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Original Research Article

Efficacy and safety of a synbiotic infant formula for the prevention of respiratory and gastrointestinal infections: a randomized controlled trial

Hugues Piloquet [1,*], Bénédicte Vrignaud [1], Florence Gillaizeau [2], Odile Capronnier [2], Kirsten Berding [3], Julia Günther [3], Christina Hecht [3], Christine Regimbart [4], on behalf of the GOLF III Study Group

[1] *Department of Pediatric Chronic Diseases, University Hospital of Nantes, Nantes, France;* [2] *Biofortis SAS, Saint Herblain, France;* [3] *HiPP GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany;* [4] *Private Medical Office, Becon-les-Granits, France*

A B S T R A C T

Background: Early life nutrition is crucial for the development of the gut microbiota that, in turn, plays an essential role in the maturation of the immune system and the prevention of infections.

Objectives: The aim of this study was to investigate whether feeding synbiotic infants and follow-on formulas during the first year of life reduces the incidence rate (IR) of infectious diarrhea compared with standard formulas. Secondary endpoints included the IR of other infectious diseases as well as fecal milieu parameters.

Methods: In this double-blind, controlled trial, 460 healthy, 1-mo-old infants were randomly assigned to receive a synbiotic [galacto-oligosaccharides (GOS)/*Limosilactobacillus fermentum* CECT 5716] (IF, $n = 230$) or a control formula (CF, $n = 230$) until 12 mo of age. A reference group of breastfed infants (HM, $n = 80$) was included. Data on infections were recorded throughout the study period and stool samples were collected at 4 and 12 mo of age.

Results: IR of infectious diarrhea during the first year of life was 0.60 (CF), 0.56 (IF), and 0.29 (HM), with no statistically significant difference between groups. The IR of lower respiratory tract infections, 1 of the secondary endpoints, however, was lower in IF than in CF [0.79 compared with 1.01, IR ratio = 0.77 (0.60–1.00)]. Additionally, fecal pH was significantly lower at 4 mo ($P < 0.0001$), whereas secretory IgA was significantly higher at 12 mo of age ($P = 0.015$) in IF compared with CF.

Conclusions: Although no difference is observed in the incidence of diarrhea, consumption of a synbiotic formula containing *L. fermentum* CECT5716 and GOS in infancy may reduce the incidence of lower respiratory tract infections and affect the immune system and fecal milieu. Additional research is warranted to further investigate the potential interaction of the gut–lung axis.

This trial was registered at clinicaltrials.gov as NCT02221687

Keywords: infant, formula, synbiotic, infections, gut microbiota, respiratory tract, gut–lung axis, *Limosilactobacillus fermentum*, GOS

Introduction

Community-acquired infections remain a major health problem during the first years of life. Worldwide, around 1.7 billion cases of childhood diarrheal disease are reported each year, with 525,000 children under the age of 5 y dying of the disease [1]. Similarly, lower respiratory tract infections (LRTIs) (e.g., bronchitis and bronchiolitis) contribute to morbidity and mortality in young children <5 y of age, accounting for ~652,000 deaths and >5 million hospital admissions annually [2]. Because of the protective effect of human milk against those infections, the WHO recommends exclusive breastfeeding until 6 mo of age and breastfeeding in conjunction with complementary feeding until 2 y of age or longer [3].

Breastfeeding provides a multitude of benefits to the infant, among which support of gut microbiota development appears to be a central aspect. During breastfeeding, the infant's gut is seeded with microbes present in human milk, and bioactive components, such as human milk oligosaccharides, further stimulate the development of health-

Abbreviations: AE, adverse event; CF, control formula (standard formula); C-section, cesarean section; FAS, full analysis set; GI, gastrointestinal; GOS, galacto-oligosaccharides; GOLF III, galacto-oligosaccharides *Limosilactobacillus fermentum* CECT5716 Study III; HM, human milk group; IF, intervention group (fed synbiotic formula); IR, incidence rate; IRR, incidence rate ratio; *L. fermentum*, *Limosilactobacillus fermentum*; LRTI, lower respiratory tract infection; PPS, per protocol set; SCFA, short-chain fatty acid; URTI, upper respiratory tract infection; UTI, urinary tract infection.

* Corresponding author.
E-mail address: hugues.piloquet@chu-nantes.fr (H. Piloquet).

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promoting gut microbiota [4–6]. This early life community of microbes plays a crucial role in the maturation of the mucosal barrier, intestinal homeostasis, and mucosal immune system of the newborn, via direct interactions with the gut epithelium and innate immune cells, as well as indirectly through the production of metabolites [7]. In turn, these beneficial effects on the developing microbiota have been linked to a reduction in community-acquired infections as well as protection against pathogenic bacteria and viruses [3,8,9].

Because of the profound beneficial effects of probiotic bacteria and prebiotic substances present in human milk, various prebiotics, probiotics or a combination of both (synbiotic) have been added to infant formula to provide the best alternative nutrition for infants who cannot be breastfed. A synbiotic is defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [10]. Among probiotic strains added to infant formula is *Limosilactobacillus fermentum* CECT5716, formerly *Lactobacillus fermentum*, which was originally isolated from human milk [11]. Preclinical studies demonstrated its antimicrobial, anti-inflammatory, and immunomodulatory properties [12,13]. Similarly, tolerance and safety of *L. fermentum* CECT5716 in combination with galacto-oligosaccharides (GOS) in an infant [14] and follow-on formula [15,16] were established in human trials, which also demonstrated the clinical benefit of this synbiotic mixture in reducing the incidence of gastrointestinal (GI) and respiratory tract infections [14,16]. However, studies investigating the effect of the consumption of the synbiotic formula over the entire first year of life are lacking.

Therefore, the primary aim of this study was to investigate whether feeding a synbiotic infant and follow-on formula (GOS/*L. fermentum* CECT 5716) during the first year of life reduces the incidence rate (IR) of infectious diarrhea compared with a standard infant and follow-on formula without synbiotics. Additionally, the impact on the incidence of other infectious diseases (including LRTIs), GI tolerance, and fecal milieu parameters were investigated as secondary objectives.

Methods

Study design and population

The study was designed as a multicenter, prospective, randomized, double-blind, parallel-group, controlled clinical trial. Healthy full-term infants (gestational age of 37–41 wk), aged 4 wk ± 1 wk, with a birth weight between 2500 and 4200 g were enrolled. Recruitment took place at 40 French and 1 Belgian site (medical offices of pediatricians and general practitioners, and pediatric clinical investigation units) between August 2014 and May 2018. Infants, who were not breastfed at the time of enrollment, because of their parents' choice, were randomly allocated to 1 of 2 study groups: control group (CF, $n = 230$) or intervention group (IF, $n = 230$). Both groups received an infant formula (1–6 mo of age) as well as a follow-on formula (6–12 mo of age) for a total intervention period of 11 mo. In addition, a reference group of infants fed human milk (HM, $n = 80$) was included. Infants eligible for the HM group were exclusively breastfed, or received no >1 bottle of infant formula per day at the time of enrollment, and mothers planned to pursue this feeding mode at least until the age of 4 mo.

