

ORIGINAL ARTICLE

Effect of a new synbiotic mixture on atopic dermatitis in infants: a randomized-controlled trial

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Summary

Background Clinical trials investigating the therapeutic effect of probiotics on atopic dermatitis (AD) show inconsistent results. Better results can possibly be achieved by combining probiotics with prebiotics, i.e. synbiotics.

Objective To investigate the therapeutic effect of a synbiotic mixture on the severity of AD in infants.

Methods In a double-blind, placebo-controlled multi-centre trial, 90 infants with AD [SCORing Atopic Dermatitis (SCORAD) score ≥ 15], aged < 7 months and exclusively formula fed, were randomly assigned to receive either an extensively hydrolysed formula with *Bifidobacterium breve* M-16V and a galacto-/fructooligosaccharide mixture (Immunofortis[®]), or the same formula without synbiotics for 12 weeks. The primary outcome was severity of AD, assessed using the SCORAD index. A secondary outcome measure was intestinal microbiota composition.

Results There was no difference in SCORAD score improvement between the synbiotic and the placebo group. The synbiotic group did have a significantly higher percentage of bifidobacteria (54.7% vs. 30.1%, $P < 0.001$) and significantly lower percentages of *Clostridium lituseburens*/*Clostridium histolyticum* (0.5 vs. 1.8, $P = 0.02$) and *Eubacterium rectale*/*Clostridium coccoides* (7.5 vs. 38.1, $P < 0.001$) after intervention than the placebo group. In the subgroup of infants with IgE-associated AD ($n = 48$), SCORAD score improvement was significantly greater in the synbiotic than in the placebo group at week 12 (-18.1 vs. -13.5 points, $P = 0.04$).

Conclusions This synbiotic mixture does not have a beneficial effect on AD severity in infants, although it does successfully modulate their intestinal microbiota. Further randomized-controlled trials should explore a possible beneficial effect in IgE-associated AD.

Keywords atopic dermatitis, infant, prebiotics, probiotics, synbiotics

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Introduction

Atopic dermatitis (AD) is a highly prevalent, chronic, itching skin disease that often presents in infancy and considerably affects the quality of life of children and their families [1]. Currently, topical corticosteroids are the mainstay treatment of this disease; however, relapses are

common and parents often fear possible side-effects, leading to non-compliance.

There is increasing evidence that the intestinal microbiota plays an important role in the development of allergic diseases [2, 3]. Indeed, microbiota composition has been shown to differ between children with and without AD [4]. Therefore, innovative treatment strategies

aiming to modulate the intestinal microbiota with probiotics, living micro-organisms with immunomodulatory effects, or prebiotics, non-digestible food ingredients that stimulate the growth and/or the activity of one or a limited number of beneficial gut bacteria [5], are now foci of interest.

Animal studies show that AD severity can be reduced by certain probiotic strains [6, 7]. Yet, clinical trials in children have conflicting outcomes [8–13], although for IgE-associated AD a modest improvement was demonstrated [14–16]. A systematic review of all these clinical trials concluded that the probiotic strains studied to date are not an effective treatment for AD; however, other strains might have a greater effect [17]. In most trials, lactobacilli were used, while it can be argued that bifidobacteria, which are also considered to be safe for use as probiotics, might be better candidates for AD treatment. Low bifidobacteria levels appear to be associated with AD, as children with AD have lower *Bifidobacterium* percentages than their healthy peers and percentages are lower in severe than in mild AD [4]. Recently, it has been shown that the specific strain *Bifidobacterium breve* M-16V reduces allergic symptoms in ovalbumin-sensitized mice more effectively than lactobacilli [18]. Moreover, in a small study, this strain has been shown to decrease AD severity in children [19]. The only study that investigated the efficacy of prebiotics as AD treatment showed a beneficial effect on AD severity [20].

Theoretically, optimal synergetic combinations of pro- and prebiotics, so-called synbiotics, are most promising for treating AD. In whey-sensitized mice, a synbiotic combination of *B. breve* M-16V and 90% short-chain galactooligosaccharide (scGOS) and 10% long-chain fructooligosaccharide (lcFOS) mixture (Immunofortis®) reduced the acute allergic skin response more effectively than either the pre- or the probiotic component alone [21].

We performed a randomized, double-blind, placebo-controlled multi-centre trial to investigate the effect of an infant formula with added synbiotics, *B. breve* M-16V and an scGOS/lcFOS mixture (Immunofortis®), on the severity of AD in infants. Additionally, we investigated the effect of this synbiotic formula on the composition and the metabolic activity of the intestinal microbiota.

Subjects and methods

Subjects

Ninety full-term infants, aged 0–7 months, fulfilling Hanifin and Rajka criteria for AD [22], were recruited between September 2005 and February 2007 from Pediatric and Dermatologic outpatient clinics of the Academic Medical Center in Amsterdam and six participating regional hospitals, regional Baby Health Clinics and through advertisements in magazines. Infants had to be exclu-

sively formula fed at the time of enrolment and the SCORing Atopic Dermatitis (SCORAD) [23] score had to be >15. Infants that had used systemic corticosteroids, antibiotics or antimycotics, calcineurin inhibitors or probiotics during the 4 weeks, or antihistamines during the 2 weeks before enrolment, were excluded. These medications were also not permitted during the study, with the exception of antibiotics. Infants with major medical problems, gastrointestinal disease or skin disease other than AD were ineligible. Written informed consent was obtained from both parents of all participants.

