

No detectable beneficial systemic immunomodulatory effects of a specific synbiotic mixture in infants with atopic dermatitis

L. B. van der Aa¹, R. Lutter², H. S. A. Heymans³, B. S. Smids², T. Dekker², W. M. C. van Aalderen¹, J. H. Sillevius Smitt⁴, L. M. J. Knippels⁵, J. Garssen⁵, A. J. Nauta⁵, A. B. Sprickelman¹ and the Synbad Study Group*

¹Department of Pediatric Respiratory Medicine and Allergy, Emma Children's Hospital, ²Departments of Respiratory Diseases and Experimental Immunology, Academic Medical Center, ³Department of Pediatrics, Emma Children's Hospital, ⁴Department of Dermatology, Academic Medical Center, Amsterdam, The Netherlands and ⁵Danone Research-Centre for Specialised Nutrition, Wageningen, The Netherlands

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Summary

Background In a murine model of allergic inflammation, *Bifidobacterium breve* M-16V has been shown to reduce IL-4 and IgE by inducing IL-10 and IFN- γ . However, it remains unknown whether this strain has the same effect in humans with allergic disease.

Objective To determine the effects of *Bifidobacterium breve* M-16V combined with a prebiotic oligosaccharide mixture (synbiotic) on atopic markers, *ex vivo* cytokine production by peripheral blood mononuclear cells (PBMCs) and circulating regulatory T cell percentage in infants with atopic dermatitis.

Methods In a double-blind, placebo-controlled multi-centre trial, 90 infants with atopic dermatitis, age <7 months, were randomized to receive an infant formula with *Bifidobacterium breve* M-16V and a mixture of short chain galactooligosaccharides and long chain fructooligosaccharides (Immunofortis[®]), or the same formula without synbiotics during 12 weeks. At week 0 and 12, plasma levels of IL-5, IgG1, IgG4, CTACK and TARC, *ex vivo* cytokine responses by PBMCs and percentage of regulatory T cells, were determined.

Results There were no significant differences between the synbiotic and the placebo group in IL-5, IgG1, IgG4, CTACK and TARC levels and *ex vivo* cytokine production by anti-CD3/anti-CD28-stimulated PBMCs. With allergen-specific stimuli, we found a decreased IL-12p40/70 and IL-12p70 production in response to egg allergen ($P = 0.04$ and $P = 0.01$, respectively) and decreased IL-12p70 production in response to peanut allergen ($P = 0.003$) in the synbiotic compared with the placebo group. Circulating regulatory T cell percentage did not significantly differ between the groups.

Conclusions and Clinical Relevance This synbiotic mixture has no detectable effect on plasma levels of the analysed atopic disease markers, *ex vivo* cytokine production and circulating regulatory T cell percentage in infants with atopic dermatitis, besides down-regulation of IL-12 production in egg- and peanut-stimulated PBMCs. These results do not support the use of this synbiotic in clinical practice.

Keywords atopic dermatitis, chemokines, cytokines, infants, prebiotics, probiotics, regulatory T cells, synbiotics

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Correspondence:

L. B. van der Aa,
Department of Pediatric Respiratory
Medicine and Allergy, Emma
Children's Hospital, Academic Medical
Center, Meibergdreef 9, 1105 AZ
Amsterdam, The Netherlands.
E-mail: l.b.vanderaa@amc.nl

*Synbad Study Group: E. K. George, MD, PhD, Department of Pediatrics, Medical Center Alkmaar, Alkmaar, The Netherlands, N. J. van den Berg, MD, Department of Pediatrics, FlevoHospital, Almere, The Netherlands, D. Bosman, MD, PhD, Department of Pediatrics, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands,

G. A. M. Tytgat, MD, PhD, Department of Pediatrics, Zaan Medical Center, Zaandam, The Netherlands, E. Ree, MD, Department of Pediatrics, Sint Lucas Andreas Hospital, Amsterdam, The Netherlands, M. A. van Houten, MD, PhD, Department of Pediatrics, Spaarne Hospital, Hoofddorp, The Netherlands.

