

REGULAR ARTICLE

Intestinal microbiota in allergic and nonallergic 1-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 1 year. The aim of this study 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 1 year.

Methods: Eighty-nine infants were eligible for this follow-up study, conducted at a Tertiary care hospital. Bifidobacteria, Chis/lit group and *E. coli* were measured by fluorescent *in situ* hybridization in faecal samples collected at age 1 year. Information on allergic and infectious diseases was previously determined by questionnaire.

Results: Seventy-two of 89 (81%) infants were participated. Prevalence of all studied species was not different between glutamine-supplemented and control groups. Allergic infants were less frequently colonized with bifidobacteria than nonallergic infants ($p = 0.04$). Between neonatal period and 1 year, prevalence of bifidobacteria was increased ($p < 0.001$), of Chis/lit group was unchanged ($p = 0.84$), and of *E. coli* was decreased ($p < 0.001$).

Conclusion: 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 colonized with bifidobacteria compared to nonallergic VLBW infants.

INTRODUCTION

Allergic diseases form an important health problem worldwide, with a high disease burden and high annual health care costs (1–4). Previously, we found a decreased incidence of atopic dermatitis in very low birth weight infants (VLBW) after glutamine-enriched enteral nutrition in neonatal period (5). The mechanism underlying the potential beneficial effect of glutamine supplementation on atopic dermatitis is not fully understood, but may include glutamine-mediated changes of intestinal microbiota.

In VLBW infants, colonization with bifidobacteria and lactobacilli is often delayed compared to healthy term breastfed infants (6). Furthermore, the numbers of potentially pathogenic bacteria in VLBW infants is generally higher during the neonatal period compared to term infants (6).

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 (7). 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 (7). In several epidemiologic studies, differences in intestinal microbiota between allergic and nonallergic children have been shown (8–10). Aerobic microorganisms were observed more frequently in allergic infants, compared to nonallergic infants (8). Bifidobacteria and lactobacilli are considered beneficial bacteria, as their presence may enhance the intestinal mucosal barrier, modulate the systemic immune response and inhibit colonization with pathogenic bacteria (11). In atopic infants, Kalliomaki et al. (10) found a higher incidence of clostridia and a tendency towards a lower incidence of bifidobacteria compared to nonatopic infants. Penders et al. (12) showed that the presence of *Escherichia coli* was associated with a higher risk of developing eczema, and infants colonized with *Clostridium difficile* were at higher risk of developing eczema, recurrent wheeze and allergic sensitization. The

three mentioned species, bifidobacteria, clostridia and *E. coli* are related to allergy; therefore, these species were studied in this study.

In our previous study (13), neonatal glutamine supplementation was not associated with changes in the prevalence of bifidobacteria, lactobacilli, *E. coli*, streptococci and clostridia in neonatal period. However, colonization with beneficial bacteria was delayed, whereas potentially pathogenic bacteria appeared rapidly after birth. Antibiotic treatment delayed the intestinal bacterial colonization (13). We hypothesized that the lower rate of atopic dermatitis in 1-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 this 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 1 year of age in VLBW infants, in relation to (i) neonatal glutamine supplementation, (ii) serious neonatal infections and (iii) allergic and infectious disease in the first year of life. Furthermore, we aimed to determine changes in colonization 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 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 (14). For further details of the study design, we refer to the study protocol (15). 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 (5). 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 this study, data at age 1 year are compared to previously published data from the neonatal period (14).

Analysis of faecal samples by fluorescent *in situ* hybridization

At the corrected age of 1 year, all participating infants visited our outpatient clinic. Faecal 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 faecal analysis, samples were treated under standardized conditions, immediately after the samples were obtained. Prior to analysis, faecal samples were thawed in ice water, diluted 10 times (w/v) in phosphate-buffered saline (PBS) at pH 7.4 and homogenized for 10 min using a stomacher. Of the homogenized faecal suspension, 750 μL was fixed in 250 μL of 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 ($0.25\text{cm}^2/\text{well}$)] and air-dried. Slides were hybridized overnight in a dark moist chamber at 50°C , with 10 ng/ μL Cy3 labelled 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 min at room temperature. Slides were automatically counted by an Olympus AX70 epifluorescence microscope. The percentage of labelled bacteria per sample was determined in 25 randomly chosen positions by counting all cells and all labelled bacteria with a DAPI filter set (SP100) and Cy3 filter set (41007), respectively (Croma Technology Corp., Brattleboro, VT, USA). Fluorescent *in situ* hybridization (FISH) is a commonly used method that is well validated and reported (16). 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 (16,17). 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 faeces. Data are expressed as the percentage of infants colonized with a certain bacterial group. All probes used in this study are listed in Table 1.