Study design

Participants were randomized, using computer-generated 4-block design lists, drawn up by a statistician, with stratification according to recruiting hospital and current use of topical steroids, to receive either an extensively hydrolysed whey-based formula (Nutrilon Pepti®, Nutricia, Zoetermeer, the Netherlands) with additional synbiotics or the same formula without synbiotics for a period of 12 weeks. Patients were enrolled by the investigator (L. B. A.) and sequentially assigned a patient number connected to a formula code. Formulas were prepared and coded by Danone Research and dispensed by the pharmacy of the Academic Medical Center. Both formulas were identical with respect to smell, taste, texture, colour and packaging. The investigator, participants' own physicians and parents were all blind to the treatment groups. Participants were clinically assessed, by one investigator (L. B. A.), at weeks 0, 4, 8 and 12. At baseline, medical history (including mode of delivery, gestational age, parental allergy and nutrition) was obtained. Intake of formula, fecal frequency and consistency, gastrointestinal symptoms, diaper dermatitis, intercurrent illness and medication use were recorded in a diary. An episode of three or more watery stools in 24 h was considered as diarrhoea. Any episode of vomiting and diarrhoea with or without fever was considered as gastroenteritis. Parents were advised not to introduce solid foods before age 4–6 months and were instructed on emollient use and bathing habits. Topical corticosteroids were used under the guidance of the participant's own physician.

The protocol was approved by the Medical Ethics Committees of all participating centres. The trial is registered in the ISRCTN register: ISRCTN69085979.

Synbiotics

Synbiotics consisted of *B. breve* M-16V (Morinaga Milk Industry Co. Ltd, Tokyo, Japan) at a dose of 1.3×10^9 CFU/100 mL and a mixture of 90% scGOS and 10% lcFOS (Immunofortis®, Nutricia Cuijk B.V., Cuijk, the Netherlands), 0.8 g/100 mL [24]. Formula was given on demand.

The product was stable for at least 18 months when stored at room temperature (20–25 °C).

Outcome measures

The primary outcome measure was change in the severity of AD after 12 weeks of intervention compared with baseline. Severity was evaluated at every visit with the SCORAD index [23]. This tool takes into account the extent of the eczema, the intensity of the lesions and subjective symptoms. One trained investigator, blinded to the treatment groups, performed all SCORAD assessments. Secondary outcome measures were: (1) change in topical corticosteroid usage, represented as change in class (class 1 = mild, class 2 = moderate, class 3 = strong) and frequency (days/week), (2) total serum IgE, specific IgE against food and inhalant allergens and serum eosinophilic granulocytes, (3) change in microbiota composition, fecal short-chain fatty acids (SCFAs), lactate and pH and (4) stool frequency and consistency (1 = watery, 2 = soft pudding-like, 3 = soft-formed, 4 = dry-formed and 5 = dry hard pellets) and occurrence of gastrointestinal symptoms, diaper dermatitis and adverse events (defined as any symptom or disease episode that occurred during the study).

Blood samples

A 2–3 mL blood sample was obtained at baseline and week 12 for determination of eosinophilic granulocyte count, liver and renal function, total IgE and specific IgE against milk (f2), peanut (f13), egg (f245), fish (fx74), cat (e1) and house dust mite (HDM) (d1), determined using the CAP FEIA system (Phadia, Uppsala, Sweden).

Immunoglobulin E-associated atopic dermatitis

A subgroup of patients with IgE-associated AD was defined as patients with AD who had elevated total and/or specific serum IgE levels at baseline. Total IgE was considered elevated if ≥ 5 kU/L in infants aged <3 months and ≥ 15 kU/L in infants aged >3 months

(reference values of the Academic Medical Center, Amsterdam). Specific IgE was considered elevated if ≥ 0.35 kU/L.

Fecal samples

At baseline, week 1 and 12 fecal samples were collected and frozen immediately by the parents, then transported to the hospital in a cold storage bag, where they were stored at -20 °C until further analysis. Upon arrival to the laboratory, frozen samples were thawed on ice water and 0.5 g was fixed with 4% para-formaldehyde (PFA) as described previously. For DNA extraction, 0.2 g fecal sample was re-suspended in 0.05 M PBS and DNA was isolated using the method described previously by Haarman and Knol [25].

Real-time polymerase chain reaction (Q-PCR). The presence of *B. breve* M-16V was analysed by quantitative PCR using the duplex 5'-nuclease assay as described earlier by Haarman and Knol [25].

Fluorescent *in situ* hybridization. The 16S rRNA-targeted probes used in this study, their sequence and their targeted bacteria as well the hybridization conditions are listed in Table 1. All probes were purchased from MWG (Ebersberg, Germany) and were covalently linked at their 5'-end Cy3. The Nucleic acid stain DAPI (Invitrogen, Leiden, the Netherlands) was used for total fecal cell counts. PFA-fixed fecal samples were hybridized with the specific probes as described previously by Zoetendal et al. [31] and then counted using an automated Olympus AX70 epifluorescence microscope equipped with a Lang LStep13 8 slides-stage (Paes Nederland bv, Zoeterwoude, the Netherlands) and an F-View II charge-coupled device (Soft Imaging System GmbH, Münster, Germany) and image analysis software. The percentage of bacteria cells was determined at 25 randomly chosen positions on each well by counting all cells using a DAPI filter set (SP100; Chroma Technology Corp., Brattleboro, VT, USA) and by counting the target bacterial group using a Cy3 filter set (41007; Chroma Technology Corp.).