Introduction

Atopic dermatitis (AD) is a highly prevalent, chronic, itching skin disease that often presents in infancy and greatly affects the quality of life of children and their families [1]. Probiotics, live microorganisms with various, strain-specific immunomodulatory effects, have been proposed as preventive or therapeutic agents in allergic, i.e. Th2-mediated diseases, such as AD. *In vitro* studies revealed that probiotics are able to down-regulate Th2 cytokine production by stimulating production of Th1 cytokines, such as IL-12 and IFN- γ [2], or that of regulatory cytokines, such as IL-10 and TGF- β [3]. The latter is accomplished by either increasing production of these cytokines by antigen-presenting cells or by driving the development of IL-10- or TGF- β -producing regulatory T cells (Tregs) [4–6].

However, *in vitro* immunological effects of probiotics seem to differ from *in vivo* effects [7]. *In vivo* studies investigating the effect of probiotics on prevention or treatment of allergic disease in children have shown inconsistent immunological effects. Although up-regulation of IL-10 [8–10] and IFN- γ [11, 12] have been described, several studies showed no beneficial effect on cytokine production [7, 13–15]. These conflicting findings could be due to probiotic strain-specific effects, but even trials with identical strains gave opposing results [7–9, 11, 17]. Possibly, the different experimental settings contribute to these inconsistent findings.

Most of these clinical trials have been performed with lactobacilli or mixtures of lactobacilli and other probiotic species. *In vitro* studies showed that bifidobacteria induce more IL-10 production than lactobacilli [4, 5, 17], and thus bifidobacteria might have a larger immunomodulatory potential. In a murine model of allergic inflammation, the specific strain *Bifidobacterium breve* M-16V effectively reduced IL-4 and IgE production through induction of IL-10 and IFN- γ [18]. Furthermore, prebiotics, non-digestible food ingredients that stimulate the growth of certain probiotic bacteria in the colon [19], have been shown to increase mucosal and systemic IL-10 production in adults [20, 21]. Therefore, synbiotics, combinations of probiotics and prebiotics, seem to be promising candidates for inducing a beneficial immunological effect.

We performed a randomized, double-blind, placebo-controlled trial to investigate the effects of *Bifidobacterium breve* M-16V combined with a prebiotic mixture consisting of short chain galactooligosaccharides (scGOS) and long chain fructooligosaccharides (lcFOS; Immunofortis®; Danone Research-Centre for Specialised Nutrition, Wageningen, the Netherlands) in infants with AD. This synbiotic had no clinical effect on eczema severity [22]. In the current study, the effect on circulating levels of the following markers for atopic disease

was analysed: IL-5, IgG1, which may be promoted by enhanced IL-5 levels [23], IgG4, which is considered to dampen IgE responses, cutaneous T cell attracting chemokine (CTACK) and thymus and activation-regulated chemokine (TARC), two chemokines that attract T cells to the skin and are related to AD severity [24–26]. In addition, *ex vivo* cytokine responses by peripheral blood mononuclear cells (PBMCs) and the percentage of circulating Tregs were determined.

Materials and methods

Participants

Details of this study have been described elsewhere [22]. Ninety full-term infants, aged 0–7 months, fulfilling Hanifin and Rajka criteria for AD [27], were recruited between September 2005 and February 2007 from Paediatric and Dermatological 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 exclusively formula fed at time of enrolment, and all kinds of formula feeding were allowed (regular, partially hydrolysed, extensively hydrolysed). Infants were ineligible if the SCORing Atopic Dermatitis [28] score was <15, if they had major medical problems or if they had used probiotics, systemic antibiotics or anti-mycotics or immunosuppressive drugs during the 4 weeks prior to enrolment. Written informed consent was obtained from both parents of all participating children.

Study design

Participants were randomized using computer-generated four-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 additional 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. Blood samples were taken at baseline and at the end of the intervention period (week 12). The protocol was approved by the Medical Ethics Committees of all participating centres. The trial is registered in the ISRCTN register: ISRCTN69085979.

