition of the colonic microflora in 1-y-old Estonian and Swedish infants were high counts of lactobacilli and eubacteria in the Estonian and increased numbers of clostridia in the Swedish infants. Bifidobacteria and anaerobic cocci prevailed equally often in both groups, while eubacteria and enterococci were parts of the predominant flora in many Estonian infants and bacteroides and clostridia, respectively, in many Swedish infants.

Thus, the current microflora of Estonian children appears to be similar to the prevailing microflora in European infants in the 1960s and 1970s (24–26). Even at that time, however, there were reports of decreasing numbers of faecal bifidobacteria in German infants between 1958 and 1978 (27).

Ordinarily, the intestinal microflora is characterized by high counts of anaerobes, predominating over the facultative microorganisms by some 100–1000-fold (23). The early predominance of bifidobacteria in breastfed infants, which was recently confirmed by specific molecular primers (28), is replaced over time by a more diverse flora, with a common predominance of bacteroides (4, 9). We have previously reported that in healthy infants up to 1 y of

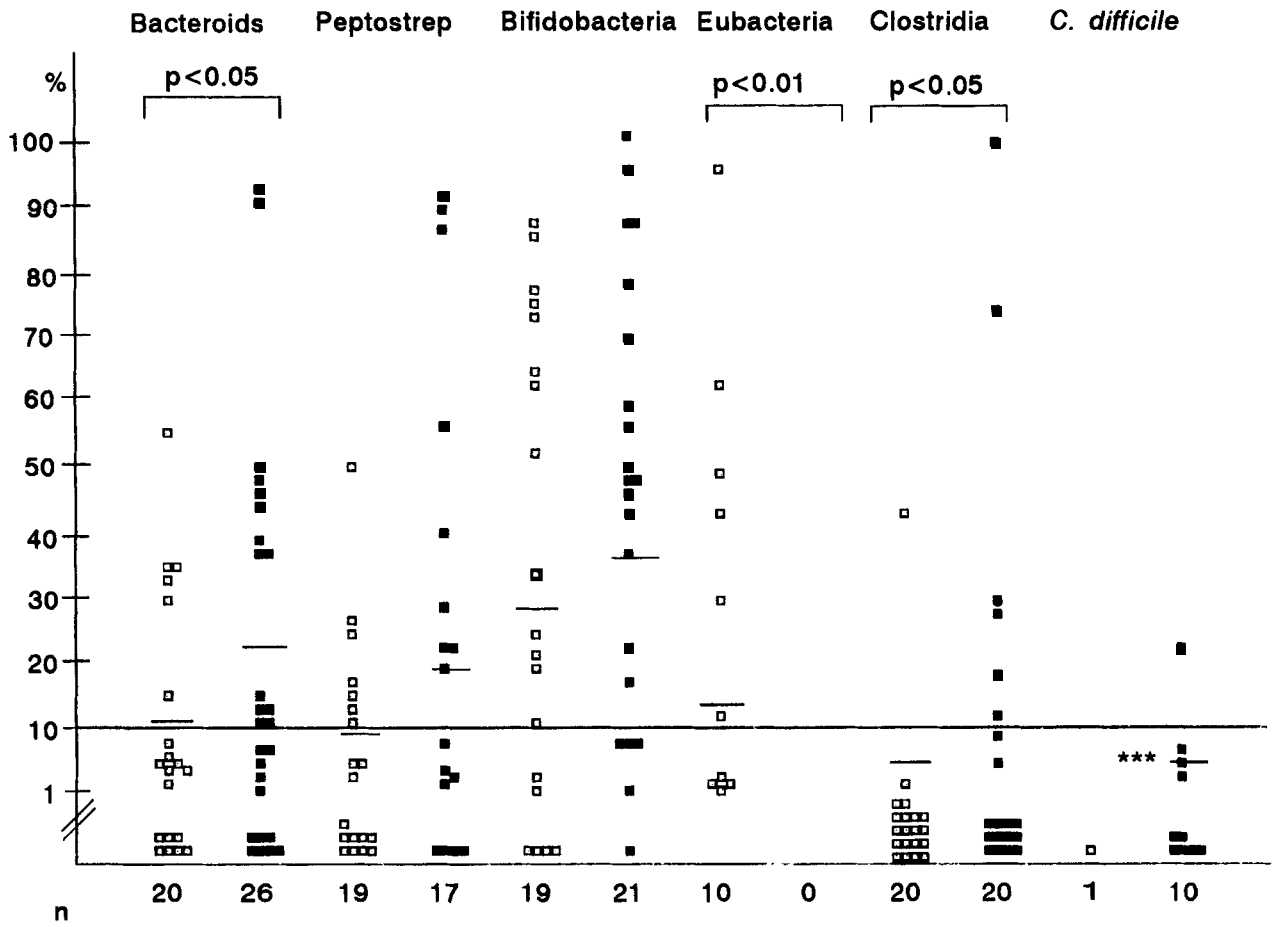

Fig. 1. Relative amounts of anaerobic microorganisms expressed as a percentage of the total microbial faecal flora in Estonian (open squares) and Swedish (filled squares) 12-month-old infants. Each symbol represents a particular microorganism in individual samples of infants. Microorganisms comprising >10% of the faecal flora were regarded as predominating species. The *p*-values (χ^2 test) indicate significant differences in the number of infants harbouring a major microorganism. Difference in the mean values: $***p < 0.001$.

age the normal intestinal microflora consists of bifidobacteria, eubacteria, bacteroides and/or anaerobic cocci, as predominating species, either singly or in combinations (29). The results of the present study, showing the individuality of the infants' anaerobic microflora, agree largely with the previous observations. An increased prevalence of *Clostridium perfringens* has for a long time been associated with rheumatoid arthritis (30) and, similarly, some relationship between the presence of bacteroides and atopy has been suggested (9).

