Chapter 5

Intestinal microbiota in allergic and non-allergic one year old very low birth weight infants after neonatal glutamine supplementation.

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Abstract

Aim: Previously, glutamine-enriched enteral nutrition in very low birth weight infants (VLBW) decreased the incidence of atopic dermatitis at age one year. The current aim was to determine whether this effect is related to changes in intestinal bacterial species that are associated with allergy, such as bifidobacteria, clostridium histolyticum, clostridium lituseburense (Chis/lit group) and *Escherichia coli* at age one year. Methods: 89 infants were eligible. Bifidobacteria, Chis/lit group and *E. coli* were measured by fluorescent in situ hybridization in fecal samples collected at age one year. Information on allergic and infectious diseases was previously determined by questionnaire. Results: 72/89 (81%) infants participated. Prevalence of all studied species was not different between glutamine-supplemented and control groups. Allergic infants were less frequently colonised with bifidobacteria than non-allergic infants (*p*=0.04). Between neonatal period and one year, prevalence of bifidobacteria increased (*p*<0.001), of Chis/lit group was unchanged (*p*=0.84), and of *E. coli* decreased (*p*<0.001). Conclusions: The beneficial effect of glutamine-enriched enteral nutrition on the incidence of atopic dermatitis in the first year of life in VLBW infants is not related to changes in bifidobacteria, Chis/lit group or *E. coli*. Allergic VLBW infants are less frequently colonised with bifidobacteria compared to non-allergic VLBW infants.
Introduction

Allergic diseases form an important health problem worldwide, with a high disease burden and high annual healthcare costs.\(^8\)\(^{-11}\) Previously, we found a decreased incidence of atopic dermatitis in very low birth weight infants (VLBW) after glutamine-enriched enteral nutrition in neonatal period.\(^8\) The mechanism underlying the potential beneficial effect of glutamine supplementation is not fully understood, but may include glutamine-mediated changes of intestinal microbiota.

In VLBW infants, colonisation with bifidobacteria and lactobacilli is often delayed compared to healthy term breastfed infants.\(^4\)\(^4\) Furthermore, the numbers of potentially pathogenic bacteria in VLBW infants is generally higher during the neonatal period compared to term infants.\(^4\)\(^4\)

In a recent review on the ‘microbiota hypothesis’ of allergic diseases, it is hypothesized that endogenous microbiota may play a significant role in the development of the immune system.\(^4\)\(^5\) This hypothesis is supported by the fact that mice can develop allergic airway responses to allergens if their endogenous microbiota is altered at the time of first allergen exposure.\(^4\)\(^5\)

In several epidemiologic studies, differences in intestinal microbiota between allergic and non-allergic children have been shown.\(^4\)\(^6\),\(^117\),\(^118\) Aerobic micro-organisms were observed more frequently in allergic infants, compared to non-allergic infants.\(^4\)\(^6\) Bifidobacteria and lactobacilli are considered beneficial bacteria, as their presence may enhance the intestinal mucosal barrier, modulate the systemic immune response and inhibit colonisation with pathogenic bacteria.\(^4\)\(^7\)

In atopic infants, Kalliomaki et al. found a higher incidence of clostridia and a tendency towards a lower incidence of bifidobacteria compared to non atopic infants.\(^118\) Penders et al. showed that the presence of *Escherichia coli* was associated with a higher risk of developing eczema, and infants colonised with *Clostridium difficile* were at higher risk of developing eczema, recurrent wheeze and allergic sensitization.\(^119\) The three mentioned species, bifidobacteria, clostridia and *E. coli* are related to allergy, therefore these species were studied in the current study. In our previous study\(^53\), neonatal glutamine supplementation was not associated with changes in the prevalence of bifidobacteria, lactobacilli, *E. coli*, streptococci and clostidia in neonatal period. However, colonisation with beneficial bacteria was delayed, whereas potentially pathogenic bacteria appeared rapidly after birth. Antibiotic treatment delayed the intestinal bacterial colonisation.\(^53\) We hypothesized that the lower rate of atopic dermatitis in one year old VLBW infants receiving glutamine-enriched enteral nutrition in the neonatal period was associated with higher levels of beneficial bacteria like bifidobacteria. The aims of the current study were to determine intestinal bacterial species associated with allergy such as bifidobacteria, Chis/lit group and *E. coli* by fluorescent in situ hybridization at one year of age in VLBW infants, in relation to 1) neonatal glutamine supplementation, 2) serious neonatal infections and 3) allergic and infectious disease in the first year of life. Furthermore, we aimed to determine changes in colonisation with these bacterial species during the first year of life in this cohort of VLBW infants.
Patients and methods