Synbiotics

The synbiotic consisted of *Bifidobacterium 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®), 0.8 g/100 mL. Formula was given on demand. The product was stable for at least 18 months when stored at room temperature (20–25°C).

Analysis of blood samples

Processing of blood. The EDTA-plasma samples were aliquoted and stored at –80°C till analyses of immunoglobulins (IgE, specific IgE, IgG1 and IgG4), CTACK and TARC. PBMCs were isolated within 2 h from heparin blood samples using standard density gradient techniques, and approximately 7.5×10^6 cells per ampoule were cryopreserved in liquid-N₂ awaiting analysis.

Determination of interleukin-5, immunoglobulin G1, immunoglobulin G4, cutaneous T cell attracting chemokine and thymus and activation-regulated chemokine. All were determined using ELISA. For IgG1 and IgG4, reagents from Sanquin (Amsterdam, the Netherlands; M1325, M1802, anti-human IgG-biotin) were used. Duosets for CTACK and TARC were obtained from and applied according to the recommendations by R&D. IL-5 was determined as described before [29].

Ex vivo cytokine production. For analysis, cryopreserved PBMCs were thawed, washed and incubated at 1.5×10^6 per mL culture medium [RPMI 1640 (Gibco, Invitrogen Ltd, Paisley, UK), containing 10% heat-inactivated FCS (Gibco), antibiotics and L-glutamine (Gibco)] in the absence or presence of anti-CD3/anti-CD28 (1XE 1 in 1000 dilution/5 µg/mL 15E8) or milk, egg or peanut allergen (25 µg/mL) for 5 days at 37°C and 5% CO₂. Supernatants were harvested and stored at –20°C until analysis. To be able to assess TGF-β, cells were incubated in serum-free medium (R&D; CCM010) instead of the RPMI medium, which was used in parallel to assess the other cytokines. The amount of several cytokines (IL-4, IL-5, IL-6, IL-10, IL-12p40/70, IL-12p70, IL-13, IL-17 and IFN-γ) produced by stimulated PBMCs, was determined using Luminex assay, according to the manufacturer's protocol (Biosource, Camarillo, CA, USA). Briefly, standard solutions and samples were added to a mixture of antibody-coated beads, whereupon cytokines, bound to their specific beads, were detected using biotinylated secondary antibodies and streptavidin-RPE (Biosource, Camarillo, CA, USA). The cytokine levels were determined by measuring the fluorescence intensity per bead per sample using the BioPlex System 100 (BioRad Laboratories, Hercules, CA, USA). TGF-β was determined using ELISA (R&D; MAB240 and BAF240;

according to the manufacturer's protocol). The amount of each cytokine in the supernatant of stimulated cultures was adjusted for background by subtracting the amount in the medium-only supernatant.

Determination of regulatory T cells. Thawed PBMCs were washed in PBS containing 0.01% (w/v) NaN₃ and 0.5% (w/v) bovine serum albumin. A total of 500 000 PBMCs were incubated for 30 min at 4°C with fluorescent-labelled conjugated monoclonal antibodies to the following surface markers (concentrations according to manufacturer's protocols): anti-CD3-PECy7, anti-CD4-FITC, anti-CD25-PE and anti-CD8-PerCP-Cy5.5, CD127-APC AF750 (BD Biosciences, San Jose, CA, USA). For intracellular FoxP3 staining, cells were fixed after staining of surface markers, and subsequently permeabilized in freshly prepared buffers from the FoxP3-staining kit (eBioscience, San Diego, CA, USA), and then incubated with anti-FoxP3-APC or rat isotype IgG2a-APC, according to the manufacturer's instructions. After staining, cells were washed and analysed using a FACS Canto flow cytometer (Becton Dickinson, Erembodegem, Belgium) and FlowJo software (v8.8.3, Tree Star Inc., Ashland, OR, USA).

