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A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers

Ting F. Leung¹ | Laurien H. Ulfman² | Marc K. C. Chong³ | Kam L. Hon¹ |
Ilse M. S. L. Khouw² | Paul K. S. Chan⁴ | Dianne J. Delsing² | Guus A. M. Kortman⁵ |
Ingeborg M. J. Bovee-Oudenhoven²

¹Department of Paediatrics, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong

²FrieslandCampina, Amersfoort, The Netherlands

³Jockey Club School of Public Health and Primary Care, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong

⁴Department of Microbiology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong

⁵NIZO, Ede, The Netherlands

Correspondence

Ting F. Leung, MD, FRCPCH, FAAAAI, Room 84043, Lui Che Woo Clinical Sciences Building, Prince of Wales Hospital, Shatin, New Territories, Hong Kong.
Email: tfleung@cuhk.edu.hk

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Abstract

Background: Bioactive proteins and human milk oligosaccharides (HMOs), important ingredients in breast milk, that protect against infections are lacking in young child formula (YCF). This study investigated the effects of new YCFs on respiratory and gastrointestinal infections in toddlers.

Methods: Four hundred and sixty one healthy Chinese children aged 1-2.5 years were recruited in this randomized, controlled, double-blind, parallel-group clinical trial of different YCFs. They were randomly assigned to either standard milk formula (YCF-Ref) or one of three new YCFs containing bioactive proteins and/or the HMO 2'-fucosyllactose (2'-FL) and/or milk fat for six months. Primary outcomes were incidence of upper respiratory tract infection (URTI) and duration of gastrointestinal tract infections (GITI).

Results: There were no significant between-group differences in primary outcomes. For secondary outcomes, subjects receiving 2'-FL-supplemented YCF had longer URTI. Subjects receiving YCF supplemented with milk fat and intact bioactive proteins, and 2'-FL at levels found in breast milk, had more GITI episodes and shorter time to first GITI but similar effects on URTI duration than YCF-Ref recipients. No effects on URTI and GITI were observed in toddlers receiving YCF with bioactive proteins at lower levels than breast milk. Occurrence of adverse events and anthropometry were similar in all groups.

Conclusions: All three YCFs supplemented with different combinations of intact bioactive proteins, 2'-FL, and milk fat are safe in toddlers. No difference is detected among YCFs on URTI incidence and GITI duration. Further studies are needed to verify these findings especially in infants who may benefit most from the immune-boosting effects of bioactive proteins and HMOs.

KEYWORDS

bioactive protein, gastrointestinal infection, human milk oligosaccharide, immunity, respiratory infection, young child formula

1 | INTRODUCTION

Respiratory tract infections (RTIs) and gastrointestinal tract infections (GITIs) are the leading causes of morbidity and mortality in young children. Breastfeeding helps protecting against infections¹ due to the presence of immunoglobulins and other bioactive proteins. Most of these immune-boosting ingredients are absent from current infant formula (IF) or young child formula (YCF).^{2,3} Protein denaturation during the processing of raw cow's milk explained the absence and loss of functionality of bioactive proteins in formula.⁴ Besides, human milk oligosaccharides (HMOs) abundant in breast milk are virtually lacking in cow's milk formulas. Numerous HMOs are present in human milk, and they are postulated to help shaping the gut microbiome by acting as prebiotics, reducing the risk of infections, modulating immune function, and enhancing brain development.⁵ Therefore, HMOs are presumed to offer added functionalities compared with prebiotics like galacto-oligosaccharides (GOS), which are typically included in IFs and YCFs.^{6,7} The most abundant HMO in breast milk is 2'-fucosyllactose (2'-FL).^{5,8} Pre-clinical studies have shown that 2'-FL has inhibitory effects on gut and airway pathogens/viruses and infections.^{9,10} Furthermore, high levels of 2'-linked fucosylated oligosaccharides (including 2'-FL) in human milk were associated with less diarrhea in infants.¹¹ Larger quantities of 2'-FL have recently become available to organize human dietary intervention studies to investigate the safety and efficacy of formulas containing 2'-FL. In addition to bioactive proteins and HMOs present in human milk, milk fat and its structural composition may also contribute to the health benefits of breastfeeding. Most current IFs contain vegetable oil, and its structural composition differs from the more closely related fat in human and cow's milk; these differences may affect fat digestibility and subsequently intestinal functionality.¹² This study investigated the effects of new YCFs containing intact bioactive proteins and/or 2'-FL and/or milk fat on upper RTIs (URTIs) and GITIs in toddlers.