The initial study, conducted at the VU University Medical Center from September 2001 to July 2003, was a randomized, double-blind placebo-controlled trial of glutamine-enriched enteral nutrition in 102 VLBW infants (gestational age <32 weeks and/or birth weight <1500 g). Infants admitted to the level III neonatal intensive care unit (NICU) of the VU University Medical Center, Amsterdam, were eligible for participation in the study. In this study, infants received enteral glutamine supplementation (0.3 g/kg/day) or an isonitrogenous placebo supplementation (alanine) between day 3 and 30 of life. For further details of the study design, we refer to the study protocol. Baseline characteristics of the follow-up cohort and their mothers were recorded in the initial study. Information by means of validated questionnaires on nutritional, environmental characteristics and allergic and infectious diseases in the first year of life was previously published. The national central committee on research involving human subjects and the medical ethical review board of our institute approved the study protocol. All parents gave written informed consent. In the current study, data at age one year are compared to previously published data from the neonatal period.

Analysis of fecal samples by FISH

At the corrected age of one year, all participating infants visited our outpatient clinic. Fecal samples were collected during the visit and directly stored in sterile tubes and stored at -20°C until analysis. To guarantee accuracy and reliability of the fecal analysis, samples were treated under standardized conditions, immediately after withdrawal. Prior to analysis, fecal samples were thawed in ice water, diluted 10 times (w/v) in phosphate buffered saline (PBS) at pH 7.4 and homogenized for 10 minutes using a stomacher. Of the homogenized fecal suspension, 750 μl was fixed in 250 μl freshly prepared 4% (w/v) paraformaldehyde in PBS and incubated overnight at 4°C. Fixed samples were aliquoted and stored at -20°C. Fixed samples were applied to gelatin coated glass slides (18-well object slides with square shaped wells [0.25cm²/well]) and air-dried. Slides were hybridized overnight in a dark moist chamber at 50°C, with 10 ng/μl Cy3 labeled group-specific 16S rDNA-targeted oligonucleotide probes in preheated hybridization buffer. For total cell counts, samples were incubated with 0.25 ng/μl 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at room temperature. Slides were automatically counted by an Olympus AX70 epifluorescence microscope. The percentage of labeled bacteria per sample was determined in 25 randomly chosen positions by counting all cells and all labeled bacteria with a DAPI filter set (SP100) and Cy3 filter set (41007) respectively (Croma Technology Corp., Brattleboro, VT, USA). FISH is a commonly use method that is well validated and reported. It has been well-demonstrated that relative differences in microflora composition between volunteers can be assessed objectively by the FISH method using similar group-specific probes. Bifidobacteria, Chis/lit group and E. coli were studied. Bifidobacteria as a marker
for beneficial bacteria, Chis/lit group as a marker for gram positive bacteria including potential pathogens and *E. coli* as a marker for gram negative bacteria including potential pathogens. Relative numbers of bacterial groups are expressed as % of total counted number of cells/g wet weighted feces. Data are expressed as the percentage of infants colonised with a certain bacterial group. All probes used in this study are listed in Table 1.

### Table 1. 16S rRNA-targeted oligonucleotide probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence from 5’ to 3’ end</th>
<th>Specificity</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bif164-mod</td>
<td>CATCCGGYATTACCACCC</td>
<td>Bifidobacterium spp.</td>
<td>Modified from Bif164</td>
<td>(129)</td>
</tr>
<tr>
<td>Chis150</td>
<td>TTATCGCGTATTAATCTYCTTTT</td>
<td>Clostridium histolyticum group</td>
<td>Applied together with Clit135</td>
<td>(130)</td>
</tr>
<tr>
<td>Clit135</td>
<td>GTTATCGCGTGTACAGG</td>
<td>Clostridium lituseburense group</td>
<td>Applied together with Chis150</td>
<td>(130)</td>
</tr>
<tr>
<td>Ec1531</td>
<td>CACCGTAGTCGCTGCATCA</td>
<td>Subset enterobacteriaceae (<em>E. coli, Shigella, Salmonella, Klebsiella</em>)</td>
<td></td>
<td>(131)</td>
</tr>
</tbody>
</table>