Immunoglobulin E-positive subgroup

A subgroup of patients with IgE-associated AD was defined as patients with AD with elevated total and/or specific IgE levels at baseline. Total IgE and specific IgE against milk (f2), peanut (f13), egg (f245), fish (fx74), cat (e1) and house dust mite (d1), were determined using the CAP FEIA system (Phadia, Uppsala, Sweden). Total IgE was considered elevated if ≥ 5 kU/L in infants aged <3 months or ≥ 15 kU/L in infants aged >3 months (reference values of the Academic Medical Center, Amsterdam, the Netherlands). Specific IgE was considered elevated if ≥ 0.35 kU/L.

Statistics

Parametric data were analysed using unpaired *t*-tests and ANCOVA with steroid use and baseline values as co-variables. Non-parametric data were analysed using the Mann–Whitney *U*-test. A *P*-value <0.05 was considered statistically significant. SPSS software (15.0, Chicago, IL, USA) was used for all analyses.

Results

Patient characteristics

Baseline characteristics of all randomized infants are shown in Table 1. There were no differences between the synbiotic and the placebo group. The mean intake

Table 1. Baseline characteristics of all randomized children

	Synbiotics (N = 46)	Placebo (N = 44)
Male subject, n (%)	31 (67.4)	28 (63.6)
Age (months), mean $\pm$ SD	5.0 $\pm$ 1.4	4.8 $\pm$ 1.5
SCORAD index, mean $\pm$ SD	35.6 $\pm$ 10.6	34.7 $\pm$ 12.6
Total serum IgE (in kU/L), median (range) [N]	11 (2.3–234) [40]	18 (2.8–631) [40]
Elevated specific IgE, n (%) [N]		
Food allergens	21 (45.7) [42]	19 (43.2) [41]
Aeroallergens	4 (8.7) [42]	6 (13.6) [41]
Parental allergy, n (%)		
Maternal	25 (54.3)	27 (61.4)
Paternal	22 (47.8)	24 (54.5)
Both	16 (34.8)	16 (36.4)
Exposure to day care, n (%)	14 (30.4)	13 (29.5)
Pets at home, n (%)	14 (30.4)	15 (34.1)

SCORAD, SCORing Atopic Dermatitis.

of formula during the study was 778 mL/day (SD: 135) in the synbiotic and 760 mL/day (SD: 148) in the placebo group. A sufficient blood sample could be drawn from 82 infants (41 in the synbiotic and 41 in the placebo group), and paired samples could be drawn of 65 infants (32 in the synbiotic and 33 in the placebo group). Some samples were re-analysed on time, which

showed that there were no large differences between the various assay runs (data not shown).

Effect of the synbiotic mixture on plasma interleukin-5, immunoglobulins G1 and G4, cutaneous T cell attracting chemokine and thymus and activation-regulated chemokine

There were no significant differences between the synbiotic and the placebo group in plasma IL-5, IgG1, IgG4, CTACK and TARC (Table 2).

Effect of the synbiotic mixture on ex vivo cytokine production by peripheral blood mononuclear cells

In unstimulated PBMCs, the difference in IL-4 production between week 12 and baseline (Δ IL-4) was significantly lower in the synbiotic group than in the placebo group [mean: 0.02 (SD: 0.59) and 0.14 (SD: 0.68), respectively, group, $P = 0.04$]. IL-5, IL-6, IL-10, IL12p40/p70, IL-12p70, IL-13, IL-17, IFN- γ and TGF- β production by unstimulated PBMCs did not differ significantly between the synbiotic and the placebo group. Results for PBMCs stimulated with anti-CD3/anti-CD28 are shown in Fig. 1. There were no significant differences between the two groups. Results of

Table 2. Effect of the synbiotic mixture on plasma IL-5, IgG1, IgG4, CTACK and TARC

