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Effect of Synbiotic on the Gut Microbiota of Cesarean Delivered Infants: A Randomized, Double-blind, Multicenter Study

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ABSTRACT

We determined the effect of short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and *Bifidobacterium breve* M-16V on the gut microbiota of cesarean-born infants. Infants were randomized to receive a standard formula (control), the same with scGOS/lcFOS and *B. breve* M-16V (synbiotic), or with scGOS/lcFOS (prebiotic) from birth until week 16, 30 subjects born vaginally were included as a reference group. Synbiotic supplementation resulted in a higher bifidobacteria proportion from day 3/5 ($P < 0.0001$) until week 8 ($P = 0.041$), a reduction of Enterobacteriaceae from day 3/5 ($P = 0.002$) till week 12 ($P = 0.016$) compared to controls. This was accompanied with a lower fecal pH and higher acetate. In the synbiotic group, *B. breve* M-16V was detected 6 weeks postintervention in 38.7% of the infants. This synbiotic concept supported the early modulation of *Bifidobacterium* in C-section born infants that was associated with the emulation of the gut physiological environment observed in vaginally delivered infants.

Key Words: *Bifidobacterium breve* M-16V, C-section, gut microbiota, prebiotics, probiotics, synbiotics

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What Is Known

- Cesarean birth has been associated with increased risk of immune and metabolic diseases later in life, likely due to the altered gut microbiota.
- The gut microbiota acts as a potential modifiable risk factor for disease development.
- Little is known about the effect of nutrition on C-section–born infants.

What Is New

- Supplementation with short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides and *Bifidobacterium breve* M-16V compensates the delayed *Bifidobacterium* colonization in C-section–delivered infants and modulates the production of acetate and the acidification of the gut. These observed physiological conditions, described as indicator of gut health, emulate the ones observed in vaginally born infants.

Epidemiological studies have indicated associations between cesarean section (C-section), immune, and metabolic disorders (1–3). The way we are born has been hypothesized to cause an epigenetic and microbial imprinting that may have consequences on our long-term health (4). Several studies depicted a delayed colonization of keystone microbial colonizers, such as *Bifidobacterium* and *Bacteroides*, in cesarean-born infants (5,6). Those early colonizers have a role in orchestrating humoral and cell-mediated immune maturation, are endowed with the genomic capability to metabolize human milk oligosaccharides, and provide colonization resistance by deterring the overgrowth of opportunistic pathogens (7,8). Members of the Enterobacteriaceae family, such as *Klebsiella* and *Enterobacter*, have been described to dominate the gut of infants born by C-section; however, little is known about their impact on the host health (5,6).

Because early life is a critical period, as the infant's immune system is still maturing and is influenced by the gut microbiota, any dysbiosis as a result of environmental factors such as delivery mode and/or antibiotic use could lead to long-lasting health effects (9). A recent study suggested that swabbing infants born by C-section immediately after birth with vaginal secretions (vaginal seeding)

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could partially restore the missing maternal microbiota (10). Alternatively, supplementation with prebiotics, probiotics, or synbiotics has been pursued as a nutritional solution to regulate immune responses through a modulation of the gut microbiota. The aim of the present study was to investigate the effect of an infant formula supplemented with short-chain galacto-oligosaccharide (scGOS)/long-chain fructo-oligosaccharides (lcFOS) and *Bifidobacterium breve* M-16V on the gut microbiota of C-section–born infants.

METHODS

This was an exploratory, randomized, double-blind, controlled study conducted between June 2011 and April 2013 in Singapore and Thailand. All participating centers obtained approval of their independent local Ethical Review Board. Written informed consent was obtained from all parents before randomization. The study was registered in the Dutch Trial Register (*http://www.trialregister.nl/NTR* Number: 2838).