All probes are 5’ Cy3-labelled

### Statistical analysis

Data are presented as mean and standard deviation (SD), and median (range) as appropriate. Infant and maternal characteristics were analyzed by Student’s t-test (for normally distributed continuous data), Mann-Whitney U test (for nonparametric continuous data), chi-square test or Fisher’s exact test (for dichotomous data). Generalized estimating equations were used to analyze changes in prevalence of bifidobacteria, Chis/lit group and *E. coli* in the first year of life. Time was treated as a categorical variable in order to analyze changes at the different time-points simultaneously in one model. We used an exchangeable correlation structure in all analyses. This choice was the most simple correlation structure that was comparable to the observed correlation matrix. A p-value <0.05 (two-tailed) was considered significant. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses.

### Results

### Participants

At the corrected age of one year, 89/102 (87%) infants were eligible to participate in the follow-up study. Of these 89 infants, 72 (81%) finally participated in the study: 34 infants received enteral glutamine supplementation and 38 received placebo supplementation during the
neonatal period. The complete trial profile is shown in Figure 1. Baseline infant and maternal characteristics are shown in Table 2. Further details of the participating infants at the age of one year have been previously described. The incidence of serious neonatal infections was lower in the glutamine-supplemented group compared to controls (p=0.04). In the glutamine-supplemented group, fewer infants had developed allergic diseases: doctor diagnosed atopic dermatitis, bronchial hyperreactivity, or milk protein allergy, in the first year of life compared to controls (p=0.03). In the glutamine-supplemented group, more infants had a urinary tract...
Intestinal microbiota, age one year

Infection during the first year of life as compared to controls (data not shown). However, all of these infants had congenital malformations of the urogenital tract.

**Intestinal microbiota as reflected by the presence of bifidobacteria, Chis/lit group and *E. coli*, at age one year and clinical outcome**

At one year of age, the prevalence of bifidobacteria, Chis/lit group and *E. coli* is not different in infants in glutamine-supplemented and control groups (**Figure 2A**). Furthermore, serious neonatal infections were not associated with differences in intestinal microbiota as reflected by the presence of bifidobacteria, Chis/lit group and *E. coli* at the corrected age of one year (**Figure 2B**). Infants with allergic disease in the first year of life were less frequently colonised with bifidobacteria compared to infants without allergic disease (**Figure 2C**). Colonisation with Chis/lit group or *E. coli* was not different in infants with and without allergic disease in the first year of life. In addition, intestinal microbiota, as reflected by bifidobacteria, Chis/lit group and *E. coli* was not different in infants with and without infectious disease in the first year of life (**Figure 2D**).
Development of intestinal microbiota as reflected by the presence of bifidobacteria, Chis/lit group and *Escherichia coli*, in the first year of life in VLBW infants

The development of the intestinal microbiota in the first year of life in both groups is expressed as proportion of infants colonised with a specific bacterial group (**Figure 3A**) and absolute number of bacteria per gram of feces (**Figure 3B**). Between the neonatal period (time points are (1) before start of the study, and at days (2) 7, (3) 14 and (4) 30 after birth respectively) and the age of one year, the prevalence of bifidobacteria increased (p<0.001), while the prevalence of *E. coli* decreased (p<0.001). No change was observed for the prevalence of Chis/lit group (p=0.84).
At none of the time points, a difference was detected between glutamine and placebo groups in bifidobacteria, *Escherichia coli* or clostridia (Figure 3A and 3B).

In additional analyses, we found that correction for mode of delivery, use of antibiotics in neonatal period, exclusive breast milk feeding until three months of age, the presence of siblings and a family history of atopy did not change the results of the primary analysis. Therefore, only the results of the primary analyses are reported.
Discussion

In this study in one year old VLBW infants, we found that the beneficial effect of glutamine-enriched enteral nutrition on atopic dermatitis during the first year of life is not related to changes in intestinal bacterial species associated with allergy such as bifidobacteria, Chis/lit group and *E. coli*, at one year of age. Furthermore, we showed that VLBW infants, who develop allergic diseases during the first year of life, are less frequently colonised with bifidobacteria compared to VLBW infants without allergic diseases. This finding is in line with previous studies in term infants, comparing microbiota in infants with and without allergic diseases. In term infants, a lower rate of allergic diseases, such as atopic dermatitis, is associated with higher levels of beneficial bacteria like bifidobacteria. Therefore, we hypothesized that the lower rate of atopic dermatitis at age one year in VLBW infants receiving glutamine-enriched enteral nutrition in the neonatal period was associated with higher levels of beneficial bacteria. So far, little information is available on the development of intestinal microbiota in the first year of life in VLBW infants. In our study, both the prevalence and the number per gram feces of bifidobacteria increased significantly between neonatal period and at one year of age. In breast-fed term infants, bifidobacteria become the predominant bacteria in the intestinal microbiota already after one week of life. In formula-fed term infants, the intestinal microbiota becomes more diverse during the first week of life. Apart from bifidobacteria, the microbiota also contains clostridia, enterobacteria, *Bacteroides spp.* and streptococci. 