Exclusion criteria for all groups included developmental delay, history of neonatal health problems, presence of any acute or chronic illness (i.e., growth-related, GI, metabolic, immune deficiency), consumption of an infant formula for special medical purposes (e.g., protein hydrolysate-based formula), or treatment with systemic antibiotics. The overall study design is shown in Figure 1 and described by Lagkouvardos et al. [17].

Study products and intervention

In the IF group, formula was enriched with a synbiotic mixture: prebiotic GOS (0.02 g/g and 0.03 g/g, respectively) and the probiotic strain *L. fermentum* CECT 5716 ($\geq 1 \times 10^6$ CFU/g and 1.5×10^6 CFU/g, respectively). Infants in the CF group received a standard infant and follow-on formula similar in all components but without the synbiotic mixture. All study formulas matched in taste, color, and odor, and supplied in similar packaging, so that group allocation would not be revealed, and complied with EC Directive 2006/141/EC. Study formulas were manufactured and provided by HiPP GmbH & Co. Vertrieb KG. Nutrient composition is shown in Supplemental Table 1.

The formulas were provided to parents or caregivers (in the following referred to as parents) as dehydrated powders, to be reconstituted in an infant bottle with water according to the instructions included on the box. Daily dosage was adapted to the infant's age, weight, and appetite throughout the study. Consumption of any other infant and follow-on formula as well as intake of prebiotics and probiotics (especially *Lactobacilli*, *Bifidobacteria*, or *Saccharomyces* species) was prohibited in compliance with the study protocol. Infants should not start food diversification before the age of 4 mo, regardless of the study group.

Randomization of the formula-fed infants occurred according to a dynamic randomization combining 2 main risk factors for infections as stratification factors: mode of birth [vaginal route or cesarean section (C-section)] and history of breastfeeding before inclusion (yes or no). The general product allocation list was generated by Biofortis SAS using the software SAS version 9.3 (SAS Institute Inc.) before the study started, using permuted random blocks of size 4 or 6. Enrollment and allocation were managed by the investigators at the sites through the randomization management interface called Interactive Web Response System. Generation of the randomization list of the products and implementation into the system was performed by independent personnel with no clinical involvement in the trial. Parents of formula-fed infants and personnel at study sites remained blinded to the group allocation throughout the study.

In the HM reference group, parents were asked not to feed any infant formula or at most ≤1 bottle of standard infant formula per day until at least the age of 4 mo.

Study visits

Infants and their parents completed 5 study visits at 1 (M1), 4 (M4), 6 (M6), 9 (M9), and 12 (M12) mo of age during the first year of life. Additionally, 4 phone interviews were conducted between visits at 2, 5, 8, and 11 mo of age to collect information on adverse events (AEs) and to assess compliance to the study product and feeding instructions (Figure 1). Parents completed a 3-d diary before each visit (except for M1) to document infant feeding, characteristics of bowel movements, GI symptoms, as well as sleep and crying durations. In addition, parents recorded any occurrence and duration of symptoms, information on diarrhea and infectious episodes, and concomitant medication in a daily diary throughout the study period, and reported the information to the investigator during visits and phone interviews. At M4 and M12, stool samples were collected. Anthropometric measurements (weight, length, and head circumference) and clinical examinations were performed during each visit at the study site.

Incidence of diarrhea (primary endpoint)

The primary endpoint was defined as the IR of infectious diarrhea (number of episodes per infant) during the first year of life (from M1 to M12). In formula-fed infants, a diarrhea episode was defined as ≥3

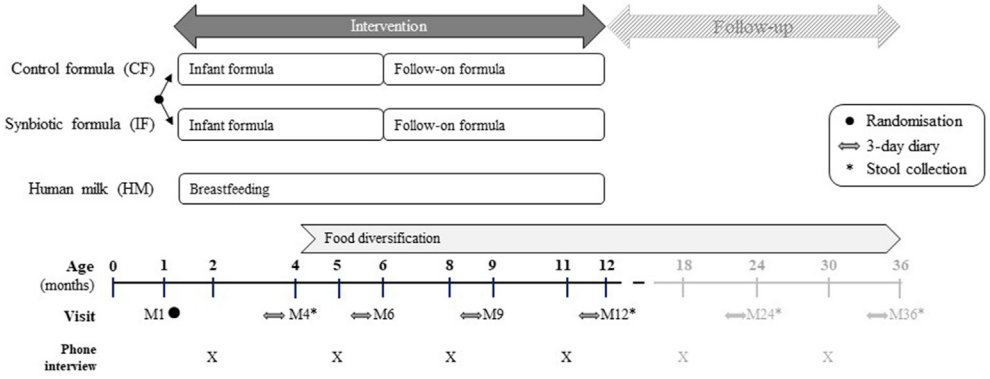

FIGURE 1. Study design. The study visit diagram illustrates study procedures over the 11-mo intervention period. Formula-fed infants received infant formula (randomly assigned to either synbiotic or control) from 1 to 6 mo of age and follow-on formula from 6 to 12 mo of age. A human milk group was included as a reference. Three-day diary data were collected at 4, 6, 9, and 12 mo of age. Stool samples were collected at 4 and 12 mo of age. Additional stool samples were collected during diarrhea episodes. Phone interviews were conducted between scheduled visits to inquire about adverse events and compliance with the study formula. In the noninterventional follow-up period, infant health was monitored (including collection of stools and 3-d diaries) until 36 mo of age (data not included in this publication).

loose or watery stools in 24 h, according to the definition by the WHO and European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) [18]. Diarrhea episodes (as reported by parents on the daily diary) were declared as AEs by the investigators during study visits and were considered to be resolved after 2 consecutive nonwatery stools or absence of stools over 24 h.

Incidence of infections and stool parameters (secondary endpoints)

As secondary endpoints, IR and duration of LRTIs, upper respiratory tract infections (URTIs), urinary tract infections (UTIs), otitis, total infections, fever, and treatments with antibiotics, as well as stool parameters (stool characteristics, pH, IgA), were assessed.

Infections were reported by parents in the daily diary and subsequently evaluated by the investigators and, if verified, classified as an AE. Parents reported characteristics (color, amount, consistency) of bowel movements using the Amsterdam Infant Stool Scale [19] in the 3-d diary before each visit.

Stool samples were collected and analyzed as previously described [17]. Briefly, stool samples were collected directly from the diaper into sterile tubes at the visits. During episodes of diarrhea, an additional stool sample was collected. Parents kept the stool samples in the refrigerator before transfer to a local medical laboratory (at most 24 h) for preparing aliquots and storage at −20°C. Samples were then transferred to the central laboratory at Biofortis (Saint Herblain, France) and stored at −80°C for later analysis.