Eligible infants were randomly assigned to receive either nonhydrolyzed cow's milk–based formula (control formula), or to the same formula supplemented with 0.8 g/100 mL scGOS/lCFOS (prebiotic formula) or to identical prebiotic formula additionally supplemented with *B. breve* M-16V (Morinaga Milk Industry Co. Ltd) at a dose of 7.5×10^8 cfu/100 mL (synbiotic formula). Nonrandomized infants born vaginally were included as a reference group. Study formulas were administered from birth (1–3 days at the latest) until 16 weeks of age (intervention period). Stool samples were collected at day 3, day 5, week 2, week 4, week 8, week 12, week 16, and week 22. All samples were frozen immediately by the parents and transported to the hospital and stored at −80°C. Fluorescent in situ hybridization and quantitative real-time polymerase chain reaction were used to assess the gut microbiota composition. The primary outcome was the determination of total fecal bifidobacteria. Secondary parameters were *Bifidobacterium* species abundance, other members of the gut microbiota, pH, short-chain fatty acid (SCFA), and lactate and safety parameters (anthropometry, gastrointestinal tolerance, adverse events [AEs]).

Statistical analyses were performed on both the modified intention-to-treat (mITT) and Per Protocol (PP) populations. The mITT consisted of all randomized subjects who provided at least 1 baseline and postbaseline stool sample. For the safety data, All Subjects Treated (AST) was used. A generalized linear mixed model (PROC GLIMMIX) with lognormal distribution and identity link function was used to evaluate the treatment effect on primary and secondary parameters (SAS 9.2, SAS Institute, NC). The model was fitted with core factors (treatment and time as categorized by week numbers, treatment-by-time interaction, and country) and selected covariates (daily formula-feeding and number of daily breast-feeding servings). Description of the clinical trial conduct, laboratory, and statistical analyses is available in the Supplemental Digital Content, Table 1 and Table 2, *http://links.lww.com/MPG/A988*.

RESULTS

A total of 153 subjects delivered by C-section were randomized to receive either the synbiotic (n = 52), prebiotic (n = 51), or the control formula (n = 50). Thirty subjects were included in the nonrandomized reference group (Supplemental Digital Content, Fig. 1, *http://links.lww.com/MPG/A988*). Baseline data did not show significant differences between control and active C-section treatment groups (Supplemental Digital Content, Table 3, *http://links.lww.com/MPG/A988*), with the exception for birth weight in Thailand where simultaneous pairwise comparisons showed a statistical significance between control (n = 6, mean = 3.27) and prebiotic (n = 6, mean = 2.94) (mean difference = 0.32; 95% confidence interval: 0.03, 0.63) (data not shown). All infants included were mixed-fed; indeed most subjects received the study product corresponding to their allocated group in addition to breast-feeding. The product intake and exposure is illustrated in Supplemental Digital Content, Table 4, *http://links.lww.com/MPG/A988*.

Although the objective of the study was not to compare the randomized intervention groups with the reference group, our data showed a striking delayed colonization by bifidobacteria in infants delivered by C-section compared to those delivered vaginally during the first 2 to 3 months of life (Fig. 1). From day 3/5 until week 8, the proportion of bifidobacteria in the mITT population was significantly higher in the synbiotic than in the control ($P < 0.0001$ and $P = 0.041$), respectively). Analysis by quantitative real-time polymerase chain reaction, revealed a significantly higher absolute gene count of *Bifidobacterium* in the synbiotic compared to the control group from day 3/5 ($P < 0.0001$) till week 12 ($P = 0.032$). In the prebiotic group, the percentage of bifidobacteria increased over the course of the intervention, but no statistically significant difference was observed when compared to the control group in both mITT and PP populations (Table 1, Fig. 1A, Supplemental Digital Content, Table 5, *http://links.lww.com/MPG/A988*).

The probiotic strain *B. breve* M-16V was detected at day 3/5 in 97.9% of infants in the synbiotic group and was still found in 38.7% of the infants 6 weeks postintervention (Fig. 1B). In addition, we monitored the distribution of other *Bifidobacterium* species, and observed that *B. breve, B. bifidum*, and *B. longum* group were the most frequently detected in all groups. At day 3/5, nearly all infants of the synbiotic group were colonized by *B. breve* and this was significantly higher compared to the control group ($P < 0.0001$). The statistical significance remained till week 16 ($P = 0.02$). The estimated mean proportion of infants with detectable *B. bifidum, B. catenulatum*, and *B. longum* group was comparable in the control, prebiotic, and synbiotic groups and across all time points (Fig. 1B–D).