Brück et al. showed that the microbiota of breast-fed healthy term infants is stable throughout the first six months of life and clearly dominated by bifidobacteria. In their review on intestinal microbiota in early infancy, Fanaro et al. showed that the predominance of bifidobacteria is slightly reduced at three months of age, but bifidobacteria remain predominant. In our study, the prevalence of bifidobacteria was low in the neonatal period, but bifidobacteria became the predominant bacterial group at one year of age in this cohort of VLBW infants, taking into account that our study was limited to three species. In our study, the prevalence of *E. coli* significantly decreases in the first year of life. This species, which also harbors pathogenic strains, dominated the intestinal microbiota during neonatal period in this cohort of VLBW infants. At one year of age, both prevalence and number of *E. Coli* per gram of feces were low in VLBW infants, which is in line with results in healthy term infants at 4 and 6 months of age. Glutamine-enriched enteral nutrition and serious infections in the neonatal period did not change intestinal microbiota, in both neonatal period and at one year of age. In a study in rats, glutamine supplementation mediated changes of the mucus layer. The mucus layer is an important site for bacterial colonisation and its composition may modulate bacterial adherence. We hypothesized that enteral glutamine supplementation in VLBW infants may change the mucus layer, which might reduce bacterial translocation and thereby reduce serious infectious morbidity in the neonatal period. Recently, van der Schoor et al. showed that enteral supplementation of glutamine in VLBW infants is used to a great extent by the splanchnic
Based on the results of the current study, the beneficial effect of glutamine on infectious morbidity in the neonatal period and allergic diseases at one year of age is not related to a direct effect on the intestinal bacterial species associated with allergy such as bifidobacteria, Chis/lit group and E. coli.

Some aspects of our study design need to be addressed. Firstly, as the sample size was based on the sample size calculation for the primary outcome of the initial trial, the sample size of the follow-up was relatively small. As a consequence, the conclusions of our study may be susceptible to a type II error. However, compared to other studies on the development of intestinal microbiota in neonates, the number of infants in our study (n=72) is relatively high. Secondly, in the initial study, the rate of neonatal infections was relatively high in both treatment groups. The relatively high rate of infections is in line with results of a recent surveillance study of nosocomial infections in our neonatal intensive care unit (NICU). We used predefined definitions for nosocomial infections in neonates that were adapted from the current definitions from the Center for Disease Control and Prevention (Atlanta, USA) for nosocomial infections in children < 1 year. The relatively high infection rate can be partially explained by our definitions of nosocomial infections and the relatively high device-associated utilization ratios in our NICU. Thirdly, in our study, intestinal microbiota was reflected by only three bacterial species: bifidobacteria, Chis/lit group and E. coli. Therefore, we can not exclude that glutamine supplementation in the neonatal period may influence the number of other bacterial species. However, previously we found that glutamine supplementation in the neonatal period did not influence the intestinal microbiota reflected by 9 bacterial species in the first month of life. Furthermore, despite the absence of differences in total numbers of bifidobacteria, the different strains of bifidobacteria were not studied. Therefore we can not exclude that a difference in one of these strains might exist.

In conclusion, to our knowledge, this is the first study into the effect of neonatal glutamine-enriched enteral nutrition in VLBW infants on intestinal microbiota, as reflected by bifidobacteria, Chis/lit group and E. coli, at one year of age. The beneficial effect of glutamine-enriched enteral nutrition on atopic dermatitis during the first year of life is not (directly) related to changes in intestinal microbiota as reflected by bifidobacteria, Chis/lit group and E. coli. However, in this cohort of VLBW infants, infants with allergic diseases were less frequently colonised with bifidobacteria compared to infants without allergic diseases. Finally, the intestinal microbiota as reflected by bifidobacteria, Chis/lit group and E. coli of these VLBW infants gradually becomes similar to the intestinal microbiota of healthy term breastfed infants.