Development of intestinal microflora during the first month of life in Estonian and Swedish infants

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Little is known about possible regional differences in the development of the intestinal microflora in infants. The aim of the present study was therefore to compare the development of the microflora in Estonian and Swedish infants during the first month of life. The intestinal microflora of one week old and one month old Estonian ($n = 20$) and Swedish ($n = 20$) infants was studied by quantitative culture of faecal samples. All newborns were delivered vaginally and fed with breast milk during the first month of life. At one week of age the counts of aerobes (coagulase negative staphylococci, enterococci, enterobacteria) were higher in Estonian than in Swedish newborns. The counts of lactobacilli and candida increased in the Estonian infants over the first month of life, while the counts of enterobacteria increased in Swedish infants. At one month of age, the Estonian infants were more frequently colonised with lactobacilli than the Swedish infants and the counts were higher. Our data indicate that previously described differences in intestinal microflora of Estonian and Swedish 1 to 2 year old children are present already at the first month of life. *Key words*: newborn, intestinal microflora, geographic differences.

INTRODUCTION

Vaginally born neonates acquire the first micro-organisms mainly from the mother (1, 2). They include aerobic or facultatively anaerobic bacteria; e.g. enterobacteria, staphylococci, streptococci and lactobacilli (3–5). The initial colonisation is followed by a gradual increase in the number of anaerobes, such as Bacteroides, Bifidobacterium, Eubacterium, anaerobic cocci and clostridia (3, 5–7). Modern obstetrical practice, including treatment of the genital tract with disinfectants may yet alter the maternal microflora, thus affecting and delaying the colonisation of the newborns (1, 8). The consequences for the infant have been little studied.

Breast-fed babies are usually colonised with high numbers of bifidobacteria (9–11), lactobacilli (10) and staphylococci (4, 9), while in bottle-fed newborns clostridia (9, 10), enterococci (9, 10) and enterobacteria (9) are more frequent. There are also studies showing a dominance of Bacteroides in both breast-fed and bottle-fed infants, and equal colonisation with bifidobacteria in breast-and bottle-fed infants (11, 12).

Differences in the composition of the intestinal microflora in early infancy between western industrialised and developing countries have been reported in several studies (13, 14). Little is known, however, about the possible differences in this respect in various regions of Europe. We recently observed differences in the composition of the intestinal microflora among one year old children in Estonian and Sweden, i.e. two geographically close countries with large differences in living conditions (15). In a subsequent study, increased numbers of aerobic bacteria were observed in allergic as compared to non-allergic two-year-old children (16). As intestinal microbes stimulate the immune system, the microbial colonisation of neonates may affect the development of the immune system in the growing infant. It would therefore be important to study this process and identify differences in the colonisation process during the neonatal period and early infancy in different environments.

The aim of the present study was thus to compare the development of the intestinal microflora in Estonian and Swedish babies during the first month of life.

MATERIALS AND METHODS

Study group

The study group comprised 20 Estonian (12 male, 8 female) babies born at the Maternity Hospital of Tartu University between February and April 1997 and 20 Swedish (13 male, 7 female) infants, born at the Linköping University Hospital between March 1996 and April 1997. They were recruited to participate in a prospective study comparing the development of immune responses to aller-

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gens in relation to environmental factors. They were all vaginally delivered at term and the perinatal period was uncomplicated. The median birth-weight was 3650 g (range 3020–4890 g) in the Estonian newborns and 3670 g (range 2850–4470 g) in the Swedish babies. All babies were breast-fed during the first month of life.

Faecal samples were collected at 5/6 days and one month of age. Approximately 1–2 g of voided stool was collected into sterile plastic containers by the ward staff and/or by the parents. Samples collected at home were kept in a domestic refrigerator at 4°C for a maximum of 2 h before transportation to the laboratory, where they were frozen at −70°C. The Swedish samples were transported to Estonia on dry ice for bacteriological analyses.

Bacteriological analyses

Weighed samples of faeces were serially diluted (10^{-2}–10^{-9}) in pre-reduced phosphate buffer (pH 7.2) in the anaerobic glove box (Sheldon Manufacturing Inc. with a gas mixture: 5% CO_2; 5% H_2, 90% N_2). A quantitative analysis of the microflora was performed using seeding serial dilutions on eleven freshly prepared media.