We assessed the abundance of 6 other major gut bacterial taxa. In all intervention groups and at all-time points, the estimated mean proportions of the main bacterial groups were suboptimal and varied from 0% to 0.6% across the *Atopobium*, *Bacteroides*, and *Clostridium histolyticum*/*C. lituseburense* and from 0.2% to 2.9% across the *Eubacterium rectale/C. coccoides* and Lactobacilli-Enterococci groups. No major effect was observed on these bacterial groups except for the Enterobacteriaceae (Table 1). In the mITT population, Enterobacteriaceae were detected at a high level at day 3/5 especially in the control group and to a less extent in the prebiotic group (15.1% vs 9.2%, respectively; $P = 0.178$). In the synbiotic group, the estimated mean proportion of Enterobacteriaceae at day 3/5 was 3.9% and this was significantly lower than in the control group ($P = 0.002$) and the difference persisted up to week 12 ($P = 0.016$). At week 2, a significantly higher level in the prebiotic than in the control group was observed ($P = 0.03$); however, no significant difference was detected between the prebiotic and control group in the PP population (Table 1, Supplemental Digital Content, Table 5, *http://links.lww.com/MPG/A988*).

Fecal pH, SCFA, and lactate were assessed as markers of the metabolic activity of the entire gut microbial ecosystem. In the mITT population, supplementation with synbiotic resulted in a lower estimated mean pH from day 3/5 until week 4, and this was significantly different from the control group ($P < 0.0001$ and $P = 0.001$, respectively) (Table 1). Acetate was the main SCFA detected and the concentration increased over the study period in all intervention groups. At day 3/5, the concentration of acetate was significantly higher in the synbiotic than in the control group in both mITT and PP populations ($P < 0.0001$) (Table 1, Supplemental Digital Content, Table 5, *http://links.lww.com/MPG/A988*). The data on butyrate, propionate, and lactate were converted into binary outcome as detected/not-detected because >30% of the subjects had values below the detection limit at one or more time points.