	Synbiotics	Placebo	P-value
IL-5, pg/mL (detection limit 0.18)			
Median (range)			
Week 0 [n]	1.80 (0.18 to 19.80) [40]	1.80 (0.18 to 17.70) [41]	0.72
Week 12 [n]	0.18 (0.18 to 10.30) [36]	0.18 (0.18 to 29.80) [38]	0.69
Δ Week 12–week 0 [n]	–0.82 (–7.42 to 7.12) [35]	–0.11 (–12.30 to 17) [38]	0.88
IgG1, mg/mL			
Median (range)			
Week 0 [n]	5.63 (0.50 to 20.10) [40]	4.51 (1.68 to 66) [41]	0.26
Week 12 [n]	7.92 (2.59 to 46.38) [36]	6.65 (0.98 to 66) [38]	0.26
Δ Week 12–week 0 [n]	1.50 (–5.39 to 37.67) [35]	1.78 (–6.02 to 26.20) [38]	0.98
IgG4, μ g/mL			
Median (range)			
Week 0 [n]	20.05 (1.40 to 406) [41]	28.43 (1.20 to 1290) [41]	0.84
Week 12 [n]	10.69 (1 to 503) [36]	13.30 (0.40 to 337) [38]	0.96
Δ Week 12–week 0 [n]	–1.83 (–363 to 147) [35]	–1.85 (–1248 to 206) [38]	0.62
CTACK, ng/mL			
Median (range)			
Week 0 [i]	17.47 (4.50 to 34.10) [39]	16.28 (10.17 to 34.90) [38]	0.42
Week 12 [n]	13.60 (7.98 to 26) [34]	13.10 (4.50 to 31.30) [35]	0.28
Δ Week 12–week 0 [n]	–3.64 (1.17) [33]	–3.93 (1.02) [35]	0.80
TARC, pg/mL			
Median (range)			
Week 0 [n]	475 (140 to 3991) [38]	382 (85 to 6641) [35]	0.75
Week 12 [n]	176 (57 to 4038) [33]	244 (80 to 7305) [33]	0.12
Δ Week 12–week 0 [n]	–248 (–3219 to 3898) [31]	–129 (–2763 to 6300) [31]	0.07

CTACK, cutaneous T cell attracting chemokine; TARC, thymus and activation-regulated chemokine.