Yeast extract agar was employed for total aerobes count, yeast extract agar with 6.5 per cent of sodium chloride for staphylococci, Endo agar for enterobacteria, Leeds acinetobacter medium (LAM) with vancomycin at 10 mg/l, cefsulodin at 15 mg/l and cephradine at 50 mg/l for acinetobacteria (17) de Man-Rogosa-Sharpe agar (MRS; Oxoid) for microaerophiles as lactobacilli and streptococci, Columbia agar with colistin sulphate and oxolinic acid supplement (COA; Oxoid) for beta-haemolytic streptococci, fastidious anaerobes agar (FAA; LAB M, Bury, UK) for total anaerobes, Schaedler agar (BBL) with vancomycin and nalidixic acid supplement (Oxoid) for gram-negative anaerobes, Columbia agar with colistin and nalidixic acid (CNA; BBL) for gram-positive anaerobes, cefoxitin-cycloserine-fructose agar (CCFA; Oxoid) with egg yolk and sodium taurocholate for *Clostridium difficile* and Sabouraud dextrose agar with penicillin (50 000 U/L) and streptomycin (40 000 U/L) for yeasts and fungi were applied in our study. The total counts of clostridia were estimated on FAA after ethanol treatment (18).

Seeding of anaerobes and incubation of FAA, Schaedler agar, CNA and CCFA for 5–6 days was performed in an anaerobic glove box. The yeast extract agar, salt-yeast-extract agar, Endo agar, LAM, COA and Sabouraud medium were incubated aerobically by 37°C and inspected after 24 and 48 h. The MRS medium was incubated in a microaerophilic atmosphere (GampyPAK Plus; Oxoid) for 72 h.

Colonies with different morphology growing on the plate with the highest dilution of bacteria were Gram stained and subjected to microscopy. The microorganisms were identified mostly on genus (coagulase negative staphylococci, enterococci, streptococci, acinetobacteria, candida, bifidobacteria, bacteroides, eubacteria, clostridia) and species level (lactobacilli, beta-haemolytic streptococci, enterobacteria, *Staphylococcus aureus*, *Clostridium difficile*).

Standard methods were used for identification of enterobacteria and other gram-negative bacteria, i.e. estimation of oxidase, indole, motility, growth/fermentation pattern on Klieger Iron agar, Simmons citrate agar, acetate and sodium malonate agar (19). A coagulase test (Oxoid) was employed for differentiation of *Staphylococcus aureus* and coagulase negative staphylococci (CONS). Streptococci and enterococci were identified by absence of catalase production and differentiated by fermentation of esculine. Beta-haemolytic streptococci were identified by their growth on COA medium and employing the latex test (Oxoid). Colony and cellular morphology and a negative catalase production identified lactobacilli when grown on selective media, they were further tested by API 50 CHL (bioMerieux, France). The anaerobes were identified up to familia or genus level by growth on selective media, colony and cellular morphology and Gram stain reaction. We diagnosed anaerobic gram-negative rods as bacteroides, gram-positive rods as eubacteria, bifidobacteria or clostridia and gram-positive cocci as anaerobic cocci. *Clostridium difficile* was identified by the ability to grow on CCFA, the colony and cellular morphology, positive gram staining, typical smell and gas liquid cromatography. All anaerobic microorganisms were tested for absence of growth under aerobic and microaerophilic conditions.

Statistical methods

A special program was used for quantitative estimation of the composition of the microflora (20). The program written for personal computers gives output data for all species of microorganisms as absolute counts (log 10; CFU/g—colony forming units per gram). The detection level of the various microorganisms was 3 log CFU/g.

Statistical analyses were performed using 'Statgraphics' (Statistical Graphics Corp.) and 'Excel' (Microsoft Corp.) software programs. The following tests were employed: Fisher's test (prevalence of colonisation); Mann-Whitney rank sum test for unpaired data and Wilcoxon rank test for paired data (counts); Spearman Rank Order Correlation (the counts between different microorganisms).