Fecal pH was measured using a pH meter [1000 L with pHenomenal 220 electrode (VWR)]. Secretory IgA was determined in duplicates using an enzyme-linked immunoassay (Secretory IgA ELISA Kit, ImmuChrom) per manufacturer's instructions. The lower limit of IgA quantification was 0.28 mg/g.

To perform viral testing of diarrhea samples, RNA was extracted (NucliSens miniMAG, bioMérieux), and viral testing was performed by qRT-PCR (CeeramTOOLS kits for norovirus GI, norovirus GII, and rotavirus, bioMérieux) according to suppliers' instructions.

Safety, tolerance, and compliance

Safety was evaluated based on growth (anthropometric data) and monitoring of AEs. Parents reported tolerance and compliance in 3-d diaries, which included infant's sleeping and crying behavior (h and min/d), frequency, and intensity of digestive symptoms (vomiting, regurgitation, flatulence, and constipation), daily drinking amount, and acceptance of the formula.

Sample size determination

The sample size was calculated based on an IR of 21% in the IF group and assuming a 40% reduction in IR of GI infections as compared with the CF group. The 40% reduction in IR was based on clinical relevance according to an expert opinion (30%) as well as previous observations reported by Maldonado et al. [15] (46%). On the basis of a 2-sided chi-square test, it was estimated that 161 infants needed to be recruited into each formula group to achieve a significance level of 5% and statistical power of 80%. Considering a 2:2:1 ratio for CF:IF:HM, 81 infants needed to be included in the HM group. Finally, with an expected dropout rate of 30% in formula groups and 20% in the HM group, it was determined that 230 infants in each formula group and 100 infants in the HM group needed to be enrolled. Because of recruitment difficulties of breastfed infants in France and Belgium, the initially planned number of infants in the HM group was reduced to 80. Breastfed infants were not randomly assigned, but served as a reference group for the gold standard of infant nutrition.

Statistical analysis

All analyses were performed using the full analysis set (FAS), except for 3 sensitivity analyses of the primary outcome [using the per protocol set (PPS)] and safety endpoints [using the safety set (SAFETY)]. The FAS was defined as all randomly assigned subjects having consumed the control or interventional formula at least once and having $\geq$1 measurement of the primary endpoint available.

The PPS included all infants of the FAS without any major protocol deviation (including no dropout before M12 or no intake of prohibited medication; for details, see Figure 2) and who did not receive a rotavirus vaccination. The SAFETY consisted of all randomly assigned infants having consumed the study formula at least once and all infants included in the HM group.

For the primary endpoint, the IR of diarrhea episodes during the first year of life (number of episodes per infant in the first year) was compared between the 2 formula-fed groups using a negative binomial

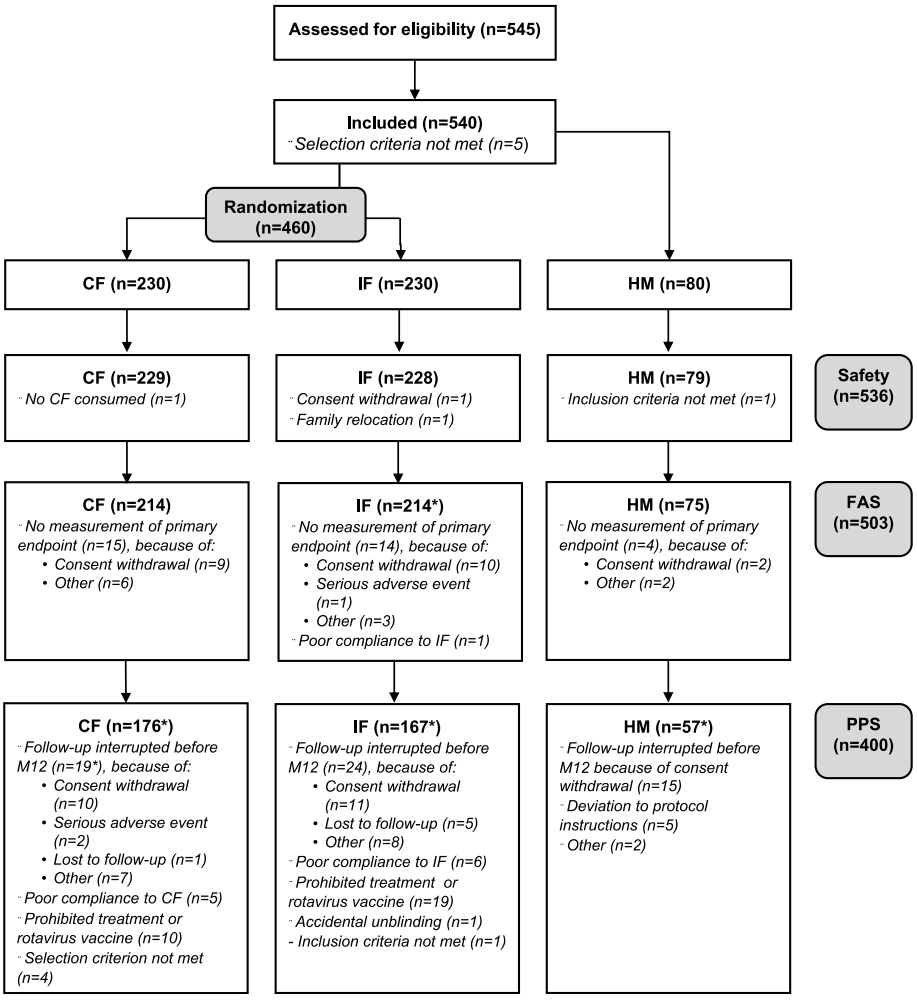

FIGURE 2. CONSORT flowchart. CF, control formula; FAS, full analysis set; HM, human milk; IF, intervention (synbiotic) formula; M12, month 12; PPS, per protocol set. Asterisk (*) denotes that a subject may have several reasons for exclusion from a dataset.

regression model with group (CF compared with IF) as the fixed factor and the 2 variables used to stratify randomization as covariates (mode of birth and history of breastfeeding). The IR was expressed as an estimated mean IR for each group as well as an incidence rate ratio (IRR), both including a 95% CI.

Two sensitivity analyses were conducted on the primary endpoint in the FAS. First, the same negative binomial regression model was used with additional confounding factors, which were collected via questionnaires at enrollment: sex (female/male), mother's educational level (categories: <high school, high school completed, high school + 2 y, license/bachelor degree, master degree, and master degree and more), smoking of mothers during pregnancy (yes/no), exposure to pets (yes/no), siblings (yes/no), daycare or at home with mother, and rotavirus vaccination (yes/no). Second, the diarrhea rate per person-time contributed was evaluated using a negative binomial regression model with an offset corresponding to the number of days at risk between M1 and M12. Moreover, 3 additional sensitivity analyses were carried out by applying the same negative binomial regression model of the primary endpoint and the 2 previous sensitivity analyses to the PPS. Lastly, to compare the time from inclusion to the first diarrhea episode, an exploratory Kaplan–Meier analysis and log-rank test were performed.