Table 1. List of 16S rRNA-targeted oligonucleotide probes used in this study

Probe name	Probe sequence (5'-3' end)	Target organism	Hybridization	
			Temperature* (°C)	Time (h)
Bif164 [26]	CATCCGGCATTACACCC	<i>Bifidobacterium</i>	50/50	16
Ec1531 [27]	CACCGTAGTGCTCGTCATCA	<i>Escherichia coli</i>	37/37	3
Erec482 [28]	GCTTCTTAGTCA(A/G)GTACCG	<i>Clostridium coccoides</i> and <i>Eubacterium rectale</i> group	50/50	16
Bac303 [29]	CCAATGTGGGGGACCTT	<i>Bacteroides/Prevotella</i> group	46/48	3
Chis150 [28]	TTATGCGGTATTAATCT(C/T)CCTTT	<i>Clostridium histolyticum</i> group	50/50	16
Clit135 [28]	GTTATCCGTGTGTACAGG	<i>Clostridium lituseburense</i> group	50/50	16
Lab158 [30]	GGTATTAGCA(C/T)CTGTTCCA	<i>Lactobacillus</i> and <i>Enterococcus</i>	50/50	16

*The values correspond to the hybridization and washing temperature, respectively.

pH measurements. Fecal samples were thawed and pH was measured directly at room temperature using a Handylab pH meter (Schott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler-Toledo, Columbus, Schwerzenbach, Switzerland).

Lactate and short-chain fatty acid measurements. The lactate (L-lactate and D-lactate) and SCFA content (acetic, propionic, *n*-butyric, iso-butyric, *n*-valeric and iso-valeric acids) of the samples were quantitatively determined by a Varian 3800 gas chromatograph (GC) (Varian Inc., Walnut Creek, CA, USA) equipped with a flame ionization detector as described earlier [32].

Statistics

A 25% difference in SCORAD score reduction between the synbiotic and the placebo group was considered clinically relevant. To detect this difference at a 5% significance level with 80% power, 35 children per group were required. To allow for a 20% dropout rate, 90 children were included. Data analysis was carried out according to a pre-established statistical analysis plan. Differences in the SCORAD score between the two treatment groups were analysed using repeated measures analysis of variance (ANOVA), with the SCORAD score at baseline, treatment and baseline topical corticosteroid use as prespecified covariables/factors. Other parametric data were analysed using paired or unpaired *t*-tests. Non-parametric data were analysed using the Mann-Whitney *U*- or the Jonckheere-Terpstra test. Binary and nominal data were analysed using the Chi-square test or Fisher's exact test. For the outcome measures 'dry stools' and 'diaper dermatitis' results were also represented as odd ratios with 95% confidence intervals. All analyses were performed on an intention-to-treat basis. A prespecified subgroup analysis of children with IgE-associated AD (children with AD and elevated total and/or specific IgE levels at baseline) was performed regarding the primary outcome measure using analysis of covariance. SPSS software (15.0) was used for all analyses.

Results

Patient characteristics

Ninety patients were randomized, of whom 82 completed the trial (Fig. 1). The mean age was 4.9 months (SD 1.4), and the mean SCORAD score was 35.1 (SD 11.6). Baseline characteristics did not differ between the synbiotic and the placebo group (Table 2). The intention-to-treat population, defined as all randomized infants that consumed at least some study formula, consisted of 89 children. Of these children, four were only assessed at baseline and therefore could not be included in the main analyses. The mean intake of formula during the study was 778 mL/day (SD 135) in the synbiotic and 760 mL/day (SD 148) in the placebo group.

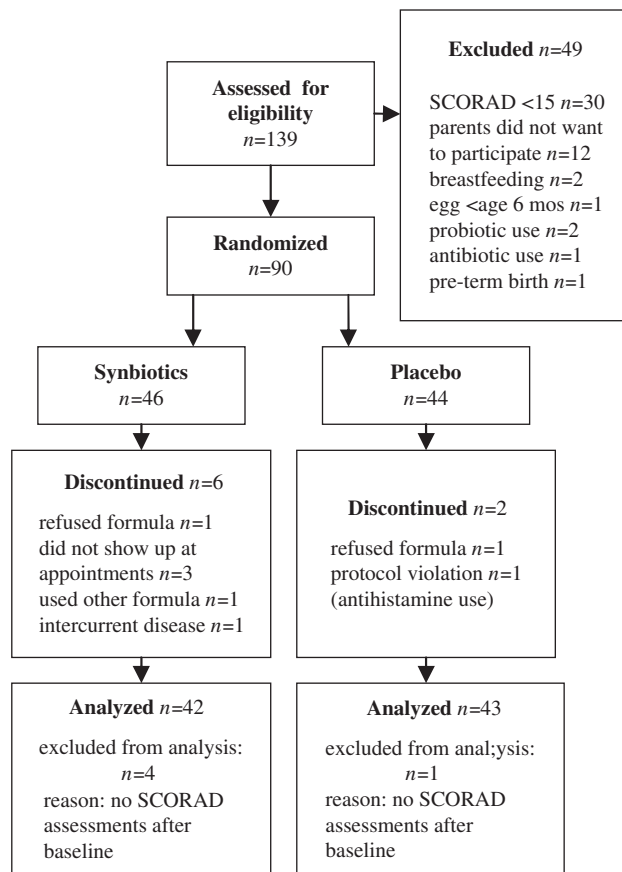


Fig. 1. Flowchart of the participants.