Among the facultative anaerobes, the main difference between Estonian and Swedish infants was high levels of enterococci and lactobacilli in the former. The increased proportion of enterococci (over 10% of intestinal populations) may reflect a disturbed microbial ecology, as the bulk of organisms in stool comprises various obligate anaerobes while enterococci still account for less than 0.01% of normal bowel flora (31). This might be caused by antibiotic administration, yet the number of children treated by antibiotics was similar in both groups. Some other factors may be involved since the microflora of Estonian children seems more similar to that of Ethiopian than of Swedish infants, as reported by Bennet et al. (13), and these changes were not attributable to antibiotic treatment.

The normal intestinal microflora protects against the establishment of various potentially harmful pathogens. For example, lactobacilli can protect against experimental colonization with *C. difficile* (32, 33) and enhance the reconstitution of the human colonic mucosa (34). This could explain the high prevalence of *C. difficile* colonization in the apparently healthy Swedish babies.

The reasons for the low prevalence of colonization with microaerophilic lactobacilli and anaerobic eubacteria and the substitution with high levels of clostridia and bacteroides in Swedish infants remain unexplained. A disturbed maternal vaginal microflora could be offered as a possible explanation (10). Alternatively, increased consumption of mostly industrially processed food as the major component of the diet in western industrialized countries (35) may affect the intestinal flora. The Estonian diet is still to a large extent based on locally produced foods and various lactic acid fermented products are part of the diet.