Secondary endpoints assessed across time were examined using mixed models for repeated measurements, either linear (continuous variables) or logistic (categorical variables), except for pH and bacterial (Wilcoxon tests) as well as viral pathogens (chi-square tests). Missing data for single visits were handled by maximum likelihood estimation (missing at random method, according to Twisk et al. [20]). For biological values, those below the limit of quantification were replaced by the lower limit of quantification divided by 2 specified for the corresponding assay. The Bonferroni–Holm adjustment was used to correct for multiple comparisons at each visit. There was no multiple testing correction for the analyses of the various secondary clinical endpoints. *Z*-for-age scores of body weight, length, body mass index, and head circumference were calculated based on WHO's standard curves [21] and compared using mixed models for repeated measurements.

For all other safety, tolerance, and compliance parameters, descriptive but not inferential statistics were performed. No inferential statistics were performed on the HM group, except for the exploratory Kaplan–Meier analysis (log-rank test) and the exploratory post hoc comparison of prevalence of diarrhea (chi-square test).

All data were analyzed using SAS software version 9.4 (SAS Institute Inc.). A P value of <0.05 was considered as the statistical level of significance. Assumptions of normality and homoscedasticity for linear models were investigated by graphic representations on residuals produced by the respective statistical models, and, if necessary, data were transformed using log10-transformation.

Ethical approval and informed consent

This clinical trial was prospectively registered on clinicaltrials.gov as "The Combiotic-Study (GOLFIII)" (NCT02221687) and conducted in accordance with the Declaration of Helsinki and standards of Good Clinical Practice, as far as they are applicable to an infant formula study. The protocol was approved by the French ethics committee (*Comité de protection des personnes Ouest IV*, Nantes, France) in April 2014, and subsequently by the Belgian ethics committee (*Comité d'éthique hospitalo-facultaire Saint Luc – Université Catholique de Louvain*, Brussels, Belgium) in March 2016. Study authorization was provided by the French health authority (*Agence nationale de sécurité du medicament et des produits de santé,* Saint Denis, France).

Written informed consent was obtained from both parents or all legal representatives of the infants before enrollment into the study. Financial compensation for travel costs to study visits as well as vouchers for the HM group was provided. Investigators and study personnel took special care throughout their communication with the families to support, protect, and not discourage breastfeeding.

Results

Baseline characteristics of infants were comparable between groups

A total of 460 infants were enrolled in formula-fed groups and 80 infants in the human milk reference (HM) group (total of 540 infants). One breastfed and 3 formula-fed infants were excluded from the SAFETY ($n = 536$) because of withdrawal of consent, no consumption of study product, family relocation, or failure to meet inclusion criteria. Thirty-seven infants were excluded from the FAS because of withdrawal of consent ($n = 22$), serious AEs ($n = 1$), or other reasons, leading to a total of 503 infants analyzed. An additional 103 infants were excluded from the PPS ($n = 400$). The main reasons for exclusion from the PPS among infants included in the FAS were withdrawal of consent ($n = 36$) and prohibited treatment or rotavirus vaccination ($n = 28$). The CONSORT flow chart outlining the study participant flow is shown in Figure 2.

Characteristics of infants at baseline are displayed in Table 1. Overall, no differences in baseline characteristics between feeding

TABLE 1
Baseline characteristics of study participants

	CF ($n = 214$)	IF ($n = 214$)	HM ($n = 75$)	FAS ($n = 503$)
Age at inclusion (d)	29.2 ± 5.50	29.1 ± 5.00	30.1 ± 5.08	29.3 ± 5.23
Sex				
Female	100 (46.7%)	101 (47.2%)	34 (45.3%)	235 (46.7%)
Male	114 (53.3%)	113 (52.8%)	41 (54.7%)	268 (53.3%)
Mode of birth				
Vaginal	187 (87.4%)	186 (86.9%)	64 (85.3%)	437 (86.9%)
C-section	27 (12.6%)	28 (13.1%)	11 (14.7%)	66 (13.1%)
Gestational age at delivery (wk)	39.7 ± 1.1	39.6 ± 1.1	39.7 ± 1.1	39.6 ± 1.1
Anthropometrics				
Weight at birth (kg)	3.34 ± 0.39	3.32 ± 0.37	3.28 ± 0.43	3.32 ± 0.39
Weight at inclusion (kg)	4.28 ± 0.48	4.21 ± 0.43	4.23 ± 0.52	4.24 ± 0.47
Weekly weight gain from birth to inclusion (g/wk)	216.7 ± 63.9	206.4 ± 48.8	224.9 ± 54.0	213.2 ± 56.67
Length at birth (cm)	50.0 ± 2.1	50.1 ± 2.1	50.0 ± 2.3	50.0 ± 2.1
Length at inclusion (cm)	53.4 ± 2.1	53.3 ± 1.9	53.8 ± 1.8	53.4 ± 2.0
Head circumference at birth (cm)	34.4 ± 1.4	34.5 ± 1.8	34.5 ± 1.3	34.4 ± 1.5
Head circumference at inclusion (cm)	37.2 ± 1.3	37.1 ± 1.2	37.1 ± 1.2	37.2 ± 1.3
History of breastfeeding				
≥1 meal	33 (15.4%)	31 (14.5%)	75 (100.0%)	139 (27.6%)
If ever breastfed, age at the time of cessation (d)	14.6 (10.53)	12.9 (10.78)	—	—
Mother's educational level				
High school not completed	38 (17.8%)	39 (18.2%)	6 (8.0%)	83 (16.5%)
High school completed	72 (33.6%)	46 (21.5%)	13 (17.3%)	131 (26.0%)
2-y post high school	37 (17.3%)	57 (26.6%)	18 (24.0%)	112 (22.3%)
License/bachelor degree	31 (14.5%)	35 (16.4%)	11 (14.7%)	77 (15.3%)
Master degree	28 (13.1%)	24 (11.2%)	13 (17.3%)	65 (12.9%)
Higher than master degree	8 (3.7%)	13 (6.1%)	14 (18.7%)	35 (7.0%)
Mother smoking during pregnancy				
Yes	46 (21.5%)	42 (19.6%)	10 (13.3%)	98 (19.5%)
No	168 (78.5%)	172 (80.4%)	65 (86.7%)	405 (80.5%)
Exposure to pets				
Yes	127 (59.3%)	114 (53.5%)	37 (49.3%)	278 (55.4%)
No	87 (40.7%)	99 (46.5%)	38 (50.7%)	224 (44.6%)
Siblings				
Yes	134 (62.6%)	130 (60.7%)	57 (76.0%)	321 (63.8%)
No	80 (37.4%)	84 (39.3%)	18 (24.0%)	182 (36.2%)
Child daycare at home with mother				
Yes	185 (86.4%)	174 (81.3%)	68 (90.7%)	427 (84.9%)
No	29 (13.6%)	40 (18.7%)	7 (9.3%)	76 (15.1%)

Data are shown as mean ± SD for continuous variables or *N* (%) for categorical variables.
Abbreviations: CF, control formula; C-section, cesarean section; FAS, full analysis set; HM, human milk (reference); IF, intervention (synbiotic) formula.

groups were observed. Slightly more boys than girls (53.3% compared with 46.7%) were enrolled in the study and only a minority was born via C-section (13.1%). Approximately 15% of formula-fed infants had a history of breastfeeding before inclusion into the study cohort, lasting for an mean of <15 d.