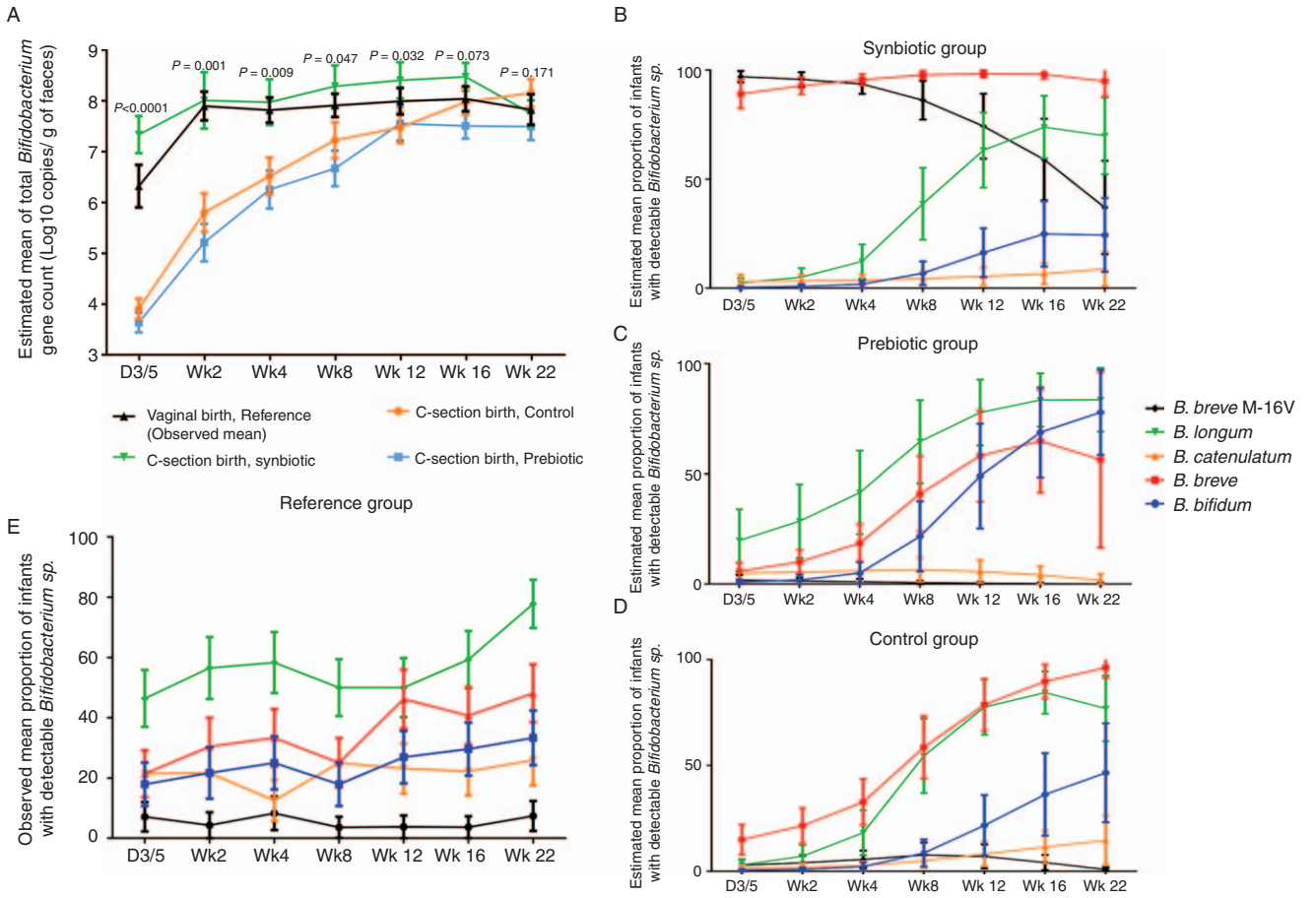

FIGURE 1. Effect of the intervention on the estimated mean of (A) total *Bifidobacterium* gene count (Log_{10} copies/g of feces) as determined by quantitative real-time polymerase chain reaction (Q-PCR), and the effect of the interventions on the colonization of the infants gut by *B. bifidum, B. breve, B. catenulatum, B. longum, and B. breve* M-16V in (B) synbiotic group, (C) prebiotic group, (D) control group, and (E) reference group. Data expressed as estimated mean proportion (SEM) of infants with detectable *Bifidobacterium* (modified intention-to-treat mITT population). The black line in (A) represents the observed (SD) mean of the vaginally delivered infants (reference group). This group was nonrandomized and was not included in the statistical model. The *P* values correspond to the comparison between the synbiotic and the control group. No statistical difference was observed for the prebiotic versus the control group.

A significantly lower number of infants with detectable amounts of butyrate were observed from week 4 until week 16 in the synbiotic compared to the control group. No significant effect on propionate and lactate was observed (data not shown).

All formulas were well tolerated and all groups showed a comparable safety profile, based on the number and severity of AEs and growth (data not shown). The proportions of subjects experiencing AEs were similar across intervention groups with no significant difference between groups for overall AEs (Chi-square = 1.78, $P = 0.62$). All of the reported AEs were not related to the study product except for one subject in the prebiotic and the control groups (irritability and constipation of mild severity, respectively). Post-hoc analysis showed a lower percentage of subjects with AEs-related skin disorders in the synbiotic compared to the control group (20% vs 42%, $P = 0.017$) (Supplemental Digital Content, Fig. 2, *http://links.lww.com/MPG/A988*). After correcting for family history of allergy, we observed that infants with reported eczema/Atopic Dermatitis (AD) were less frequent in the synbiotic than in the control group; however, the number of subjects is low to draw any biological conclusion.