Statistical analysis

Data are presented as mean and standard deviation (SD), and median (range) as appropriate. Type of distribution was

Table 1 16S rRNA-targeted oligonucleotide probes

Probe*	Sequence from 5' to 3' end	Specificity	Notes	Reference
Bif164-mod	CATCCGGYATTACACCC	Bifidobacterium spp.	Modified from Bif164	(30)
Chis150	TTATGCGGTATTATCTYCCTTT	Clostridium histolyticum group	Applied together with Clit135	(31)
Clit135	GTTATCCGTGTGTACAGG	Clostridium lituseburense group	Applied together with Chis150	(31)
Ec1531	CACCGTAGTGCCCTCGTCATCA	Subset enterobacteriaceae (<i>Escherichia coli</i> , <i>Shigella</i> , <i>Salmonella</i> , <i>Klebsiella</i>)		(32)

*All probes are 5' Cy3-labelled.

tested prior to analysis. Infant and maternal characteristics were analysed 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 analyse changes in prevalence of bifidobacteria, *Chis/lit* group and *E. coli* in the first year of life (18). Time was treated as a categorical variable to analyse 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 1 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 1 year have been previously described (5). 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,

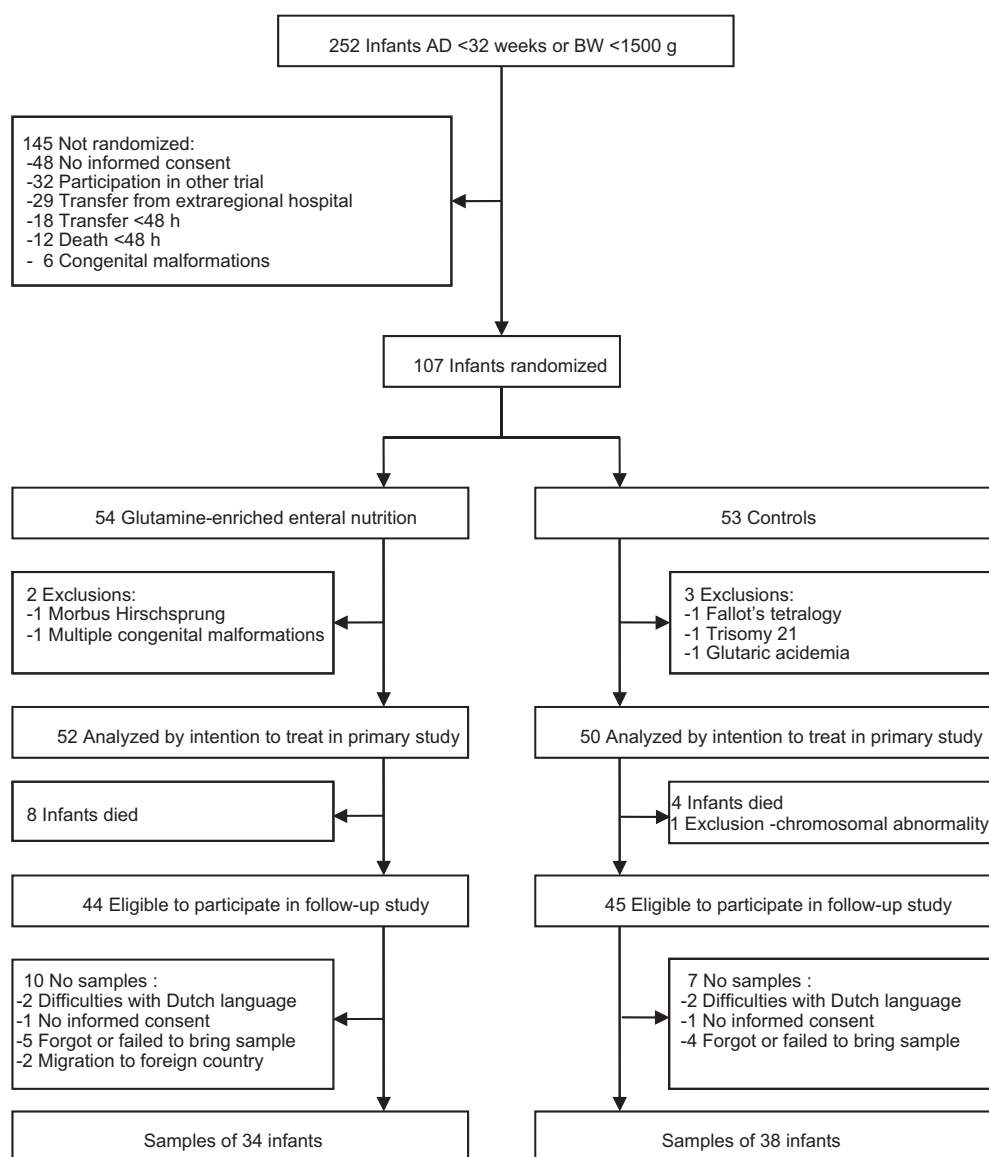


Figure 1 Trial profile.

Table 2 Baseline characteristics

	Glutamine group (n = 34)	Control group (n = 38)	p*
Neonatal period			
Vaginal delivery (%)	19/34 (56%)	17/38 (45%)	0.96
Gestational age (weeks)	29.3 (1.7)	29.1 (1.7)	0.42
Birth weight (kg)	1.19 (0.38)	1.20 (0.32)	0.77
Sex (% male)	16/34 (47%)	17/38 (45%)	0.84
≥1 serious infection in the neonatal period	18/34 (53%)	29/38 (76%)	0.04
Antibiotic treatment during hospital stay	24/34 (71%)	32/38 (84%)	0.17
Age at discharge from hospital (days)	61 (35–180)	65 (35–162)	0.52
1 year of age			