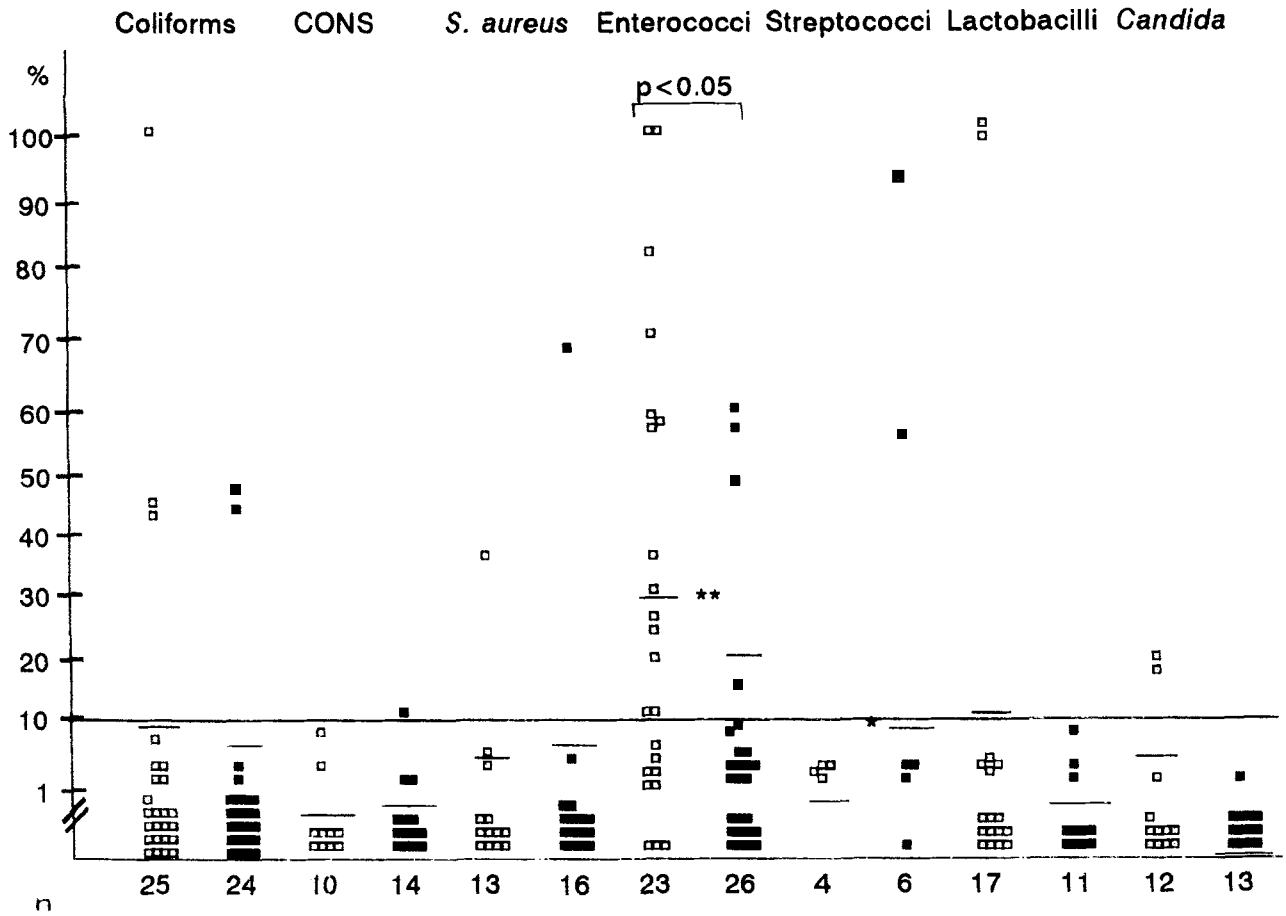

Fig. 2. Relative amounts of aerobic microorganisms, expressed as a percentage of the total microbial faecal flora in Estonian (open squares) and Swedish (filled squares) 12-month-old infants. Each symbol represents a particular microorganism in individual samples of infants. For explanations see Fig. 1. Difference in the mean values:*$p < 0.05$, **$p < 0.01$.

Reduced microbial stimulation during infancy and early childhood has been associated with the increasing prevalence of asthma and allergy in children and young adults in developed countries (14, 15). This would result in a slower postnatal maturation of the immune system and thus a delayed achievement of an optimal balance between Th1- and Th2-like immunity (36). According to this hypothesis, the incidence of sensitization in early life would be similar. Environmental factors, including infections and other microbial exposure, would then enhance "immune deviation" towards Th1-like immunity. The similar prevalence of sensitization in Estonian and Swedish infants and the low prevalence in Estonian children and young adults lend some support to this hypothesis. It is tempting to suggest that the differences in the indigenous intestinal flora, e.g. lactobacilli and eubacteria, may affect the development and priming of the immune system in early childhood. An increased proportion of either clostridia or bacteroides could be a reflection of a disturbed microbial balance. The role of the intestinal microflora in relation to the development of infant immunity needs to be analysed in prospective studies.

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