Effect of synbiotics on atopic dermatitis

Severity of atopic dermatitis. In both groups, the SCORAD score decreased significantly during the intervention period: 12.7 points in the synbiotic group (paired-samples *t*-test: $P < 0.001$) and 14.5 points in the placebo group (paired-samples *t*-test: $P < 0.001$). However, there was no statistically significant difference in SCORAD change between the two groups at any of the time-points (Table 3).

Use of topical corticosteroids. At baseline, 25 out of 45 children (55.6%) in the synbiotic group and 22 out of 44 (50%) in the placebo group used topical corticosteroids. In week 12, this was 22 out of 41 (53.7%) in the synbiotic and 24 out of 42 (57.1%) in the placebo group. The mean class and frequency of topical corticosteroid use did not differ statistically between the two groups at any time-point during the study (data not shown).

Serum immunoglobulin E and eosinophilic granulocytes

Total serum IgE increased significantly in both the synbiotic ($P = 0.001$) and the placebo group ($P = 0.001$) during the study, but there was no significant difference between

the two groups (Table 4). Also, the concentrations of specific IgE against HDM, cat, cow's milk, peanut and egg did not differ significantly between the two groups after 12 weeks (data not shown). Serum eosinophilic granulocytes decreased significantly in the entire study population (625×10^6 – $455 \times 10^6/L$, $P = 0.01$), but there was no difference between the two groups (Table 4).

Subgroup immunoglobulin E-associated atopic dermatitis

Fifty infants had IgE-associated AD. These children had a significantly higher baseline SCORAD score than the IgE-

negative children (38.5 vs. 30.2, unpaired t -test: $P = 0.002$). In the IgE-positive subgroup, the mean baseline SCORAD score did not differ between the synbiotic (37.6, SD 11.7) and the placebo group (39.5, SD 13.9). The interaction between treatment and IgE was significant for change in SCORAD at 12 weeks compared with baseline ($P = 0.01$). In the univariate ANOVA hydrolysate use before start of the study was added as a cofactor because this predictor of outcome was not equally distributed between the two treatment groups. In the subgroup of children with IgE-associated AD improvement in the SCORAD score at week 12 compared with baseline was significantly greater in the synbiotic than in the placebo group ($P = 0.04$, Table 5).

Table 2. Baseline characteristics of all randomized children

	Synbiotics ($n = 46$)	Placebo ($n = 44$)
Male, n (%)	31 (67.4)	28 (63.6)
Age (months), mean $\pm$ SD	5.0 $\pm$ 1.4	4.8 $\pm$ 1.5
Gestational age (weeks), mean $\pm$ SD	39.7 $\pm$ 1.9	39.9 $\pm$ 1.5
Delivered by caesarean section, n (%)	8 (17.4)	8 (18.2)
SCORAD index, mean $\pm$ SD	35.6 $\pm$ 10.6	34.7 $\pm$ 12.6
Eczema severity, n (%)		
Mild: SCORAD < 25	5 (10.9)	9 (20.5)
Moderate: SCORAD 25–50	37 (80.4)	30 (68.2)
Severe: SCORAD > 50	4 (8.7)	5 (11.4)
Corticosteroid potency, n (%)		
Unknown	1 (2.2)	0 (0.0)
None	20 (43.5)	22 (50.0)
Mild, class 1	16 (34.8)	15 (34.1)
Moderate, class 2	5 (10.9)	4 (9.1)
Potent, class 3	4 (8.7)	3 (6.8)
Elevated specific IgE, n (%)		
Food allergens	21 (51.2), $n = 41$	19 (47.5), $n = 40$
Aeroallergens	4 (9.5), $n = 42$	6 (14.6), $n = 41$
Parental allergy, n (%)		
Maternal	28 (60.9)	27 (61.4)
Paternal	24 (52.2)	24 (54.5)
Both	16 (34.8)	16 (36.4)
Breastfed before study period, n (%)	34 (73.9)	32 (72.7)
Total duration (weeks), median (range)	8.0 (1–24)	8.0 (0–22)
Exclusively (weeks), median (range)	6.5 (0–20)	6.0 (0–20)
Exposure to day care, n (%)	14 (30.4)	13 (29.5)
Pets at home, n (%)	14 (30.4)	15 (34.1)