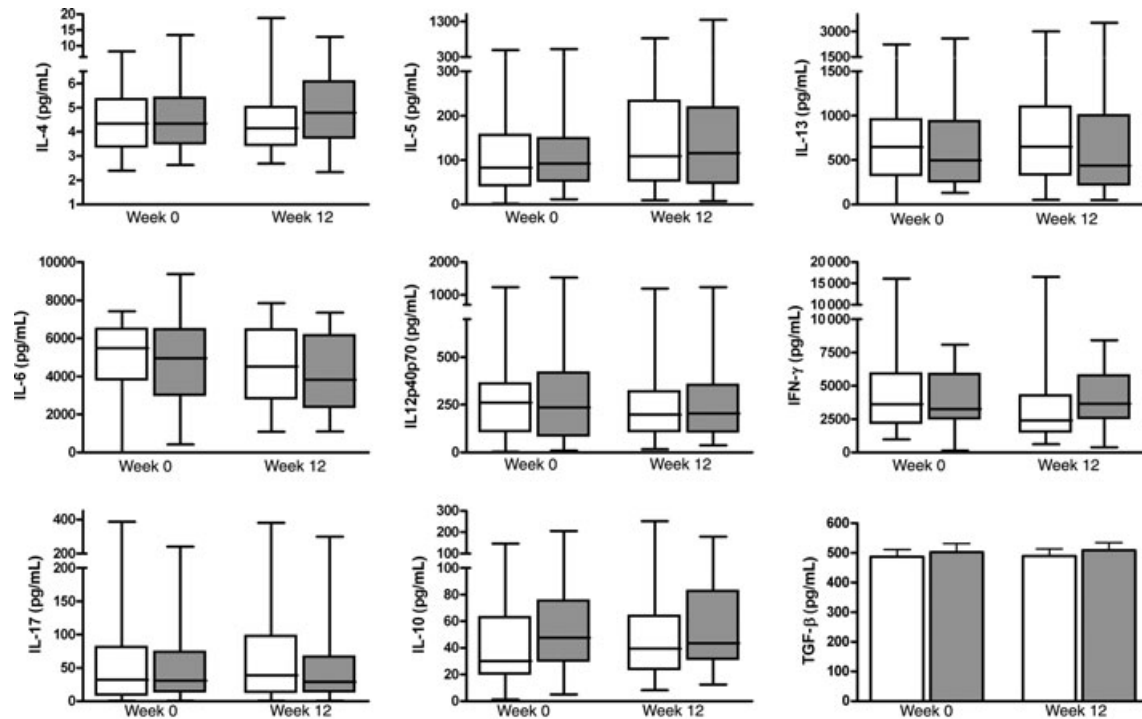


Fig. 1. Ex vivo cytokine production by peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3/anti-CD28 in the synbiotic group (white bars) and the placebo group (grey bars) at week 0 and after 12 weeks of intervention (median, interquartile range and range, comparison between the two groups using Mann-Whitney *U*-test).

PBMCs stimulated with egg, peanut or cow's milk allergen are shown in Table 3. PBMCs stimulated with egg allergen showed a significantly greater increase in IL12p40/p70 and IL-12p70 production in the placebo group than in the synbiotic group ($P = 0.04$ and $P = 0.01$, respectively). The same was true for IL-12p70 ($P = 0.003$) in PBMCs stimulated with peanut allergen, but not for IL-12p40/p70 ($P = 0.89$). In PBMCs stimulated with cow's milk allergen, there were no significant differences between the two groups.

Effect of the synbiotic mixture on regulatory T cells

There were no significant differences between the two groups in percentages of CD3⁺, CD4⁺CD8⁻ and FoxP3⁺CD25⁺ cells at baseline and after 12 weeks of intervention (data not shown). Also, the mean percentage of FoxP3⁺CD25⁺CD127⁻ cells (Tregs) did not differ between the synbiotic and the placebo group (2.5%, SD: 0.8, vs. 2.5%, SD: 0.9, respectively, $P = 0.75$).

Immunoglobulin E-positive subgroup

There were 39 children with IgE-associated AD, 20 in the synbiotic group and 19 in the placebo group. In the synbiotic group, there were four children with elevated specific IgE against milk, seven against peanut and 11 against egg. In the placebo group, this was four against

milk, 12 against peanut and 10 against egg. In the subgroup of infants with IgE-associated AD, there were no significant differences between the synbiotic and the placebo group in plasma IL-5, IgG1, IgG4, CTACK and TARC, *ex vivo* cytokine production by unstimulated and anti-CD3/anti-CD28 stimulated PBMCs and circulating Treg percentage (data not shown). For PBMCs stimulated with egg, peanut and cow's milk allergen, there were no significant differences except for Δ IL-12p40/p70 in egg-stimulated PBMCs (median: -0.11 pg/mL, range: -2.74 to 13.7 , in the synbiotic and 0.91 pg/mL, range: -1.31 to 323 , in the placebo group, $P = 0.03$).

Discussion

In this study, we did not show a beneficial effect of the combination of *Bifidobacterium breve* M-16V and a specific scGOS/lcFOS mixture on circulating markers of atopic disease (IL-5, IgG1, IgG4, CTACK and TARC), *ex vivo* cytokine production by PBMCs in response to unspecific and allergen-specific stimuli and circulating Treg percentage in infants with AD.

Our results do not confirm the results of a study with a murine model of allergic inflammation, which showed that *Bifidobacterium breve* M-16V effectively reduced IL-4 and IgE production through induction of IL-10 and IFN- γ [18]. So far, no other trials have studied the immunological effects of synbiotics in children with AD.