Ethical considerations

Informed consent was obtained from the parents of the babies. The study was approved both by the Institutional Review Boards of Tartu and Linköping universities.

RESULTS

At 5–6 days of age, the intestinal tract of all newborns was colonised with aerobes and various anaerobes (Table I),

except for a lack of anaerobes in four Estonian and two Swedish newborns. The higher counts of aerobes in the Estonian than in the Swedish newborns were due to high counts of CONS, enterococci and enterobacteria. Furthermore, there was a positive correlation between the counts of enterobacteria and enterococci in Estonian, but not in Swedish newborns at the end of the first week of life ($n = 20$, $r = 0.531$, $p < 0.01$).

The prevalence of *E. coli*, *Enterobacter spp*., *Klebsiella spp*., *Enterobacter spp*. and *Citrobacter spp*. was similar in

Table I

Faecal aerobes of Estonian and Swedish one-week and one month old infants (counts-log CFU/g and prevalence of colonisation-%)

Microorganisms	Estonian infants $n = 20$			Swedish infants $n = 20$		
	median	range (log)	%	median	range (log)	%
Total aerobes						
one week	11.5***	8.4–12.3	100	10.4***	8.4–12.0	100
one month	11.4	9–12.3	100	11.2	8.9–12.2	100
CONS						
one week	10.5***	<3.0–11.1	90	7.0***	<3.0–10.3	75
one month	6.5	<3.0–11.4	60	<3.0	<3.0–10.8	40
S. aureus						
one week	7.2	<3.0–11.7	60	5.3	<3.0–10.3	60
one month	9.2	<3.0–11.3	85	6.3	<3.0–11.7	80
Enterococci						
one week	10.7**	<3.0–11.8	90	8.9**	<3.0–10.6	95
one month	9.9	<3.0–12.2	85	10.3	<3.0–11.7	95
Enterobacteria						
one week	10.9**	4.8–11.8	100	9.9**	<3.0–12.0	80
one month	11.0	<3.0–11.9	95	10.4	<3.0–11.9	95
Streptococci						
one week	<3.0	<3.0–10.8	10	<3.0	<3.0–9.6	5
one month	<3.0	<3.0–11.3	10	<3.0	<3.0–8.6	5
Lactobacilli						
one week	<3.0	<3.0–10.0	20	<3.0	<3.0–8.6	5
one month	7.7**	<3.0–10.8	80[a]	<3.0**	<3.0–10.8	30[a]
Candida						
one week	<3.0	<3.0–7.3	15	<3.0	<3.0–6.6	25
one month	5.9*	<3.0–10.3	65	<3.0*	<3.0–10.9	35
Total anaerobes						
one week	10.5	<3.0–11.8	80	8.5	<3.0–12.1	90
one month	9.6	6.3–11.8	100	9.5	3.0–11.6	100
Anaerobic cocci						
one week	<3.0	<3.0–10.9	35	<3.0	<3.0–10.6	35
one month	<3.0	<3.0–11.7	35	<3.0	<3.0–10.9	15
Bifidobacteria						
one week	<3.0	<3.0–11.7	45	<3.0	<3.0–10.7	40
one month	9.1	<3.0–10.7	65	3.1	<3.0–10.9	50
Eubacteria						
one week	<3.0	<3.0–10.3	10	<3.0	<3.0–10.6	10
one month	<3.0	<3.0–10.9	10	<3.0	<3.0–10.3	10
Bacteroides						
one week	<3.0	<3.0–11.6	45	<3.0	<3.0–12.0	40
one month	3.9	<3.0–11.7	55	<3.0	<3.0–11.6	45
Clostridia						
one week	<3.0	<3.0–11.7	40	<3.0	<3.0–10.1	25
one month	3.0	<3.0–7.9	50	<3.0	<3.0–8.1	20
C. difficile						
one week	<3.0	<3.0–7.3	5	<3.0	<3.0–5.3	5
one month	<3.0	<3.0–8.1	5	<3.0	<3.0–6.0	5

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney test); [a] $p < 0.05$ (Fisher test).

the Estonian and Swedish infants. No acinetobacteria and beta-haemolytic streptococci were found in any of the faecal samples.