Table 3. Effect of synbiotics on the severity of AD

	Synbiotics ($n = 42$)	Placebo ($n = 43$)	Difference Syn vs. plac (95% CI)	P -value
SCORAD change week 4 – baseline, EMM $\pm$ SE	–4.4 $\pm$ 1.5	–4.2 $\pm$ 1.5	–0.2 (–4.4 to 4.0)	0.93
SCORAD change week 8 – baseline, EMM $\pm$ SE	–11.5 $\pm$ 1.6	–9.6 $\pm$ 1.6	–1.9 (–6.4 to 2.6)	0.40
SCORAD change week 12 – baseline, EMM $\pm$ SE	–12.7 $\pm$ 1.6	–14.5 $\pm$ 1.5	1.8 (–2.5 to 6.2)	0.40

Repeated measures analysis of variance with cofactors treatment and topical steroid use at baseline and covariate SCORAD score at baseline. EMM, estimated marginal mean; AD, atopic dermatitis; SCORAD, SCORing Atopic Dermatitis; Syn, synbiotics; Plac, placebo.

Effect of synbiotics on a fecal microbiota

In the synbiotic group, a fecal sample was obtained of 41 infants at baseline and 39 infants at week 1 and week 12. In the placebo group, this was 40 at baseline, 36 at week 1 and 38 at week 12.

Detection of *Bifidobacterium breve* M-16V

At baseline, *B. breve* M-16V was detected in none of the fecal samples. At week 1, *B. breve* M-16V was detected in 12 of 30 children (40%) in the synbiotic group and three of 27 (11.1%) in the placebo group (Fisher's exact test: $P = 0.02$). In week 12, this was nine of 34 (26.5%) and one of 31 (3.2%), respectively (Fisher's exact test: $P = 0.01$).

Effect of synbiotics on microbiota composition

The median percentages of bifidobacteria, *Clostridium lituseburense*/*Clostridium histolyticum*, *E. coli*, lactobacilli–enterococci and *Eubacterium rectale*/*Clostridium coccoides* at 0, 1 and 12 weeks are shown in Fig. 2. The percentage of *Bacteroides-Prevotella* was below the detection limit for most children in both groups (data not shown). After 12 weeks of intervention, children in the synbiotic group had a significantly higher percentage of bifidobacteria (54.7 vs. 30.1, $P < 0.001$) and significantly lower percentages of *C. lituseburense*/*C. histolyticum* (0.5 vs. 1.8, $P = 0.02$) and *E. rectale*/*C. coccoides* (7.5 vs. 38.1, $P < 0.001$) than children in the placebo group.

Table 4. Effect of synbiotics on serum IgE and eosinophilic granulocytes

	Synbiotics	Placebo	P-value*
IgE (kU/L) baseline, median (range), (n)	11.0 (2.3–234), [40]	18.0 (2.8–631), [40]	0.32
IgE (kU/L) week 12, median (range), (n)	19.5 (2.2–391), [37]	32.2 (2.0–617), [39]	0.42
Eosinophilic granulocytes ($\times 10^6/L$) baseline, median (range), (n)	610 (50–5570), [39]	630 (60–4980), [41]	0.92
Eosinophilic granulocytes ($\times 10^6/L$) week 12, median (range), (n)	440 (50–4100), [36]	475 (20–2050), [38]	0.95

*Comparison between groups using the Mann–Whitney *U*-test.

Table 5. Effect of synbiotics on the severity of IgE-associated AD

	Synbiotics (n = 24)	Placebo (n = 24)	Difference Syn vs. plac (95% CI)	P-value
SCORAD change week 4 – baseline, EMM $\pm$ SE	–5.2 $\pm$ 2.1	–3.5 $\pm$ 2.2	–1.7 (–7.9 to 4.4)	0.57
SCORAD change week 8 – baseline, EMM $\pm$ SE	–13.7 $\pm$ 2.2	–9.3 $\pm$ 2.3	–4.4 (–10.6 to 2.0)	0.17
SCORAD change week 12 – baseline, EMM $\pm$ SE	–18.1 $\pm$ 1.6	–13.5 $\pm$ 1.6	–4.6 (–9.1 to –0.1)	0.04

Univariate analysis of variance with cofactors treatment, topical steroid use at baseline and previous use of hydrolysate formula and covariate SCORAD score at baseline.

EMM, estimated marginal mean; AD, atopic dermatitis; SCORAD, SCORing Atopic Dermatitis; Syn, synbiotics; Plac, placebo.