Age at follow-up (years)	1.3 ± 0.1	1.3 ± 0.2	0.84
Age at follow-up – 1 year of corrected age (weeks)	0.9 (–5.4–12.2)	0.9 (–4.8–22.0)	0.72
Exclusively breast milk until 3 months corrected age	18/34 (53%)	24/38 (63%)	0.47
Presence of siblings	15/34 (44%)	20/38 (53%)	0.49
Allergic diseases [†]	7/34 (21%)	17/38 (45%)	0.03
Infectious diseases [‡]	24/34 (71%)	23/38 (61%)	0.37

Data are mean (SD), median (ranges) or number (%).

*Student's *t*-test, Mann–Whitney *U*-test and chi-square or Fisher's exact test for continuous normally distributed, nonparametric continuous and dichotomous data, respectively.

[†]Allergic diseases are defined as: atopic dermatitis (skin rash on ≥1 typical location: flexural sites or around eyes and ears), bronchial hyperreactivity (≥3 of the following doctor-diagnosed symptoms: dyspnoea, wheezing, humming/sawing breath sounds or nightly dry cough without a cold) or milk protein allergy.

[‡]Infectious diseases are defined as: upper respiratory tract infection (≥1 episode of flu/serious cold, throat infection or otitis media), lower respiratory tract infection (≥1 episode of bronchitis, bronchiolitis or pneumonia), gastrointestinal tract infection, urinary tract infection or sepsis/meningitis.

bronchial hyperreactivity or milk protein allergy (5), in the first year of life compared to controls ($p = 0.03$). In the glutamine-supplemented group, more infants had a urinary tract infection during the first year of life when compared to controls (data not shown). However, all 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 1 year and clinical outcome

At 1 year of age, the prevalence of bifidobacteria, *Chis/lit* group and *E. coli* is not different in infants in glutamine-supplemented and control groups (Fig. 2A). Furthermore,