The mean duration of study participation within the intervention period was 314 ± 71.4 d, and was comparable between the 3 study groups.

In both formula groups, the median volume of formula consumed before study enrollment at each meal was 120 mL and the median number of meals per day was 6, as reported by the parents at M1.

The incidence of diarrhea episodes was not reduced by synbiotic intervention

As shown in Table 2, 270 episodes of infectious diarrhea were reported in the FAS until the 12-mo visit, corresponding to an overall IR of 0.537. Of those, 129 episodes occurred in the CF (IR = 0.603), 119 in the IF (IR = 0.556), and 22 in the HM group (IR = 0.293). IRR comparing IF with CF was 0.92 (0.70–1.22; $P = 0.58$). However, a majority of infants did not experience any diarrhea episode at all during the 11-mo intervention period, namely 56.5% (CF), 63.1% (IF), and 77.3% (HM). No statistically significant difference in the IR of diarrhea episodes was observed between IF and CF from the primary analysis performed on the FAS or in any of the sensitivity analyses (Table 2). Additionally, there was no effect of mode of birth or history of breastfeeding on the incidence of diarrhea. Only mothers' educational level had a statistically significant effect on the IR, with fewer diarrhea episodes reported in infants of females who completed high school compared with those in infants of those with higher education ($P = 0.01$, data not shown).

Exploratory post hoc analysis revealed that the proportion of infants who experienced ≥1 diarrhea episode was significantly higher in the CF group than in the HM group ($P = 0.02$), whereas no difference was observed between IF and HM ($P = 0.18$; Supplemental Figure 1). In addition, there was an overall significant difference between groups ($P = 0.02$) when comparing the time until the occurrence of the first diarrhea episode (Figure 3), driven by a delayed onset in the HM group compared with the formula-fed groups. However, the time until the onset of the first diarrhea episode did not differ between formula-fed groups.

Synbiotic intervention reduced the incidence of LRTIs

The incidence of other infectious diseases, including infections of the respiratory tract, as well as fever episodes and treatments with antibiotics, are presented in Table 3 and Figure 4. Overall, the IR of all infections until the age of 12 mo was higher in the CF than in the IF, and was lowest in the HM group. However, no statistically significant differences were detected in the IR of all infections between the formula-fed groups (Table 3 and Figure 4A).

Regarding LRTIs, a total of 48.5% of infants (IR = 0.88) in the FAS experienced ≥1 LRTI during the first year of life. In the IF group, an IR of 0.79 was observed, whereas the CF group had an IR of 1.01, demonstrating a statistically significant 23% reduction [IRR = 0.77 (0.60–1.00), $P = 0.049$] in the IR of LRTIs over the first year in the IF compared with the CF group (Table 3 and Figure 4B).

URTIs occurred in 68.2% of all infants (IR = 1.61), whereas otitis was diagnosed in 32.8% (IR = 0.56). No significant difference in the IR of these infections was observed between formula-fed groups (Table 3). Although the incidence of UTIs was significantly higher in the IF group than in the CF group, overall UTIs were rarely reported (2.8% of all infants; IR = 0.03) and only occurred in 3 infants in the CF (1.4%) and 10 infants in the IF group (4.7%). Lastly, no statistically significant difference between IF and CF groups in the incidence of fever episodes and treatments with antibiotics (Table 3), nor in the mean duration of infectious diseases, fever episodes, and treatments with antibiotics was observed (data not shown).

Synbiotic intervention shifted fecal milieu parameters closer to breastfed infants

Irrespective of group allocation, pH increased with age ($P < 0.0001$ for the M4–M12 difference in each formula-fed group). Fecal milieu parameters per group (pH and secretory IgA) at M4 and M12 are shown in Figure 5. Comparison of formula-fed groups revealed that fecal pH (Figure 5A) was significantly higher in CF than in IF at M4 (6.1 ± 0.68

TABLE 2
Characteristics of diarrhea episodes during the first year of life

	CF ($n = 214$)	IF ($n = 214$)	HM ($n = 75$)	IF vs. CF
				IRR[1]
No. of episodes	129	119	22	
Incidence rate	0.603	0.556	0.293	0.92 (0.70, 1.22)[2] 0.86 (0.65, 1.14)[3]
Incidence rate per infant-year	0.685	0.648	0.367	0.94 (0.71, 1.24)[4]
Total no. of days with diarrhea (d)	6.9 ± 6.17	6.5 ± 4.44	5.5 ± 3.91	0.02 (−0.09, 0.12)[5]
Duration of episodes (d)	5.3 ± 4.81	4.8 ± 3.56	4.6 ± 3.52	−0.01 (−0.10, 0.08)[5]
No. of episodes per infant				
0	121 (56.5%)	135 (63.1%)	58 (77.3%)	
1	66 (30.8%)	52 (24.3%)	12 (16.0%)	
≥2	27 (12.7%)	27 (12.7%)	5 (6.7%)	

Data are shown for the full analysis set, as mean ± SD for continuous variables or *N* (%) for categorical variables.
Abbreviations: CF, control formula; HM, human milk (reference); IF, intervention (synbiotic) formula; IRR, incidence rate ratio (95% CI).
[1] Difference tested with a negative binomial model.
[2] Adjustment for mode of birth and history of breastfeeding (primary analysis).
[3] Adjustment for mode of birth, history of breastfeeding, sex, mother's educational level, mother who smoked during pregnancy, exposure to pets, siblings, daycare at home with mother, rotavirus vaccination (sensitivity analysis).
[4] Sensitivity analysis including person-time contribution of subjects, adjusted for mode of birth and history of breastfeeding.
[5] Estimated difference of log-transformed data (95% CI). Difference tested with a fixed effect model, adjusted for mode of birth and history of breastfeeding.