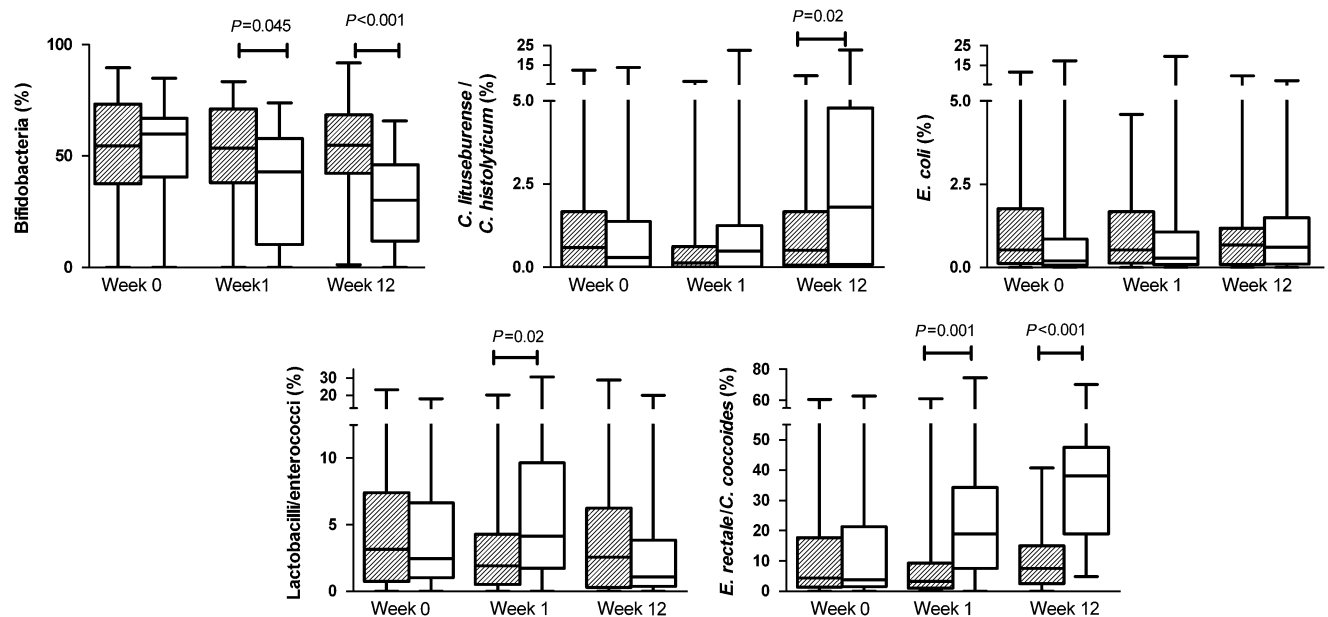


Fig. 2. Amount of bifidobacteria, *Clostridium lituseburense*/*Clostridium histolyticum*, *E. coli*, lactobacilli/enterococci and *Eubacterium rectale*/*Clostridium coccoides*, represented as percentage of the total amount of bacteria in the synbiotic (striped bars) and the placebo group (white bars) at baseline (week 0) and after 1 and 12 weeks of intervention (median, interquartile range and range, comparison between the two groups with the Mann–Whitney *U*-test).

Effect of synbiotics on fecal pH, lactate and short-chain fatty acids

Fecal pH was significantly lower and L-lactate and D-lactate concentrations were significantly higher in the synbiotic group than in the placebo group after 1 and 12 weeks of intervention (Fig. 3). Figure 3 also shows the effect of synbiotics on SCFA percentages. After 12 weeks, the synbiotic group had significantly lower percentages of butyric, isobutyric and isovaleric acid, but there were no

differences in acetic and propionic acid percentage between the two groups. Valeric acid was below the detection limit for almost all children in both groups at weeks 0, 1 and 12 (data not shown).

Gastrointestinal symptoms, diaper dermatitis and adverse events

Gastrointestinal symptoms at baseline and during the intervention are shown in Table 6. Fecal frequency did

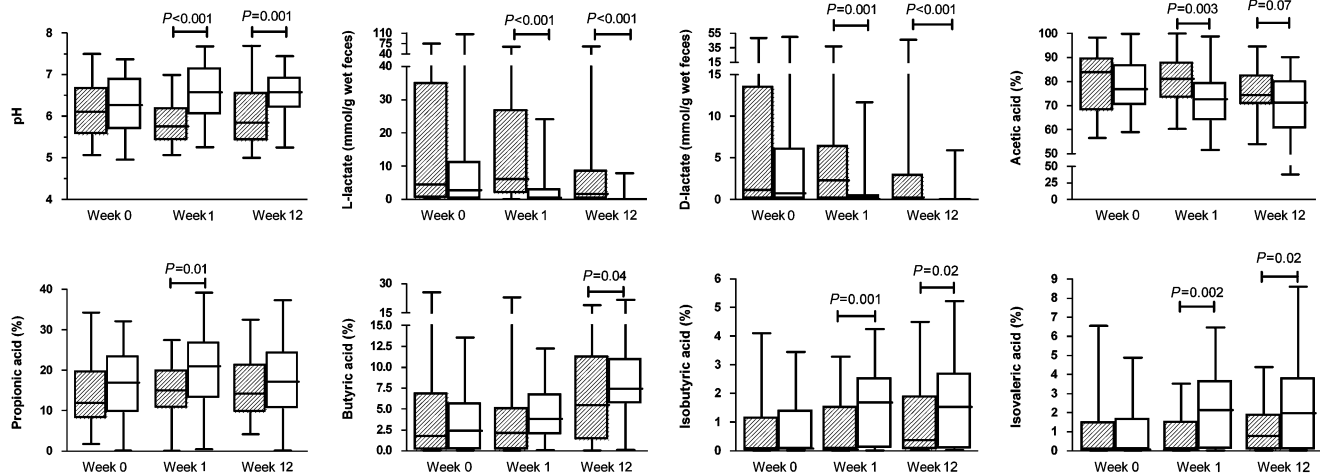


Fig. 3. Fecal pH, fecal L-lactate and D-lactate concentrations and fecal short-chain fatty acids (SCFAs) (represented as percentages of the total amount of SCFAs) in the synbiotic (striped bars) and the placebo group (white bars) at baseline (week 0) and after 1 and 12 weeks of intervention (median, interquartile range and range, comparison between the two groups with the Mann-Whitney U-test).