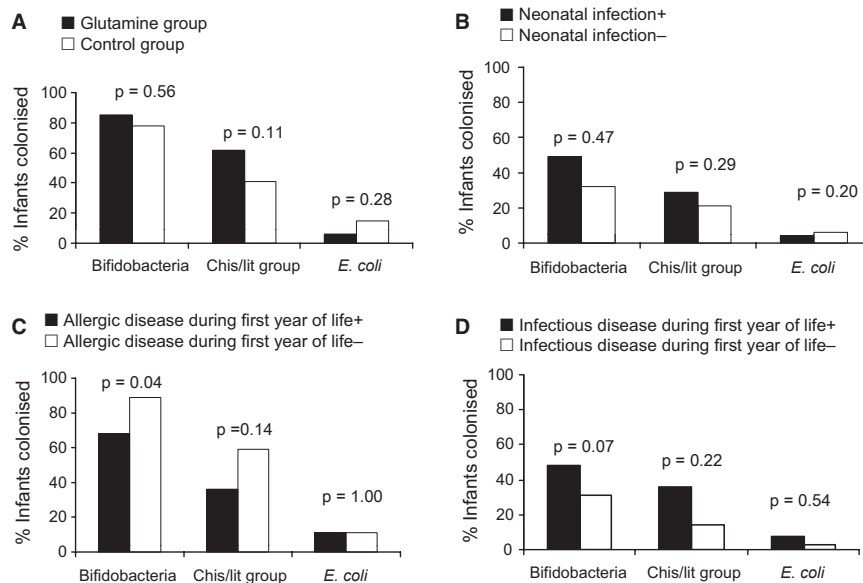


Figure 2 Intestinal microbiota, as reflected by bifidobacteria, *Chis/lit* group and *Escherichia coli*, at age 1 year and clinical outcome. Chi-square test and Fisher's exact test were used in data analysis. A. Intestinal microbiota at age 1 year in glutamine and control group. B. Neonatal infections: Sepsis, meningitis, pyelonephritis, pneumonia or arthritis as diagnosed by a combination of clinical signs and a positive culture. C. Allergic diseases: atopic dermatitis (skin rash on ≥1 typical location: flexural sites or around eyes and ears), bronchial hyperreactivity (≥3 of the following doctor-diagnosed symptoms: dyspnoea, wheezing, humming/sawing breath sounds or nightly dry cough without a cold) or milk protein allergy. D. Infectious diseases: upper respiratory tract infection (≥1 episode of flu/serious cold, throat infection or otitis media), lower respiratory tract infection (≥1 episode of bronchitis, bronchiolitis or pneumonia), gastrointestinal tract infection, urinary tract infection or sepsis/meningitis.

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 1 year (Fig. 2B). Infants with allergic disease in the first year of life were less frequently colonized with bifidobacteria compared to infants without allergic disease (Fig. 2C). Colonization 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 infants without infectious disease in the first year of life (Fig. 2D).

Development of intestinal microbiota as reflected by the presence of bifidobacteria, Chis/lit group and *E. 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 colonized with a specific bacterial group (Fig. 3A) and absolute number of bacteria per gram of faeces (Fig. 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 1 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 all time points, both percentages of colonized infants and numbers per gram faeces of either bifidobacteria, *E. coli* or the Chis/lit group were not different between glutamine and control groups (Fig. 3A,B).

In additional analyses, we found that correction for mode of delivery, use of antibiotics in neonatal period, exclusive breast milk feeding until 3 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.