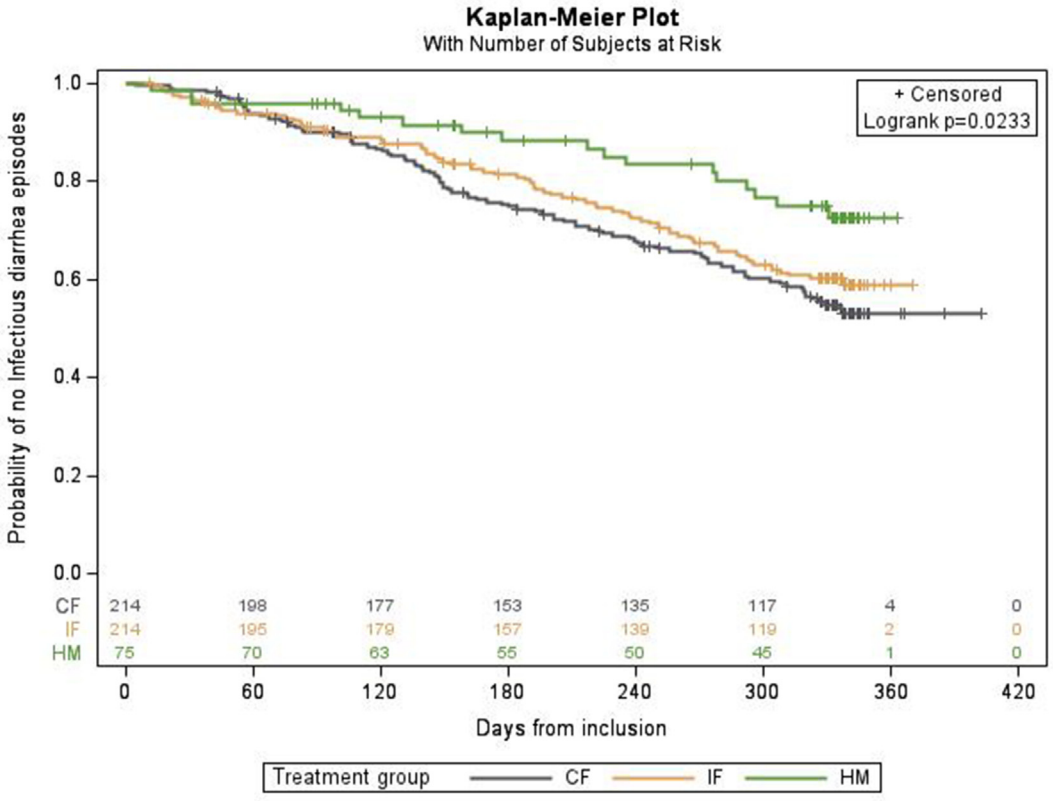

FIGURE 3. Kaplan–Meier curve showing time until occurrence of the first diarrhea episode. Kaplan–Meier curve describing the time from inclusion to first infectious diarrhea episode. Censored infants are indicated with tick marks. Log-rank test to a group-effect. Analysis for full analysis set. CF, control formula; HM, human milk; IF, intervention (synbiotic) formula.

TABLE 3
Incidence of infections, fever, and antibiotic treatment during the first year of life

Incidence rate	CF ($n = 214$)	IF ($n = 214$)	HM ($n = 75$)	IF vs. CF
				IRR[1]
Lower respiratory tract infection	1.01 ± 1.26	0.79 ± 1.15	0.79 ± 1.18	0.77 (0.60, 1.00)
Upper respiratory tract infection	1.76 ± 1.88	1.60 ± 1.58	1.20 ± 1.42	0.91 (0.75, 1.11)
Otitis	0.64 ± 1.04	0.50 ± 0.94	0.48 ± 0.94	0.81 (0.57, 1.13)
Urinary tract infection	0.01 ± 0.12	0.06 ± 0.27	0.01 ± 0.12	3.95 (1.04, 15.03)
All infections[2]	4.54 ± 3.25	4.07 ± 3.38	3.16 ± 2.80	0.89 (0.77, 1.04)
Fever episode	2.61 ± 2.53	2.53 ± 2.49	2.07 ± 2.29	0.97 (0.80, 1.19)
Treatments with antibiotics	1.43 ± 1.75	1.24 ± 2.09	1.11 ± 1.76	0.87 (0.67, 1.13)

Data are shown for the full analysis set as mean ± SD.
Abbreviations: CF, control formula; HM, human milk (reference); IF, intervention (synbiotic) formula; IRR, incidence rate ratio (95% CI).
[1] Values are mean estimates adjusted for mode of delivery, history of breastfeeding, and sex. Difference tested with a negative binomial model.
[2] Sum of all adverse events classified as infections.

compared with 5.8 ± 0.69; $P < 0.0001$) and tended to be higher at M12 (6.5 ± 0.61 compared with 6.3 ± 0.67; $P = 0.07$). At both time points, pH in IF was numerically closer to HM (5.5 ± 0.62 at M4, 6.1 ± 0.70 at M12) than CF was to HM.

In addition, there was a significant change over time in the concentration of fecal secretory IgA, decreasing from M4 to M12 in formula-fed groups ($P < 0.0001$). The levels were statistically significantly higher in IF than in CF at M12 (estimated mean difference with log10-transformation: 0.13 mg/g (0.03–0.22), $P = 0.015$; Figure 5B), and numerically closer to those of HM in IF than in CF at M4 and M12.

Diarrhea episodes were mostly caused by rota- or norovirus infections

During diarrhea episodes, 112 additional stool samples (CF: 55, IF: 51, HM: 6) were collected from 95 infants (CF: 46, IF: 43, HM: 6) to be tested for viral pathogens. Rotaviruses (CF: 39.2%; IF: 36.4%; HM: 40.0%), and noroviruses genogroup 1 or 2 (CF: 39.2%; IF: 40.9%; HM: 80.0%) were detected in a portion of the samples, with no significant difference between CF and IF. The 55 samples (CF: 27, IF: 27, HM: 1) that tested negative for rotaviruses and noroviruses were further tested for the presence of bacterial pathogens.