DISCUSSION

Our findings revealed a delayed intestinal colonization by *Bifidobacterium* species in C-section–delivered infants, consistent with previous studies (5,6). This delayed settlement of bifidobacteria was apparent from the first days of life and persisted until 2 to 3 months of age. Early supplementation with scGOS/lcFOS and *B. breve* M-16V resulted in an immediate colonization by *Bifidobacterium* suggesting that the first 3 months of life represent a window of opportunity for a fast recovery of *Bifidobacterium* colonization in C-section–delivered infants. The relevance of preventing the delayed colonization by *Bifidobacterium* in C-section born infants lies in the recognized contributory role of *Bifidobacterium* in early life immune programming (7,11). Members of the genus *Bifidobacterium* have been depicted as microbial biomarker of immune fitness in healthy nonatopic infants (12). A recent study indicated that *B. breve* abundance was correlated with a protection against the development of eczema and immune sensitization in infants (13). The synbiotic mixture used in the present study provided an exogenous strain that promoted intraspecies diversity, a biological phenomenon, which was translated through the detection of

TABLE 1. Effect of the intervention the study outcome (modified intention-to-treat population)

		% Bifidobacteria[†]	Bifidobacteria count (Log10 copies/g)[‡]	% Enterobacteriaceae[†]	% Bacteroides distasonis/fragilis group[†]	% Atopobium group[†]	% *Clostridium histolyticum/Clostridium lituseburense* group[†]	% *Eubacterium rectale/Clostridium coccoides* group[†]	% *Lactobacillus-Enterococcus* group[†]	Acetate (mmol/kg feces)	pH
Reference[§]	Day3/5	25.4 (4.7)	6.3 (0.4)	17.6 (3.3)	1.4 (0.5)	1.2 (0.6)	2.7 (2.0)	0.7 (0.2)	2.3 (0.9)	41.1 (5.8)	5.9 (0.1)
	Week 2	35.2 (6.4)	7.9 (0.3)	16.5 (3.3)	ND	ND	ND	ND	ND	43.7 (4.6)	6.0 (0.1)
	Week 4	46.2 (5.3)	7.8 (0.2)	7.7 (1.8)	2.2 (0.7)	4.1 (1.3)	0.7 (0.2)	1.8 (0.6)	2.5 (1.6)	52.9 (6.3)	6.0 (0.1)
	Week 8	38.0 (4.3)	7.9 (0.2)	5.4 (1.6)	0.7 (0.2)	3.6 (1.0)	1.0 (0.4)	4.3 (1.4)	1.7 (0.5)	55.7 (4.3)	6.0 (0.1)
	Week 12	43.4 (5.4)	8.0 (0.3)	4.3 (1.4)	1.2 (0.3)	4.6 (1.5)	0.7 (0.2)	4.9 (1.7)	1.4 (0.4)	55.3 (5.4)	6.0 (0.1)
	Week 16	43.9 (5.5)	8.0 (0.2)	7.4 (2.0)	2.1 (0.6)	4.6 (1.3)	0.8 (0.3)	5.4 (1.5)	3.2 (0.9)	57.9 (5.5)	6.1 (0.1)