2 | METHODS

2.1 | Study design

This randomized, double-blind, parallel-group, controlled clinical trial was conducted between January 2015 and October 2016 in Hong Kong. The Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee approved the study protocol; additional advice was obtained from Medical Ethics Committee of Wageningen University, the Netherlands. All parents signed written informed consent for their child's participation, and this study strictly adhered to the Declaration of Helsinki and Good Clinical Practice guidelines.

Key Message

This double-blind, randomized, controlled, parallel-group nutritional intervention study of 461 healthy Chinese toddlers showed that young child formulas (YCFs) supplemented with different combinations of intact bioactive proteins, the human milk oligosaccharide (HMO) 2'-fucosyllactose, and milk fat are safe in these toddlers. However, there were no differences in the incidence of upper respiratory tract infection and duration of gastrointestinal infection between new and standard milk formulas. Further studies are needed to verify efficacy outcomes on common childhood infections particularly in infants who would benefit most from formulas with immune-boosting bioactive proteins and HMOs.

2.2 | Subjects

Chinese children aged 1-2.5 years were recruited from hospital's pediatric clinics and several open sources (social media, advertisements, and public-parenting events). These families were invited to a screening visit at which subject eligibility was assessed based on the following inclusion criteria: Hong Kong-based, apparently healthy, Chinese ethnicity, and able to drink 400 mL YCF/d (two servings of 200 mL). Exclusion criteria are shown in Supporting Information.

2.3 | Interventions

Subjects were assigned randomly to receive: (a) YCF-Ref; reference milk formula; (b) YCF-A; milk formula containing immunoglobulins, lactoferrin, and transforming growth factor- β (TGF- β) at levels typical for breast milk, 2'-FL (at a level found in human milk) and milk fat; (c) YCF-B; milk formula containing lower levels of the bioactive proteins than in YCF-A; or (d) YCF-C, milk formula containing 2'-FL at the same level as in YCF-A (Table S1).

2.4 | Randomization and blinding

Eligible children were randomly allocated by computer-generated sequence to one of the YCF groups. Randomization was stratified by gender and age (1.00-1.50 years [60%]; 1.51-2.00 years [30%]; 2.01-2.50 [10%] years). Details of subject randomization and YCF labeling and preparation were described in Supporting Information. During the 6-month