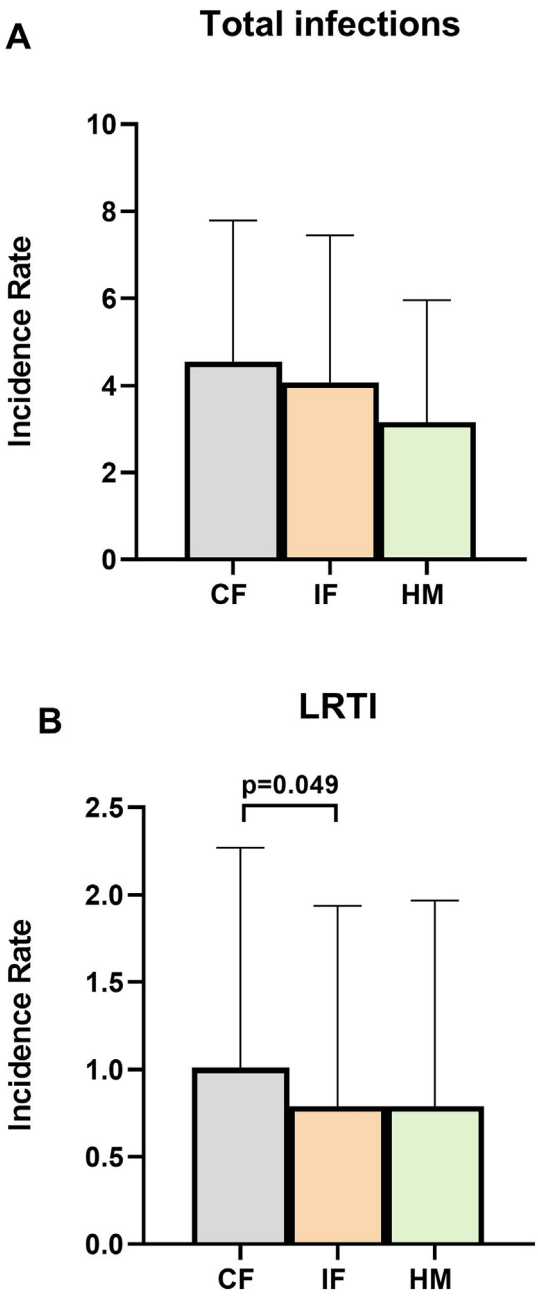

FIGURE 4. Incidence rate of (A) total infections and (B) lower respiratory tract infections until 12 mo of age. A. No statistically significant difference was observed for the rate of total infections between formula-fed groups. (B) The synbiotic intervention decreased the rate of LRTIs compared with CF. CF ($n = 214$); IF ($n = 214$); HM ($n = 75$). Data are shown as mean and SD in the full analysis set. Difference between CF and IF tested with a negative binomial model adjusted for mode of delivery, history of breastfeeding, and sex, without adjustment for multiple secondary outcome analyses. CF, control formula; HM, human milk; IF, intervention (synbiotic) formula; LRTI, lower respiratory tract infection.

Clostridioides difficile was detected in 19.2% of all samples, *Clostridium perfringens* in 32.7%, *Campylobacter jejuni* in 1.9%, and *Staphylococcus aureus* in 17.3%. Although *Escherichia coli* was detected in all samples, *Salmonella enterica* was not detected in any of them. Again, no significant difference in abundance between IF and CF was observed (data not shown).

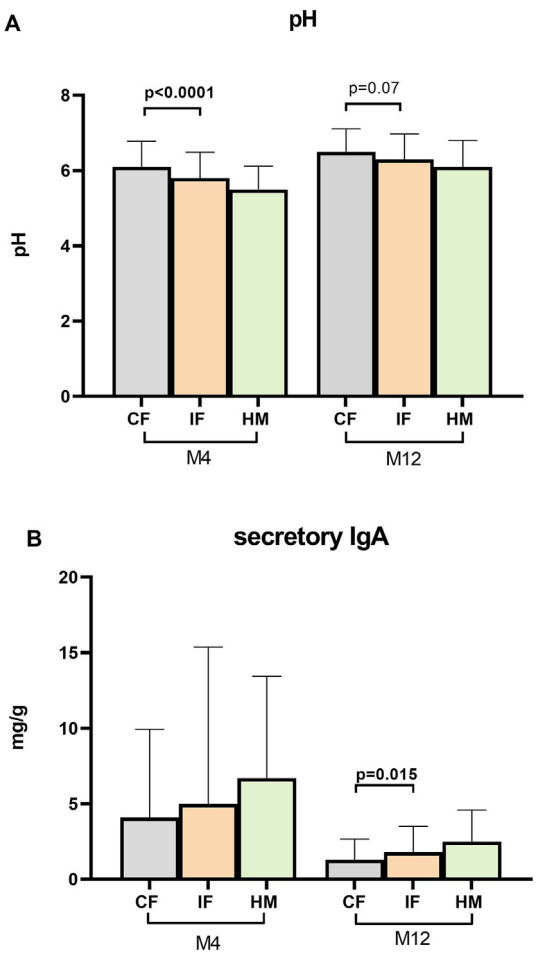

FIGURE 5. (A) Fecal pH and (B) secretory IgA concentrations at 4 and 12 mo of age. Fecal milieu parameters (pH, IgA) are shown for 4 and 12 mo of age. (A) The synbiotic intervention significantly reduced pH at 4 mo of age compared with CF. pH increased over time in all groups. (B) Secretory IgA was significantly higher at 12 mo of age in IF than in CF. Concentrations decreased over time in all groups. Data are shown as mean and SD for (A) pH and IgA, in the full analysis set. pH was analyzed with a Wilcoxon test. Secretory IgA was analyzed with a linear mixed model for repeated measurements. The Bonferroni–Holm adjustment was used to correct for multiple comparisons at each visit (IgA). Number of stool samples analyzed: 416 at M4 (CF: 185; IF: 183; HM: 48) and 378 at M12 (CF: 160; IF: 172; HM: 46). CF, control formula; HM, human milk; IF, intervention (synbiotic) formula.

Consumption of infant and follow-on formulas was safe and well tolerated

Infant growth (body weight, length, and head circumference) for all groups in the first year of life was within common ranges for this age group, and comparable between IF and CF (no statistically significant difference was detected for any standardized *z*-score of anthropometric parameters, data not shown). Descriptive values showed a lower weight of breastfed infants throughout the first year of life compared with CF and IF, but length and head circumference were similar.

The overall number of AEs throughout the study was comparable between IF and CF and did not raise particular safety concerns related to the consumption of the study formula. Likewise, sleeping and crying durations were similar between IF and CF (Supplemental Table 2). Parents reported a good acceptance of the infant and follow-on study formulas by their infants, and drinking amounts of the study formulas

were comparable (Supplemental Table 3). Lastly, stool parameters (amount, color, and consistency) and digestive tolerance of the formulas were largely comparable between IF and CF groups and did not show any peculiarities (data not shown).

Discussion

For infants who cannot be exclusively breastfed, infant formulas present an alternative way for nutrition. Thereby, the goal is to mimic the composition of human milk as much as possible, including the addition of probiotics and prebiotics commonly to support healthy infant development. Thus, the GOLF III study, a large randomized, double-blind multicenter trial, was designed to evaluate the effects of a synbiotic (GOS and *L. fermentum* CECT5716) compared with a standard formula on infant health when consumed over the first year of life. Although the addition of the synbiotic mixture to infant formula did not affect the incidence of diarrhea, consumption of the synbiotic formula decreased the incidence of LRTI by 23% compared with the standard formula and resulted in an increase in fecal secretory IgA concentration and a lower fecal pH.