	Week 22	43.9 (3.7)	7.8 (0.3)	4.3 (0.8)	ND	ND	ND	ND	ND	77.0 (7.1)	5.9 (0.2)
Control	Day3/5	1.2 (0.4)	3.9 (0.2)	15.1 (4.8)	0.6 (0.2)	0.0 (0.0)	0.2 (0.1)	0.2 (0.1)	0.5 (0.2)	17.7 (2.0)	6.2 (0.1)
	Week 2	10.6 (2.5)	5.8 (0.4)	18.2 (3.3)	ND	ND	ND	ND	ND	39.9 (4.1)	6.2 (0.1)
	Week 4	11.7 (3.4)	6.5 (0.4)	5.0 (1.1)	0.2 (0.1)	0.1 (0.0)	0.5 (0.1)	0.6 (0.2)	2.7 (0.8)	43.6 (4.4)	6.4 (0.1)
	Week 8	14.1 (4.5)	7.2 (0.4)	3.3 (0.7)	0.1 (0.0)	0.1 (0.0)	0.3 (0.1)	0.8 (0.2)	1.7 (0.5)	50.2 (5.4)	6.1 (0.1)
	Week 12	24.3 (5.8)	7.5 (0.3)	2.5 (0.6)	0.2 (0.0)	0.2 (0.0)	0.2 (0.1)	1.5 (0.5)	1.6 (0.4)	60.1 (6.0)	6.1 (0.1)
	Week 16	27.7 (6.4)	8.0 (0.3)	1.7 (0.4)	0.5 (0.2)	0.3 (0.1)	0.3 (0.1)	2.9 (0.9)	1.7 (0.5)	64.8 (7.0)	6.0 (0.1)
	Week 22	26.7 (4.6)	8.1 (0.3)	3.7 (0.7)	ND	ND	ND	ND	ND	68.5 (7.2)	6.1 (0.1)
Synbiotic	D3/5	13.7 (4.5)*	7.3 (0.4)*	3.9 (1.3)**	0.3 (0.1)	0.0 (0.0)	0.1 (0.0)	0.2 (0.1)	0.2 (0.1)	32.3 (3.7)*	5.7* (0.1)
	Week 2	29.0 (7.4)*	8.0 (0.6)**	9.4 (1.9)**	ND	ND	ND	ND	ND	45.2 (4.8)	5.9** (0.1)
	Week 4	26.4 (8.5)**	8.0 (0.5)**	3.2 (0.7)**	0.2 (0.1)	0.1 (0.0)	0.2 (0.1)	0.3 (0.1)	0.4 (0.1)	45.2 (4.5)	5.9** (0.1)
	Week 8	29.3 (9.6)**	8.3 (0.4)**	2.0 (0.5)**	0.1 (0.0)	0.2 (0.0)	0.1 (0.0)	0.4 (0.1)	1.4 (0.4)	46.8 (5.1)	6.0 (0.1)
	Week 12	37.7 (9.9)	8.4 (0.4)**	1.4 (0.3)**	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)	1.3 (0.5)	1.5 (0.4)	63.0 (6.7)	5.9 (0.1)
	Week 16	36.2 (8.0)	8.5 (0.3)	1.6 (0.4)	0.3 (0.1)	0.3 (0.1)	0.2 (0.10	1.7 (0.5)	1.2 (0.4)	67.0 (7.3)	5.9 (0.1)
	Week 22	32.0 (5.1)	7.7 (0.3)	3.5 (0.6)	ND	ND	ND	ND	ND	75.3 (7.4)	6.1 (0.1)
Prebiotic	D3/5	0.5 (0.2)**	3.6 (0.2)	9.2 (3.6)	0.2 (0.1)	0.0 (0.0)	0.2 (0.1)	0.2 (0.1)	0.8 (0.4)	18.0 (2.2)	6.1 (0.1)
	Week 2	6.6 (1.7)	5.2 (0.4)	27.7 (5.5)**	ND	ND	ND	ND	ND	35.2 (3.8)	6.1 (0.1)
	Week 4	12.0 (4.1)	6.2 (0.4)	6.1 (1.4)	0.3 (0.1)	0.1 (0.0)	0.2 (0.1)	0.4 (0.2)	1.5 (0.5)	46.4 (4.7)	5.9* (0.1)
	Week 8	11.0 (3.8)	6.7 (0.4)	2.3 (0.6)	0.1 (0.0)	0.1 (0.0)	0.2 (0.1)	0.9 (0.3)	2.1 (0.7)	44.8 (5.1)	6.1 (0.1)
	Week 12	24.5 (6.6)	7.5 (0.3)	2.2 (0.6	0.2 (0.1)	0.2 (0.1)	0.2 (0.0)	1.4 (0.5)	1.6 (0.4)	53.4 (5.9)	6.0 (0.1)
	Week 16	22.0 (5.3)	7.5 (0.3)	2.8 (0.7)**	0.4 (0.1)	0.3 (0.1)	0.3 (0.1)	1.6 (0.5)	1.5 (0.4)	57.0 (6.5)	6.0 (0.1)
	Week 22	28.7 (5.0)	7.5 (0.3)	3.3 (0.6)	ND	ND	ND	ND	ND	64.8 (7.1)	6.2 (0.1)