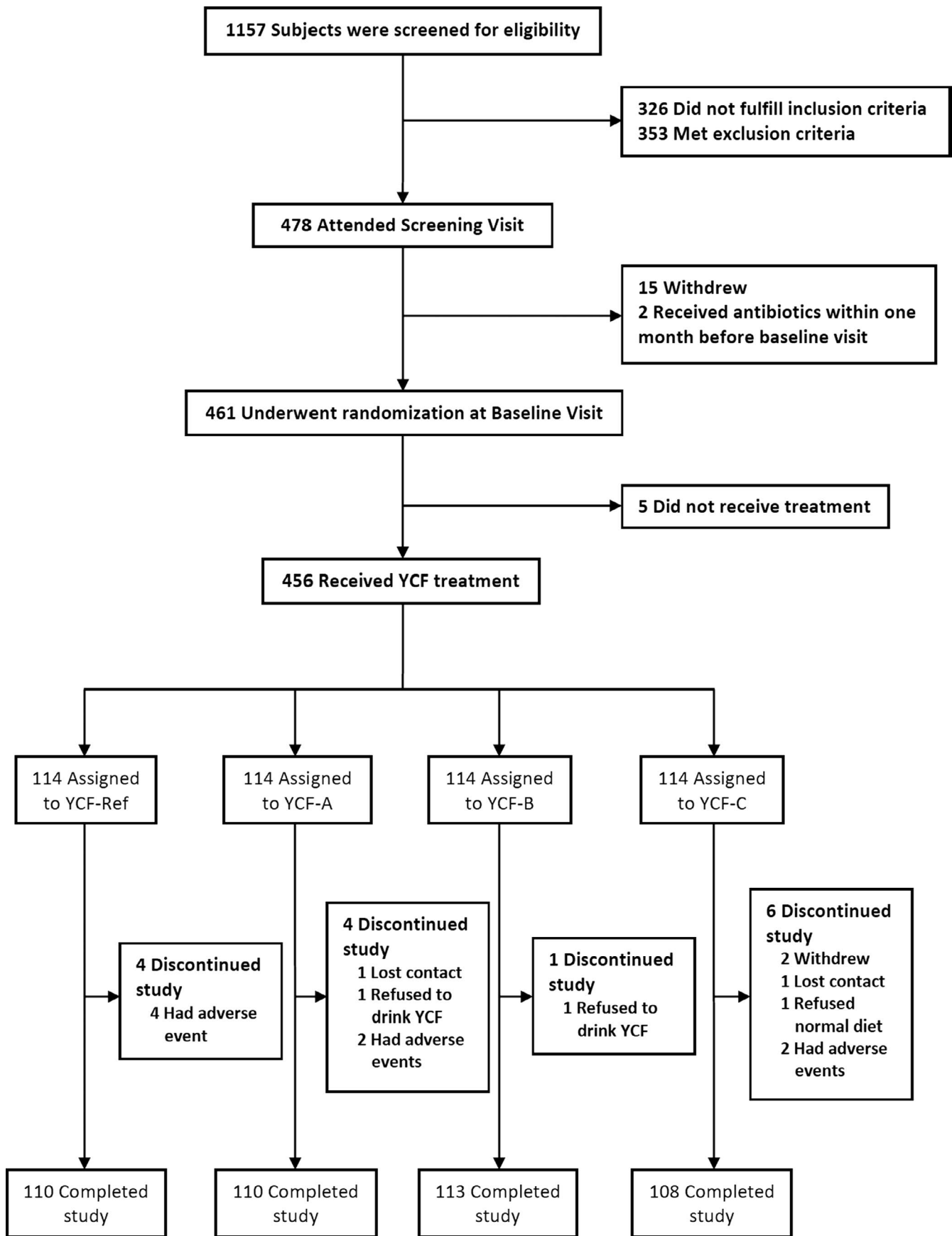


FIGURE 1 Flow diagram of subjects

Feature	YCF-Ref (n = 114)	YCF-A (n = 114)	YCF-B (n = 114)	YCF-C (n = 114)
Age, months	17.7 ± 4.8	17.6 ± 4.7	17.7 ± 4.7	17.9 ± 4.7
Male	60 (52.6)	57 (50.0)	57 (50.0)	58 (50.9)
Birthweight, kg	3.10 ± 0.42	3.18 ± 0.42	3.15 ± 0.39	3.18 ± 0.43
Neonatal problems ^b	3 (2.6)	7 (6.1)	5 (4.4)	4 (3.5)
With older siblings	48 (42.1)	42 (36.8)	36 (31.2)	42 (36.8)
Paternal age, y	35.9 ± 5.5	35.7 ± 5.2	34.8 ± 5.2	35.9 ± 5.6
Paternal education at college or above	45 (39.5)	62 (54.4)	56 (49.1)	61 (53.5)
Maternal age, y	32.5 ± 4.6	32.5 ± 4.3	32.0 ± 4.4	32.9 ± 4.8
Maternal education at college or above	55 (48.2)	66 (57.9)	64 (56.1)	67 (58.8)
Body weight, kg	10.7 ± 1.4	10.8 ± 1.5	10.9 ± 1.6	10.9 ± 1.5
Standing height, cm	80.6 ± 4.6	80.6 ± 4.5	81.1 ± 4.8	81.3 ± 4.5
Breastfeeding ever	100 (87.7)	104 (91.2)	104 (91.2)	103 (90.4)
Duration of breastfeeding, mo	3.3 ± 4.4	3.7 ± 4.4	4.9 ± 5.1	3.1 ± 4.9
Current household smokers	36 (31.6)	34 (29.8)	37 (32.5)	34 (29.8)
Current day-care attendance	41 (36.0)	46 (40.4)	39 (34.2)	42 (36.8)
Intake of nutritional supplements	25 (21.9)	26 (22.8)	27 (23.7)	24 (21.1)
Personal history of allergy	14 (12.3)	21 (18.4)	14 (12.3)	16 (14.0)

Note: Results are expressed as mean ± standard deviation or number (percentage).

^aAll measured subject baseline characteristics were similar between groups (all $P > .05$).

^bMain neonatal problems included neonatal jaundice, clinical sepsis, congenital heart disease, and pneumonia. However, none of these problems was considered to be clinically significant at the time of subject recruitment.

TABLE 1 Demographics and clinical information of study participants at baseline^a

intervention period, milk consumption was restricted to two servings of 200 mL/d for the allocated YCFs. Subjects preferring to drink more than two daily servings were also provided with regular YCF (similar composition to YCF-Ref), to be consumed only after consumption of the allocated YCF.