Our data are in contrast with previously published reports of randomized controlled trials [14,15,22] as well as a recent meta-analysis [23], demonstrating a preventative effect of *L. fermentum* CECT5716 on GI infections. Although no specific factor explaining the differing results in IR of GI infections could not be identified from this study, we hypothesize that country-specific differences related to climate, attendance of childcare during the first year of life, lifestyle and dietary habits, exposure to pets or antibiotics could be reasons why the synbiotic formula reduced the incidence of diarrhea in a Spanish, but not a French population of infants. Furthermore, diarrhea rates could vary considerably from year to year, making comparisons between studies difficult. Lastly, previous studies highlighted that specifically infants delivered by C-section exhibited a reduced rate of GI infections through synbiotic supplementation [24]. In our GOLF III study, the rate of infants born via C-section was low, limiting the comparability between cohorts. Thus, future studies investigating the impact of *L. fermentum* CECT5716 on the incidence of diarrhea in other cohorts are warranted.

Most notably, we observed a statistically significant 23% reduction in the incidence of LRTIs in the synbiotic compared with the control group, resulting in similar incidences between the synbiotic and human milk group and confirming observations reported in previous studies [15]. In the context of frequent exposure to viruses (such as respiratory syncytial virus) and lack of efficient treatment, breastfeeding still stands as the best primary prevention strategy against acute LRTI-related morbidity and mortality [25]. However, prebiotics and/or probiotics could be useful in alleviating the burden of LRTIs when infants cannot be breastfed. Therefore, the results presented herein contribute to the growing evidence reported in the literature regarding the possible benefit of probiotic therapy on respiratory tract infections [26]. Although this beneficial effect highlighted by our study is encouraging, it remains to be confirmed in future cohorts, as previous studies showed an effect on upper (but not lower) respiratory tract infections [22] or did not consistently report such an effect [14]. Whether the protective effect is maintained in the long term will be explored in the follow-up phase of this study, which followed the children until 3 y of age.

Surprisingly, we found a higher IR of UTIs in the IF group than in the CF group. Although this difference was statistically significant, it should be noted that the IR was very low in all groups (overall only 13 of 503 infants experienced a UTI) and results of culturing (confirming a UTI infection) were only available in 4 cases by reported culturing, limiting the interpretability of the statistical analysis.

The immune system develops rapidly in early life and is shaped by various exogenous factors, including the mode of feeding. IgA, a marker for immune health, plays a key role in strengthening the regulatory and tolerogenic immune system of infants [27]. Delayed production of IgA and the associated delayed maturation of the immune system were suggested to be linked with the development of atopy [28]. Besides provision through human milk, IgA production can be stimulated through commensal microbiota [29,30]. Although previous studies have shown lower concentrations of fecal secretory IgA in exclusively formula-fed infants [29,31], significantly higher concentrations of IgA were observed in the synbiotic group of this cohort at 12 mo of age. Preclinical studies have demonstrated higher concentrations of IgA in the milk of pregnant rats [32] as well as in the feces of mice [33] after *L. fermentum* CECT516 supplementation, pointing to the potential of this strain to stimulate IgA secretion. Moreover, some studies have also indicated that the addition of *Bifidobacteria* or prebiotics to infant formula might increase IgA concentration in feces [34–36]. Intriguingly, we have recently shown in our GOLF III study that the addition of GOS and *L. fermentum* CECT5716 exerts a bifidogenic effect [17], potentially providing an additional link between gut and immune maturation in these infants. However, this association warrants further investigation.

Over the last decade, evidence has accumulated indicating the effect of the gut microbiota on respiratory health [9] through the gut–lung axis, and, some studies have shown the potential of synbiotics to modulate the gut–lung axis in infants [26,37], possibly mediated through microbial metabolites [i.e., short-chain fatty acids (SCFAs)] or the immune system [38]. Although the data reported herein do not allow us to draw conclusions regarding underlying mechanisms, we did observe an increase in secretory IgA as well as changes in SCFAs and higher amounts of *Lactobacilli* and *Bifidobacteria* closer to those observed in breastfed infants (as shown in the research by Lagkouvardos et al. [17]) in the stools of infants receiving the synbiotic formula, leading to the intriguing hypothesis that the preventative effect of the synbiotic mixture on LRTIs might be because of gut microbiota maturation and activation of the gut immune system. Furthermore, *L. fermentum* CECT5716 may contribute to antioxidant defenses of the gut and reduced intestinal inflammation by increasing glutathione concentration, and may facilitate absorption of iron through the secretion of hydroxyphenyllactic acid [13], which may support local and systemic defense against pathogens.

Although this study included a large, well-powered cohort, we acknowledge its limitations in interpreting the findings. History of breastfeeding was not an exclusion criterion for enrollment in the formula-fed groups. Even if breastfeeding was limited (low rate, short duration) and well-balanced between the randomly assigned groups, we cannot exclude any carry-over effect that may have blurred the effect of the intervention. Furthermore, complementary feeding, which started around 4 mo of age, was not evaluated as a potential confounding factor in the incidence of infections. Statistical results of secondary outcomes, including infections, were neither corrected for multiple testing nor adjusted for study centers. It should also be mentioned that the HM group was not randomly assigned, giving rise to potentially important covariates; thus, results presented from statistical analyses including the HM group should be regarded as

exploratory. Nevertheless, our results confirm the benefit of human milk to protect infants from infectious diseases [3,39,40]. Lastly, it is important to note that the effects of the synbiotic intervention were observed in a population of healthy full-term infants and cannot be extrapolated to sensitive populations, such as preterm infants or infants with underlying diseases.

In summary, we observed no beneficial effect of the synbiotic intervention on the primary outcome of infectious diarrhea in infancy, but did note a reduction in the incidence of LRTIs and the effect on the fecal milieu. Future preclinical experiments or data integration projects are required to explore causality and decipher possible mechanistic pathways of this gut–lung interaction. Additionally, further clinical trials are needed to confirm findings on respiratory health, especially because this was a secondary outcome in the present study that was not corrected for multiple comparisons. Nevertheless, the results hold promise for infants who cannot be breastfed.

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Author contributions

The authors' responsibilities were as follows – HP, CH: contributed, among others, to the design of the research (project conception, development of overall research plan, and study oversight); CH: coordinated the project as project lead; FG: performed statistical analysis; OC, KB, JG: wrote the manuscript; CR, BV: contributed to infant recruitment; and all authors: interpreted the data, reviewed and revised the manuscript, and read and approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Conflict of interest

FG and OC are employees at Biofortis. CH, KB, and JG are employees at HiPP Research and Development. HP received consultation fees from HiPP as the medical expert and supervisor of the study. CR received fees as an investigator. BV reports no conflict of interest.

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Data availability

The relevant data presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2024.03.005.

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