Table 6. Effect of synbiotics on gastrointestinal symptoms and diaper dermatitis

	Synbiotics (n = 45)	Placebo (n = 44)	P-value
Fecal frequency/day, median (range)			
Baseline	1.36 (0.29–3.43)	1.43 (0.43–4.83)	0.91*
Week 1–4	1.36 (0.50–3.61)	1.29 (0.29–3.54)	0.69
Week 5–8	1.27 (0.50–3.46)	1.54 (0.39–3.89)	0.36
Week 9–12	1.39 (0.57–3.43)	1.52 (0.30–3.24)	0.68
Fecal consistency [†] , median (range)			
Baseline	2.00 (1.00–3.50)	2.00 (1.00–4.36)	0.76 [‡]
Week 1–4	2.00 (1.00–4.35)	2.57 (1.30–4.63)	0.002
Week 5–8	2.26 (1.00–4.05)	2.69 (1.26–3.25)	0.02
Week 9–12	2.48 (1.28–4.09)	2.85 (1.90–3.77)	0.05
Diarrhoea, n (%)	17 (37.8)	12 (27.3)	0.37 [§]
≥1 episode of dry stools, n (%)	10 (22.2)	26 (59.1)	0.001 [§]
Constipation, n (%)	0 (0)	6 (14.0)	0.01 [¶]
Gastroenteritis (%)	6 (13.3)	2 (4.5)	0.27 [¶]
Diaper dermatitis			
Baseline, n (%)	10 (24.4)	11 (25.6)	0.90 [§]
During study, n (%)	27 (60)	38 (86.4)	0.008

*Mann-Whitney U-test.

[†]1 = watery, 2 = soft pudding-like, 3 = soft-formed, 4 = dry-formed, 5 = dry hard pellets.

[‡]Jonckheere-Terpstra test.

[§]Chi-square test.

[¶]Fisher's exact test.

not differ between the two groups at any time-point, but fecal consistency was significantly softer in the synbiotic group during the intervention period. Diarrhoea and gastroenteritis occurred equally in both groups. Also, there were no group differences in parent-reported bowel cramps, flatulence and regurgitation (data not shown).

Fewer children in the synbiotic group had episodes of dry stools (OR: 0.20, 95% CI 0.08–0.50, $P = 0.001$) and parents reported less constipation ($P = 0.01$). During the intervention period, diaper dermatitis was less prevalent in the synbiotic group (OR: 0.24, 95% CI: 0.08–0.68, $P = 0.008$).

The percentage of patients experiencing any adverse event was similar in the synbiotic and the placebo group (91.1% vs. 84.1%, Chi-square test: $P = 0.35$). There were two serious adverse events (hospitalization because of respiratory syncytial virus bronchiolitis and because of severe cow's milk allergy) in the synbiotic and none in the placebo group. None of the reported adverse events were considered to be treatment-related. Growth, weight, renal and liver function did not differ between the two groups (data not shown). In the synbiotic group, one child used antibiotics during the study (2.2%, for infected AD), and in the placebo group, five children (11.4%), for infected AD, upper respiratory infection, fever of unknown origin, lymphadenitis and otitis (Fisher's exact test: $P = 0.11$).

Discussion

We showed that an infant formula with added synbiotics, *B. breve* M-16V and a specific scGOS/lcFOS mixture (Immunofortis®), has no effect on eczema severity, corticosteroid usage, serum IgE concentration or eosinophil count in infants with AD. However, it does significantly modulate the composition and metabolic activity of the intestinal microbiota of these children, potentially explaining the beneficial effects on constipation and diaper dermatitis prevalence that we observed. The synbiotic mixture was well tolerated and did not cause any adverse events.

This is the first clinical trial of synbiotics for the treatment of infant AD. A study of synbiotics as AD

treatment in older children compared a combination of *Lactobacillus rhamnosus* with prebiotics to prebiotics alone and found no difference between the groups [33]. Unfortunately, the study did not include a placebo group and did not report results for IgE-associated AD.

In agreement with our results, a number of randomized trials investigating the effect of probiotics on AD also did not find a positive effect in the total study populations [8–10, 14–16]. In some of these studies, a statistically significant improvement of AD was observed in a subgroup of IgE-sensitized children in the intervention groups [14–16], similar to our findings. However, SCORAD improvement in the IgE-positive subgroups was modest; therefore, clinical relevance remains to be determined.

We were not able to confirm the results of a small study with *B. breve* M-16V [19] and three studies with other probiotic strains (LGG, *B. lactis* Bb-12 and *Lactobacillus fermentum*), which all showed a positive effect in children with both IgE-associated and non-IgE-associated AD [11–13]. The discrepancy between these studies and our study could be due to differences in the study populations, e.g. number, age and baseline SCORAD score of the participants. It may also be due to differences in the dosages and types of bacterial strains that were used, since probiotic effects are strain specific [34]. Moreover, in some of these studies, the results were only significant in intragroup analysis [11, 13].