2.5 | Data collection

At baseline, trained senior nutritional science students assessed each child's habitual food intake in the past month using an age-appropriate Chinese Food Frequency Questionnaire.¹³ Using a diary, parents/caretakers of subjects recorded left-over milk (or amount of extra YCF consumed), and any signs of illness or medication used. Parents/caregivers also recorded daily defecation patterns (time, frequency, and stool's visual appearance), and stool consistency [using the Bristol Stool Scale (BSS)] (Figure S1).¹⁴ Subjects returned for scheduled clinic visits every 2 months, during which they were examined physically and their study diaries reviewed for clarity/completeness including any adverse events (AEs). Nasal swabs and stool samples were collected at baseline and final visits for microbiota analyses (see Supporting Information).

Parents/caregivers were instructed to report newly observed illness symptoms immediately. If subjects developed respiratory or gastrointestinal symptoms, they were instructed to return to the clinic for physical examination by a pediatrician. An additional nasal swab and/or stool sample was collected closest to the second day from the onset of infection episode (see Supporting Information). AEs were recorded using the International Classification of Diseases, 10th Revision codes (<http://apps.who.int/classifications/apps/icd/icd10online/>). Severity and likelihood of the relationship between AEs and intervention were scored by a pediatrician and evaluated by an independent data safety monitoring board (DSMB) consisting of pediatrician, nutritionist, and biostatistician.

2.6 | Study outcomes

Primary outcomes were the incidence of URTIs and duration of GITIs as recorded by parents/caregivers on study diaries and verified by investigators and nurses over a 6-month period. The incidence of URTIs was defined as the number of new episodes per child over the 6-month period, with URTI being defined as runny nose and/

TABLE 2 Summary of compliance with interventions and reasons for study discontinuation

Parameter	YCF-Ref (n = 114)	YCF-A (n = 114)	YCF-B (n = 114)	YCF-C (n = 114)
Subjects completed study	110 (96.5)	110 (96.5)	113 (99.1)	108 (94.7)
Per-protocol subjects ^a	100 (87.7)	101 (88.6)	97 (85.1)	98 (86.0)
Product compliance (%)	85.8 ± 17.6	87.3 ± 16.5	88.2 ± 12.4	85.0 ± 16.3
Subjects compliant with YCF intake ^a	103 (90.4)	106 (93.0)	101 (88.6)	104 (91.2)
Intake of extra YCF (serving per 6 mo)	215 ± 109	192 ± 110	185 ± 102 [†]	177 ± 109 [†]
Completion of primary outcome data	89.2 ± 15.0	89.8 ± 13.9	90.2 ± 7.9	88.4 ± 14.5
Primary outcome compliance ^a	105 (92.1)	105 (92.1)	106 (93.0)	102 (89.5)
Reasons for dropout				
Withdrawal of consent	0	0	0	2 (1.8)
Family lost to follow-up	0	1 (0.9)	0	1 (0.9)
Disliked study formula	0	1 (0.9)	1 (0.9)	0
Protocol violation	0	0	0	0
Adverse event	4 (3.5)	2 (1.8)	0	2 (1.8)
Others	0	0	0	1 (0.9)

Note: Results are expressed as mean ± standard deviation or number (percentage).

^aPer-protocol subjects refer to those providing ≥80% of primary outcome data in the study diaries (primary outcome compliance) and consuming ≥70% of supplied YCF (product compliance) during the study period.

[†]*P* = .034 and [‡]*P* = .008 when compared with YCF-Ref.

or cough for ≥2 days.¹⁵ A new episode of URTI was defined when at least a 7-day interval separated the previous and new episodes.¹⁶ GITI was defined as ≥2 loose/liquid stools in 24 hours,¹⁵ and the duration of GITI was defined as the number of days from first until last excretion of liquid or semi-liquid stools (BSS score 6 or 7) that was not followed by another abnormal stool within 2 days.^{17,18} The mean duration of GITI episodes per child was calculated as the total number of diarrheal days divided by the number of episodes.

Secondary/tertiary outcomes included the following: (a) mean duration of URTI, defined as the mean number of days per URTI episode; (b) incidence of GITI, defined as the mean number of new GITI episodes per subject per 6 months; (c) incidence and duration of GITI, defined by WHO criteria (≥3 liquid/semi-liquid stools within 24 hours)¹⁸; and (d) anthropometric data.

Methods used for pathogen identification and microbiome analyses in nasal and stool samples were described in Supporting Information.