A generalized linear mixed model with lognormal distribution and identity link function was used and intervention effects were compared using z test.
ND = not determined.
[†]Data obtained by fluorescent in situ hybridization (FISH) and reported as estimated mean (SEM) and as % total bacteria.
[‡]Data obtained by quantitative real-time polymerase chain reaction (Q-PCR) and reported as estimated mean (SEM).
[§]Observed data expressed as mean (SD) of the reference group (vaginally delivered infants). These infant were not randomized and not included in the statistical model.
*$P < 0.0001$.
**$P < 0.05$.

indigenous *B. breve* that appeared slowly over the time. At the end of intervention, the proportion of subjects with detectable infant type *Bifidobacterium* species was comparable across all the intervention groups, indicating that supplementation with a unique probiotic strain did not impair the development of the indigenous bifidobacterial community over time. Furthermore, synbiotic provides a well-defined and safe alternative for the recently described vaginal seeding, which is rather undefined and may contain less desired biological constituents (10). Supporting the development of the gut microbiota in early life and the typical *Bifidobacterium* species found in healthy vaginally born and breast-fed infants may improve certain immune phenotypes that are particularly relevant for C-section born infants. Hong et al (12) observed that C-section–born infants with eczema featured a delayed colonization by *Bifidobacterium* and an enrichment of Enterobacteriaceae.

In addition, we showed that the synbiotic formula resulted in reduced abundance of Enterobacteriaceae that was likely due to the acidic intestinal milieu generated by the synbiotic modulation of the gut microbiota and increase of acetate. The production of SCFA such as acetate by obligate anaerobic bacteria was depicted as a mechanism for metabolic exclusion of Enterobacteriaceae from the large bowel (14). Recently, Matsuki et al demonstrated in vaginally born infants, that acetate derived from the metabolism of *Bifidobacterium* sp. was the main SCFA detected in feces. This production of acetate contributed to the acidification of the intestinal milieu. Both physiological parameters have been described as key health indicators of the gut ecosystem milieu (15). Data from a murine infection model provided some clues on the putative role of acetate-producing *Bifidobacterium* sp. in enhancing immunity to bacterial infection (16). This may be a beneficial modulation, as many species belonging to Enterobacteriaceae are, under specific conditions, potentially pathogenic and also known to produce inflammatory lipopolysaccharides (17). We measured other bacterial taxa besides *Bifidobacterium* and Enterobacteriaceae; however, the proportion of these bacterial groups were suboptimal (0%–3%), which did not allow us to confirm the delayed colonization by *Bacteroides* in C-section–born infants and to depict the effect of the synbiotic on the entire microbial community (5). We are currently leveraging 16S rRNA sequencing to get more insight in the whole gut microbiota community in this cohort.

Recently, 2 studies indicated a strong association between C-section and the development of eczema/AD in early life (1,2). In addition, a clinical study demonstrated that probiotic intervention had a preventive effect on immunoglobulin E–associated allergic disease in cesarean-delivered children (18). We previously showed that scGOS/lcFOS and *B. breve* M-16V had a positive effect in improving the SCORAD of infants with immunoglobulin E–mediated AD and may had a protective effect on asthma (19,20). Although the present study was not designed to measure any clinical endpoint as primary outcome, the AEs data revealed a significantly lower incidence of skin disorders specifically eczema/AD in infants supplemented with the synbiotic mixture. A well-designed clinical study is warranted to confirm the biological significance of this observation.

CONCLUSIONS

Our study provides pioneering evidence that supplementation with scGOS/lcFOS and *B. breve* M16-V in C-section–born infants allows a fast colonization by bifidobacteria from the first days of life. The rapid settlement of this keystone infant type species contributes to emulate the gut physiological conditions (production of acetate and acidic gut milieu) observed in vaginally born infants. These biological phenomena have been depicted as an indicator of gut health.

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