Table 3. Effect of the synbiotic mixture on *ex vivo* cytokine production by PBMCs stimulated with egg, peanut and cow's milk allergen

Cytokines (pg/mL) Week 12–baseline (Δ), median (range)	Synbiotics ($N = 32$)	Placebo ($N = 33$)	<i>P</i> -value
Egg			
Δ IL-4, mean (SD)	−0.08 (0.66)	0.29 (0.59)	0.12
Δ IL-5	−0.37 (−29.8 to 91.5)	0.21 (−86 to 91.3)	0.46
Δ IL-6	−1.05 (−1083 to 5143)	1.23 (−152 to 540)	0.94
Δ IL-10	−0.11 (−8.9 to 9.5)	0.60 (−5.7 to 20.2)	0.07
Δ IL-12p40/70	0 (−175 to 2213)	0.98 (−12.5 to 323)	0.04
Δ IL-12p70	−0.50 (−7.8 to 11.7)	1.95 (−5.3 to 26.7)	0.01
Δ IL-13 [<i>n</i>]	−1.95 (−64 to 99.6) [29]	0.54 (−109 to 130) [31]	0.70
Δ IFN- γ	0.60 (−34 to 112)	1.78 (−9.5 to 73.7)	0.16
Peanut			
Δ IL-4	0.04 (−32.8 to 0.99)	0.08 (−0.64 to 1.1)	0.30
Δ IL-5	−1.34 (−40.8 to 119)	1.85 (−80.2 to 38.5)	1
Δ IL-6	−1.87 (−618 to 1382)	−1.50 (−584 to 411)	0.11
Δ IL-10	−0.40 (−15.6 to 15.8)	0.21 (−14 to 28.4)	0.63
Δ IL-12p40/70	0.04 (−55.1 to 59.8)	0.30 (−7.2 to 60.5)	0.89
Δ IL-12p70	−0.98 (−182 to 8.7)	1.32 (−1.6 to 13.3)	0.003
Δ IL-13, mean (SD)	3.66 (45.4)	−1.73 (39.8)	0.70
Δ IFN- γ	−1.32 (−80.5 to 129)	−0.17 (−70.4 to 123)	0.86
Cow's milk			
Δ IL-4	−0.08 (−0.79 to 1.3)	−0.04 (−0.55 to 1.6)	0.76
Δ IL-5	0.29 (−22.8 to 29.2)	1.02 (−13 to 103)	0.08
Δ IL-6	−225 (−5355 to 15375)	−117 (−7619 to 5231)	0.87
Δ IL-10	−0.44 (−15.7 to 10)	0.21 (−19 to 12.7)	0.49
Δ IL-12p40/70	31.9 (−2031 to 2740)	−2.16 (−3387 to 2083)	0.81
Δ IL-12p70	0 (−7.3 to 17.5)	0.67 (−5.6 to 14.8)	0.30
Δ IL-13 [<i>n</i>]	0.30 (−45.7 to 28.8) [28]	1.39 (−62 to 41.7) [28]	0.94
Δ IFN- γ	0.93 (−599 to 1052)	5.79 (−189 to 716)	0.18

PBMCs, peripheral blood mononuclear cells.

Available evidence from different studies examining the effects of *probiotics* in children with AD shows inconsistent results with respect to immunological effects. Consistent with our results, several studies investigating the therapeutic effect in children with AD [14–16] and two studies investigating the preventive effect in children at high risk for allergic disease [7, 13], could not demonstrate a beneficial effect of probiotics on cytokine production. One prevention trial even showed a decrease in regulatory cytokines in the probiotic group [31].

In contrast to the present results, some trials with probiotics in children with or at high risk for AD did show a positive effect on the production of regulatory and/or Th1 cytokines [8–12]; however, many of these studies do have some methodological issues. One study showed an increase in IL-10 production, serum and PBMCs, after treatment with *Lactobacillus rhamnosus* GG (LGG), but had only nine participants and no placebo group [8]. Viljanen *et al.* [9] demonstrated increased plasma IL-10 and IL-6 levels after treatment with a probiotic mixture or LGG; however, in 60–80% of the samples, IL-10 and IL-6 values were below the detection limit. In another study, increased IFN- γ

responses by PBMCs after LGG administration were shown in a subgroup of children with IgE-associated AD, but the number of samples was small, and baseline IFN- γ responses differed considerably between the treatment and the placebo group [11]. Prescott *et al.* [12] also showed increased IFN- γ responses in PBMCs of children treated with *Lactobacillus fermentum*; however, this difference was not significant in between-group analysis, and in this study also, baseline IFN- γ responses differed significantly between the two groups.