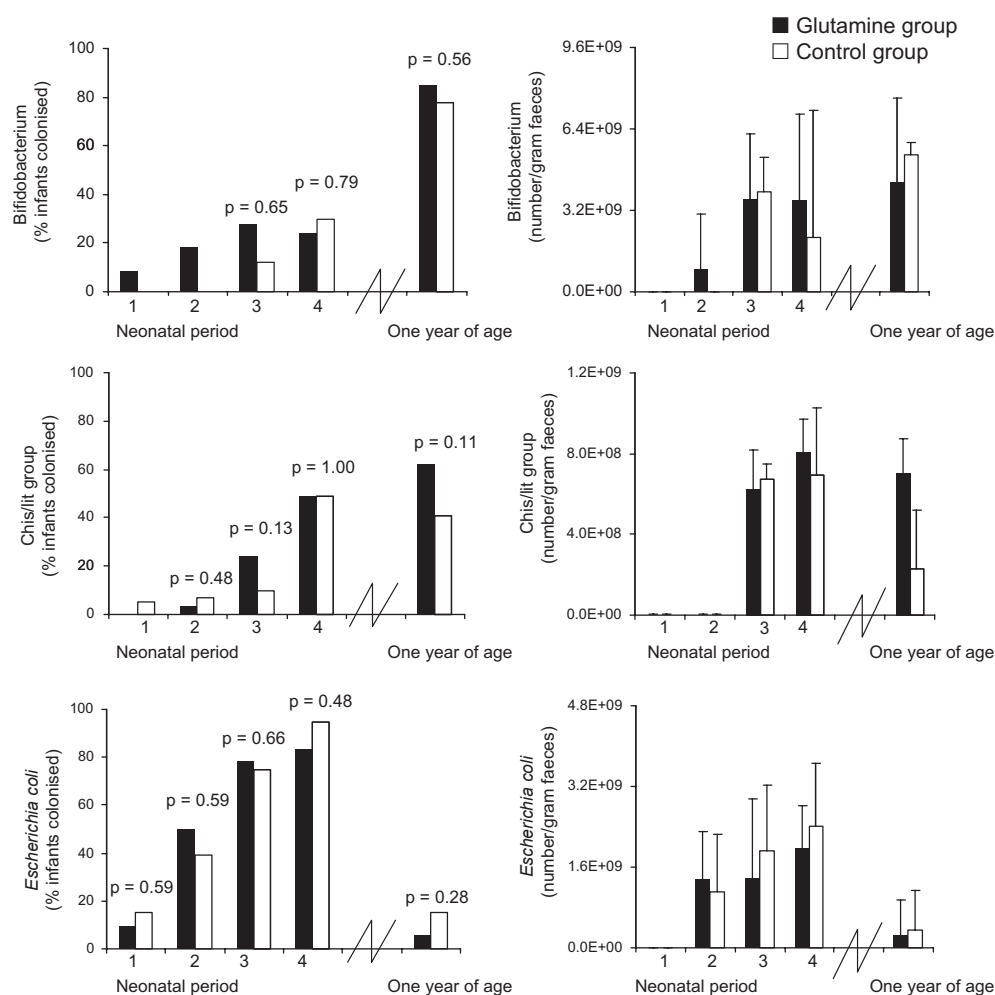


Figure 3 Development of intestinal microbiota, as reflected by bifidobacteria, Chis/lit group and *Escherichia coli*, in the first year of life. A. Percentages of infants colonized with a specific bacterium. Generalized estimating equations were used to assess differences between glutamine-supplemented and control group in the neonatal period and at 1 year of age. Time points in neonatal period are (1) before start of the study, at day 7 (2), at day 14 (3) and 30 (4) after birth. Small numbers of bifidobacteria (measurement 1 and 2) and Chis/lit group (measurement 1) in the neonatal period did not allow statistical analysis. B. Bars indicate median values and error bars 75th percentiles. When a bacterial group was present in >5 infants, data on absolute numbers/gram faeces were provided.

DISCUSSION

In this study in 1-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 1 year of age. Furthermore, we showed that VLBW infants, who develop allergic diseases during the first year of life, are less frequently colonized 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 (8,9,19).

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 1 year in VLBW infants receiving glutamine-enriched enteral nutrition in the neonatal period (5) 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 faeces of bifidobacteria increased significantly between neonatal period and at 1 year of age. In breast-fed term infants, bifidobacteria become the predominant bacteria in the intestinal microbiota already after 1 week of life (20,21). 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 (22–24). Brück et al. (25) showed that the microbiota of breast-fed healthy term infants is stable throughout the first 6 months of life and clearly dominated by bifidobacteria (25). In their review on intestinal microbiota in early infancy, Fanaro et al. (26) showed that the predominance of bifidobacteria is slightly reduced at 3 months of age, but bifidobacteria remain predominant (26). In our study, the prevalence of bifidobacteria was low in the neonatal period, but bifidobacteria became the predominant bacterial group at 1 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 harbours pathogenic strains, dominated the intestinal microbiota during neonatal period in this cohort of VLBW infants. At 1 year of age, both prevalence and number of *E. coli* per gram of faeces were low in VLBW infants, which is in line with results in healthy term infants at 4 and 6 months of age (26).

Glutamine-enriched enteral nutrition and serious infections in the neonatal period did not change intestinal microbiota, in both neonatal period (13) and at 1 year of age. In a study in rats, glutamine supplementation mediated the changes of the mucus layer (27). The mucus layer is an important site for bacterial colonization, 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 tissues (28). Based on the results of this study, the beneficial effect of glutamine on infectious morbidity in the neonatal period and allergic diseases at 1 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. First, 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 (6). Secondly, in our study, intestinal microbiota was reflected by only three bacterial species: bifidobacteria, Chis/lit group and *E. coli*. Therefore, we cannot 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 nine bacterial species in the first month of life (13). Furthermore, despite the absence of differences in total numbers of bifidobacteria, the different strains of bifidobacteria were not studied (29). Therefore, we cannot exclude that a difference in one of these strains might exist. Thirdly, the technology used in our study was FISH. Even though FISH is a commonly used method, that is well validated and reported, it might not rule out all differences in microbiota caused by early glutamine supplementation, because only three bacterial species were studied. Conventional cultivating techniques were not used for both logistic reasons and the fact that only a limited number of bacteria may be detected by this technique. FISH analyses provide quantitative data on the relative amount of the different bacterial groups, while cultivating techniques are insufficiently selective and unsuitable for quantitative analysis (17,22).

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 1 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 colonized 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.

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