At one month of age the Estonian infants were more frequently colonised with lactobacilli and the counts were higher than in the Swedish infants, as were the counts of candida (Table I). There was a positive correlation between the counts of enterobacteria and enterococci, in the Swedish infants at this age ($n = 20$, $r = 0.507$, $p < 0.05$), similar to the findings in the Estonian newborns at one week.

Over the first month of life, in both groups of infants some significant changes took place yet in different groups of bacteria. There was an increase in the counts of lactobacilli ($p < 0.01$) and candida ($p < 0.01$) in the Estonian infants from one week to one month of age, while in the Swedish infants the counts of enterobacteria ($p < 0.05$) increased.

DISCUSSION

High counts of aerobic bacteria (CONS, enterobacteria and enterococci) were detected in the Estonian babies already during the first week of life, while in Swedish newborns the counts of enterobacteria increased later and a correlation with enterococci was not seen until at one month. At this age, 80% of the Estonian, but only 30% of the Swedish newborns were colonised with lactobacilli.

These observations agree with previously reported differences between neonates in Sweden and developing countries. Thus, Ethiopian infants are more frequently colonised with enterobacteria, enterococci and lactobacilli (13) and in Pakistani babies there is a big diversity of enterobacteria (14). The counts of enterobacteria and enterococci differ between Estonian and Swedish infants at 12 months (15). The correlation between the two microbial groups in Estonians already during the first week of life may indicate that they are achieved at the same time in conjunction with birth. This relationship was true at one month in the Swedish infants. There were slight differences in the storage and transport of the Estonian and Swedish samples. This would not explain the differences in bacterial counts of Estonian and Swedish children, e.g. total aerobes and anaerobes at the age of one week, since the counts were similar at one month. The high counts of aerobic bacteria at one month, both in Estonian and Swedish neonates were unexpected at that age as the higher counts of anaerobes are usually reported at that age (7). The reasons for the differences remain still unexplained.

The early microflora may represent the principal driving force for a normal post-natal maturation of immunological functions (21). Possibly, high numbers of aerobic microorganisms soon after birth could facilitate the induction of tolerance and thus be associated with the low prevalence of allergy in non-industrialised countries and in the former socialist countries of Central and Eastern Europe (22, 23).

The numbers of lactic acid producing bacteria, such as lactobacilli, increased in Estonian infants during the first month of life, while this was less common in the Swedish infants. Thus, the previously reported low content of lactic acid bacteria in Swedish infants (15) seems to be present very early in life. It is possible that some aerotolerant bacteria such as bifidobacteria and lactobacilli are acquired both from the mother and from the environment (12, 24). Karvonen et al. (25) detected lactobacilli in breast milk, vaginal mucus and stools of mothers, while in babies the frequency of colonisation with lactobacilli increased also with time of breast feeding, indicating that lactobacilli are transmitted from mother to infant during birth-giving or via breast milk. The reasons for the lower counts of aerobes and unfrequent colonisation by lactobacilli in Swedish, compared to Estonian newborn babies are unknown but may be related to different levels of hygiene during delivery and handling, and also to differences in the maternal diet.

Lactic acid bacteria, such as bifidobacteria and lactobacilli suppress the growth of enterobacteria and other potentially pathogenic microorganisms in vitro (26, 27). This may be explained by the acidic environment created by lactobacilli (26). Nonfrequent colonisation of lactobacilli may also possibly explain an increased prevalence of clostridia in one year old Swedish as compared to Estonian infants (15). However, there are controversial data about the suppressive role of bifidobacteria and lactobacilli against enterobacteria in intestinal ecosystem. A low isolation rate of bifidobacteria in Japanese babies was associated with high numbers of gram-negative bacteria (6). Contrarily, we have observed that during the administration of *Lactobacillus* GG, associated with increase in lactobacilli counts, also the counts of enterobacteria were increasing (28). In this study no correlation between the counts of lactobacilli, bifidobacteria and enterobacteria was revealed.

In conclusion, our data indicate that the differences in intestinal microflora in Estonian and Swedish children are present already during the first month of life. Maternal and some environmental factors influencing the intestinal microbial colonisation, and as a consequence the maturation of the immune system are apparently operating already during the neonatal period.

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