In contrast with the studies mentioned above, we performed fecal analyses to detect the given probiotic strain and to assess the effect of the synbiotic mixture on microbiota composition. Although we detected the M-16V strain in significantly more infants in the synbiotic than in the placebo group, the detection rate was relatively low. However, we did demonstrate a significant modulation of intestinal microbiota composition in the synbiotic group, towards a composition with more bifidobacteria and less potential pathogenic bacteria such as clostridia-related species. These changes in the microbiota composition were also reflected in the metabolic profile that we observed in the synbiotic group (low pH, high lactate and low butyric, isobutyric and isovaleric acids). This metabolic profile resembles that of breastfed infants [32, 35]. These changes in microbiota composition indicate that compliance was good. Therefore, the reason for the relatively low detection rate of *B. breve* M-16V is not fully understood. Possibly, the real-time PCR assay, which was validated *in vitro*, was not sensitive enough to detect the M-16V strain *in vivo*.

High levels of bifidobacteria are considered to be beneficial for human health. Bifidobacteria have several effects, such as aiding in the digestion of food ingredients, synthesis of vitamins, immunomodulatory effects and inhibition of growth of potentially pathogenic bacteria [5, 36]. Since studies show an association between low levels of bifidobacteria, as well as high levels of clostridia, and

atopic disease, increasing intestinal *Bifidobacterium* levels could be beneficial for children at risk for or with atopic diseases, such as AD [4, 37, 38]. However, although the synbiotic combination of *B. breve* M16-V and the scGOS/lcFOS mixture significantly increased the percentage of bifidobacteria compared with placebo, we observed no clinical effect on AD severity. The synbiotic formula did soften fecal consistency and reduced the prevalence of dry stools and parent-reported constipation, in line with the results of studies where prebiotics were added to infant formulas [24, 39]. Also, there was a lower prevalence of parent-reported diaper dermatitis in the synbiotic group, which has never been described before. Important irritants involved in the pathogenesis of diaper dermatitis are fecal enzymes, such as urease, proteases and lipases [40]. Because the activity of these enzymes is increased by a high pH [41], it is likely that the lower stool pH in the synbiotic group resulted in the lower prevalence of diaper dermatitis. However, this should be further studied.

A possible limitation of our study is that participants were allowed to use topical corticosteroids. Because corticosteroids are the mainstay treatment of AD, it would have been unethical to withhold this treatment option in children. Theoretically, this could have concealed a synbiotic treatment effect. However, we stratified for corticosteroid use at randomization and corrected for it in the statistical analyses. Moreover, corticosteroid use was carefully monitored during the intervention period. If the synbiotic mixture would have had a positive effect on AD severity, one would expect to see a reduction in the class and/or the frequency of corticosteroid use in the synbiotic group compared with placebo, which was not the case.

In vitro studies show that probiotics are able to down-regulate Th2 cytokine production by stimulation of regulatory cytokines, such as IL-10 and TGF- β . This is accomplished by either increasing the production of these cytokines by antigen-presenting cells or by driving the development of regulatory T cells [36, 42–44]. Although these immunosuppressive effects seem to be promising for the treatment of AD and other allergic diseases, immunological results of clinical trials in children with AD are inconsistent [15, 45] (immunological analyses of our trial are underway). It has been suggested that probiotic administration during sensitization is essential for adequate immune modulation [46]. Possibly, the window of opportunity to modulate the immune system with pro- or synbiotics lies very early in life, before allergic diseases have developed, and the effect on already established allergic disease is limited. This hypothesis is supported by the decrease in AD incidence that is seen in several prevention studies with pro-, pre- and synbiotics [34, 47–49].

In our study, infants with IgE-associated AD showed a greater reduction in the SCORAD score after 12 weeks of

intervention than those who received placebo. Although our subgroup analysis was planned in advance, our study was not powered to determine whether synbiotic treatment is effective in the subgroup of patients with IgE-associated AD. However, this does suggest the hypothesis that infants with IgE-associated AD are the most likely to benefit from synbiotics. The mechanism for such a benefit is not clear, as we did not find an effect of synbiotics on serum IgE levels. In AD, a distinction can be made between the IgE-associated form and the non-IgE-associated form [50, 51]. Patients with IgE-associated AD have elevated levels of total or allergen specific serum IgE or a positive skin prick test, in contrast to patients with non-IgE-associated AD who do not display evidence of sensitization. In infants, IgE sensitization cannot yet be detected in up to 57% [52]; however, as IgE increases with age, a definite diagnosis of IgE-associated or non-IgE-associated AD in young children is difficult to make. It is conceivable that children who are already IgE-positive at a young age represent a different entity of AD patients, with more severe and persistent disease and a different cytokine pattern. Indirectly, this is confirmed by our results, which show that IgE-positive infants had a significantly higher SCORAD score than IgE-negative infants. Further exploration of cytokine patterns may reveal whether this is indeed true.

In conclusion, the synbiotic combination of *B. breve* M-16V and a specific scGOS/lcFOS mixture does not have a beneficial effect on the severity of infant AD, despite successfully changing the composition and the metabolic activity of the intestinal microbiota. A possible beneficial effect of this synbiotic mixture in IgE-associated AD should be explored in future randomized-controlled trials, adequately powered to address this group of infants.

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