2.7 | Statistical analysis

Sample size was calculated based on mean number of URTIs and duration of GITI episodes. Based on the literature^{2-4,19} and our pilot data, the average URTI incidence and GITI duration were 3.5 ± 2.8 and 3.0 ± 2.4 days/episode, respectively, in the reference group. We expected URTI to reduce by one episode and GITI to shorten by 1 day

with our intervention. With a pre-set level of 5% significance, 80% power allowing 2-sided testing, and 4% dropouts and non-compliant cases, this study needed to recruit 98 and 116 subjects per trial arm for URTI incidence and GITI duration, respectively. Therefore, this clinical trial aimed to recruit 464 children (116 per trial arm). Details of statistical analyses were reported in Supporting Information.

3 | RESULTS

A total of 1157 children were screened for eligibility, and informed consent was obtained for 478 children who attended the screening visit (Figure 1). The final intention-to-treat (ITT) group comprised 456 children (114/group) who received YCF. The percentage of dropouts during the 6-month study was 1%-5%. All study groups were comparable with respect to baseline characteristics (Table 1) and nutrient intake (results not shown). Per-protocol (PP) analysis was possible in 85%-89% of subjects (Table 2).

3.1 | Primary outcomes: URTI incidence and GITI duration

Table 3 summarizes the main efficacy outcomes. The average numbers of new URTI episodes for YCF-A, YCF-B, YCF-C, and YCF-Ref were 1.9, 1.7, 2.0, and 1.9, respectively (not statistically different).

TABLE 3 Summary of study outcomes (ITT analysis)

Outcome	YCF-Ref (n = 114)	YCF-A (n = 114)	YCF-B (n = 114)	YCF-C (n = 114)
Primary outcomes				
Total URTI episodes per subject	1.9 ± 1.5	1.9 ± 1.5	1.7 ± 1.5	2.0 ± 1.6
Duration of GITI episodes (Agustina), d	3.3 ± 2.3	3.1 ± 1.9	3.5 ± 1.7	3.2 ± 2.0
Secondary outcomes				
Duration of URTI, d	7.3 ± 4.6	8.1 ± 7.4	8.6 ± 6.0	8.9 ± 5.6 [†]
Total GITI episodes (Agustina) per subject	0.3 ± 0.7	0.6 ± 0.9 [†]	0.4 ± 0.8	0.5 ± 0.9
Total GITI episodes (WHO) per subject	0.2 ± 0.5	0.3 ± 0.6	0.2 ± 0.5	0.3 ± 0.5
Duration of GITI episodes (WHO), d	4.3 ± 2.4	3.1 ± 1.8	4.4 ± 3.5	3.4 ± 2.2
Tertiary outcomes				
Time to first URTI, d	81.5 ± 63.8	84.7 ± 65.6	95.5 ± 67.9	84.1 ± 64.0
Time to first GITI (Agustina), d	165.1 ± 53.5	146.1 ± 64.3 [†]	159.9 ± 57.5	149.7 ± 60.3 [†]
Modified Vesikari clinical severity scores of GITI	5.1 ± 4.1	4.1 ± 2.3	5.0 ± 3.2	4.2 ± 3.1
Number of days with hard stool	9.5 ± 17.1	7.8 ± 15.1 [†]	9.9 ± 18.8	8.0 ± 14.0 [†]
Number of LRTI ^a	0 (0-4)	0 (0-4)	0 (0-3)	0 (0-4)
Number of cough episodes	9.3 ± 10.9	9.1 ± 12.0	9.7 ± 14.0	12.6 ± 14.9 [†]
Number of runny nose episodes	11.2 ± 13.4	11.1 ± 13.0	10.5 ± 12.8	13.7 ± 15.8 [†]
Antibiotic prescriptions	0.1 ± 0.7	0.3 ± 1.4 [†]	0.2 ± 1.0 [†]	0.1 ± 0.8
Total number of days with fever	1.9 ± 2.7	1.5 ± 1.9 [†]	1.9 ± 2.6	2.5 ± 3.1 [†]
≥3 URTI episodes	39 (34.2)	37 (32.5)	34 (29.8)	47 (41.2)
Otitis media	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)

Note: Results are expressed as mean ± standard deviation, number (percentage) or median (range).

Abbreviations: GITI, gastrointestinal tract infection; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection.

^aConsisted of the physician diagnoses of pneumonia, bronchitis, bronchiolitis, and asthma.