In vitro and animal studies have shown that probiotics induce Tregs [5, 30]; however, in the present study it was not possible to demonstrate an effect on circulating Treg percentage. Similar to our results, a prevention study on the effect of *Lactobacillus acidophilus* in high-risk infants did not demonstrate an effect on Treg proportion [31].

A strong point of our study is that we exercised great care to process plasma and isolate PBMCs, in a standardized manner, particularly as it is known that prolonged storage may affect various T cell populations differently [32]. In addition, samples from week 0 and

12 were analysed in parallel to limiting interassay variation. Furthermore, some samples were re-analysed on time, which showed that there were no large differences between the various assay runs.

In our study, no immunomodulatory effects could be demonstrated, except a decreased IL-12 production after stimulation of PBMCs with egg and peanut allergen in the synbiotic group compared with placebo. This means that *ex vivo*, this specific synbiotic seems to down-regulate production of a Th1 cytokine, and not, as was shown in an animal study, of Th2 cytokines [18]. Certain probiotic strains have been shown to suppress IL-12 secretion [33], and this is a beneficial effect in auto-immune diseases; however, the implication of this effect in allergic disease remains less clear, as these diseases are Th2 driven. One has to keep in mind that the IL-12 results might be the result of type I error due to multiple testing.

The present immunological results are in line with previous results of our trial, as we did not find a beneficial effect of this synbiotic formula on the severity of infant AD, eosinophilic granulocyte count or serum IgE levels after 12 weeks of intervention [22]. We did show a significant beneficial effect of synbiotics on the severity of AD in the subgroup of infants with IgE-associated AD [22], but could not demonstrate an explanatory immunomodulatory effect in this subgroup. The reason for the lack of systemic immunomodulatory effects of pro- and synbiotics seen in the current and several other clinical studies, as opposed to those studies that showed differences, remains to be elucidated. Earlier, we showed that this specific synbiotic mixture significantly changes the intestinal microbiota composition of infants with AD [22]. Although one would expect such a change to be associated with immunological effects, it could be that these effects are limited to the gut-associated lymphoid tissue and possibly other mucosal sites, and therefore cannot be detected systemically.

Another hypothesis might be that the immunological effects elicited by pro- and synbiotics are transient. It has been shown that commensal bacteria elicit a specific IgA-response upon initial colonization, after which the number of bacteria that translocate the epithelial barrier and stimulate the local immune system drops [34]. Therefore, probiotics possibly only elicit an

immune response for a short period after initial administration, before the IgA-response has fully developed.

Furthermore, although we investigated an extensive panel of atopic disease markers and cytokines, it might be that other biomarkers are more sensitive. One can speculate that in this group of patients, other immunological mechanisms like immunoglobulin free light chains (Ig-fLCs), which were recently demonstrated to be important markers of atopic disease, are involved in the hypersensitivity response [35].

Finally, it could also be that immunomodulatory effects of pro- and synbiotics are most evident after a 'hit' with a specific allergen. In most experimental studies, animals are sensitized to an allergen and then challenged with that specific allergen, after which symptoms and cytokine responses are measured [36]. In studies in children with AD, no *in vivo* challenges are administered. Not only would this be unethical, it would also be difficult to choose the right allergen, as children with AD are often sensitized to multiple allergens without clear clinical consequences [37]. Moreover, approximately half of the infants with AD have no evidence of IgE-sensitization [38]. In this and several other studies, PBMCs were challenged *in vitro* with food allergens; however, such *in vitro* challenges might not reveal probiotic and synbiotic immunomodulatory effects.

In conclusion, we did not demonstrate a detectable beneficial effect of the specific synbiotic combination of *Bifidobacterium breve* M-16V and a scGOS/lcFOS mixture on plasma levels of the analysed atopic disease markers, *ex vivo* cytokine production by PMBCs and circulating Tregs percentage in infants with AD. These results do not support the use of this synbiotic in clinical practice.

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