[†] $P < .05$, ^{††} $P < .01$, [‡] $P < 0.001$, and ^{†††} $P = .002$ compared with YCF-Ref.

Changes in the mean weekly URTI incidence also showed no discernible between-group patterns or differences (data not shown). GEE revealed a trend for more URTI episodes among subjects aged 1.00–1.50 years receiving YCF-C vs YCF-Ref ($\beta = 0.303$, 95% confidence interval [CI] 0–0.606; $P = .050$). Subjects receiving YCF-A, YCF-B, YCF-C, and YCF-Ref had similar mean duration of GITI episodes (3.1, 3.5, 3.2, and 3.3 days, respectively). PP analysis showed similar findings (results not shown). GITI duration results remained insignificant after subgroup analysis for breastfeeding at infancy (<2 months vs ≥2 months; Table S4).

3.2 | Secondary/tertiary outcomes

The mean URTI duration was longer with YCF-C than YCF-Ref (8.9 vs 7.3 days; $P < .05$). The time to first URTI was similar among all four groups (Table 3). Subjects in the YCF-C group also had more episodes of cough and runny nose than YCF-Ref ($P < .001$ for both) and days with fever ($P < .01$), although total fever days (2.5 ± 3.1 days) were very low during follow-up. Furthermore, YCF-C recipients had fewer days with hard stool than YCF-Ref ($P < .001$). YCF-A showed fewer days with fever ($P < .01$) and hard

TABLE 4 Summary of main adverse events^a included in the ITT analysis

Adverse event	YCF-Ref (n = 114)	YCF-A (n = 114)	YCF-B (n = 114)	YCF-C (n = 114)
All adverse events	97 (85.1)	100 (87.0)	98 (86.0)	100 (87.7)
Any serious adverse events ^b	15 (13.2)	17 (14.8)	15 (13.2)	10 (8.8)
Respiratory				
URTI	88 (77.2)	88 (77.2)	85 (74.6)	91 (79.8)
Acute bronchiolitis	6 (5.3)	6 (5.3)	9 (7.9)	6 (5.3)
Acute bronchitis	10 (8.8)	6 (5.3)	6 (5.3)	13 (11.4)
Acute otitis media	3 (2.6)	2 (1.8)	2 (1.8)	2 (1.8)
Gastrointestinal				
GITI ^c	22 (19.3)	45 (39.5)	29 (25.4)	35 (30.7)
Acute gastritis	1 (0.9)	4 (3.5)	1 (0.9)	5 (4.4)
Vomiting	1 (0.9)	0	3 (2.6)	1 (0.9)
Others				
Isolated fever	5 (4.4)	8 (7.0)	6 (5.3)	9 (7.9)
Acute pharyngitis	3 (2.6)	5 (4.4)	2 (1.8)	1 (0.9)
Eczema	5 (4.4)	3 (2.6)	0	3 (2.6)
Hand-foot-mouth disease	3 (2.6)	2 (1.8)	2 (1.8)	3 (2.6)
Roseola infantum	6 (5.3)	8 (7.0)	6 (5.3)	7 (6.1)
Viral infection ^d	2 (1.8)	1 (0.9)	3 (2.6)	3 (2.6)

Note: Results are expressed in number (percentage).

Abbreviations: GITI, gastrointestinal tract infection; URTI, upper respiratory tract infection.

^aListed are only adverse events that occurred in ≥ 3 subjects in any group.

^bSerious adverse events in this study refer to adverse events that required hospitalization.

^cOne hundred and thirty one GITI episodes were reported by families during the study, whereas detailed analysis of relevant symptoms on study calendars and re-grouping of the nature for adverse events following study completion revealed that there were a total of 192 GITI episodes.

^dJudged by investigators to have viral etiologies because of compatible clinical features (eg, isolated fever, skin rash, lymphadenopathy), but did not meet the criteria for URTI and GITI.

stool ($P < .001$) vs YCF-Ref. GITI episodes were more frequent with YCF-A vs YCF-Ref (0.6 vs 0.3; $P < .01$). Subgroup analysis revealed that subjects in YCF-A who were breastfed for < 2 months had more GITIs ($P = .009$; Table S4).

GEE confirmed an independent association between YCF-A and the incidence of GITI, defined by Agustina criteria ($P = .006$), but not by WHO criteria ($P = .075$). Compared with YCF-Ref, GEE also showed more GITI (Agustina) among subjects aged 12-17 months receiving YCF-A ($P = .004$) and YCF-B ($P = .040$). The time to first GITI episode was significantly shorter among subjects receiving YCF-A than YCF-Ref ($P = .002$; Table 3).

Changes in anthropometric data noted no remarkable between-group differences (Figure S2). Outcomes from pathogen identification and microbiome analyses in nasal and stool samples are described in Supporting Information.

3.3 | Adverse events (AEs)

All four YCFs had similar frequencies of AEs (85.1%-87.7%) and serious AEs (8.8%-14.8%) (Table 4). There were no reported cases of product-related AEs as judged by investigators and confirmed by the DSMB. Compared to YCF-Ref (21 cases), subjects consuming YCF-A (45 cases) had a higher GITI count ($P = .001$).

3.4 | Effect of YCFs on fecal microbiome

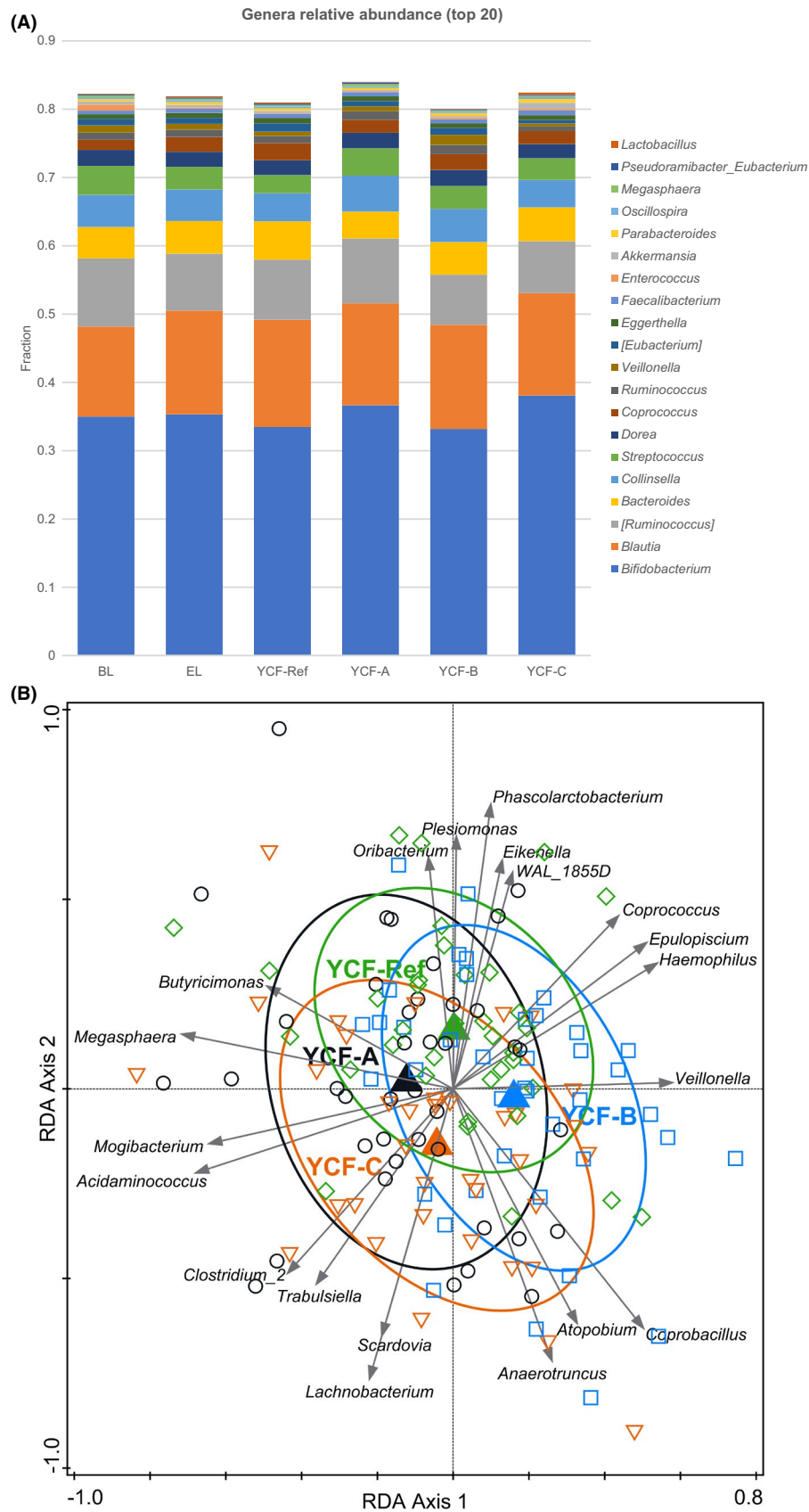
Figure 2 shows the relative abundance of the top 20 most abundant microbial genera, which did not reveal any significant differential effect of YCF-A, YCF-B, or YCF-C on fecal microbiota compared with YCF-Ref. Phylogenetic diversity analysis indicated that within-sample diversity was significantly higher in older than younger toddlers ($P < .0001$).

4 | DISCUSSION

The current trial, being the first RCT on efficacy outcomes among toddlers, showed no benefit for three new YCFs containing varying combinations of bioactive proteins and/or 2'-FL and/or milk fat on the primary outcomes of URTI incidence and GITI duration in Chinese toddlers compared to YCF-Ref. Additionally, the occurrence of AEs and changes in subjects' anthropometry were similar in all treatment arms, indicating that all YCFs were safe and supported normal growth.

The non-beneficial results observed for the 2'-FL group were unexpected. Although 2'-FL concentrations in human milk are generally between 2 and 4 g/L,^{8,20} a recent study in Chinese and Malaysian lactating women indicated that levels decline to ~ 0.8 g/L at 1 year.²¹ High 2'-FL levels in human breast milk are associated with protection against *Campylobacter jejuni* diarrhea.¹¹ Infants receiving breast milk lacking 2'-FL were also shown to have delayed establishment of gut bifidobacteria.²² Pre-clinical research indicated bifidogenic and anti-pathogenic effects to be the two most important functions of 2'-FL.^{9,10}

More clinical research on 2'-FL and other HMOs has been undertaken with their increasing availability. Levels of 2'-FL in different



formulas studied varied as follows: Two studies used 0.2 g/L, while two studies tested up to 1.0 g/L (combined with lacto-N-neotetraose [LNnT] 0.5 g/L in one study).²³⁻²⁵ Our YCF-C and YCF-A contained

3.0 g 2'-FL/L (the reported mean level in mature human milk).^{8,20} All studies showed that 2'-FL was safe and well tolerated in infants. These RCTs also found lower circulating inflammatory cytokines

FIGURE 2 Effects of study YCFs on subjects' fecal microbiome. (A) Relative abundance of the top-20 most abundant genera in fecal samples collected at baseline (BL) and end line (EL), grouped and differentiated per YCF treatment arm; and (B) multivariate redundancy analysis (RDA) plot showing no significant YCF treatment effect on the fecal microbiota profile at end line (explained 0% variance). Small colored symbols indicated individual samples and YCF group membership, while large colored symbols represented sample centroids of the YCF groups. Ellipses indicate the 66% quantile of the approximated 2D-normal density distribution function for each YCF group. Gray arrows represent the 20 best-fitted genera

with 2'-FL,²⁶ less lower respiratory tract infections (LRTIs) and medication use with 2'-FL/LNnT,²⁴ and reduced respiratory infections with 0.2 g/L 2'-FL (but no effect of 1.0 g/L 2'-FL).²⁵ In contrast, we could not find any effect of 2'-FL on URTI incidence (primary outcome) among toddlers. Remarkably, 2'-FL YCF prolonged URTI by an average of 1.6 days (secondary outcome). Because nasal microbiome was similar between groups, this finding in YCF-C was not caused by altered microbial composition or pre-disposition to certain pathogens. It is interesting to test whether such was related to the reported suppressive effect of 2'-FL on circulating inflammatory cytokines.²⁶

Considering the interest in supplementing formulas with 2'-FL and other HMOs, more studies particularly in infants are needed to substantiate postulated health benefits. We cannot exclude the possibility that the effects of nutritional support might differ with age, given that the immune system is more immature in infants.²⁷ Infants rely on breast milk and/or formula, whereas toddlers have a more varied food intake pattern. Therefore, potential effects of 2'-FL supplied in milk might less likely be noticeable in toddlers.

Interestingly, the unexpected finding of 2'-FL on URTI duration in YCF-C was not observed with YCF-A, which also contained 2'-FL 3.0 g/L. We speculate that bioactive proteins and/or milk fat in YCF-A contributed to prevention of prolonged URTI duration. Bioactive proteins such as bovine immunoglobulins were shown to be bactericidal, escape gastrointestinal digestion, bind human pathogens and allergens, interact with human Fc receptors, and enhance antigen presentation.¹⁹ It is thus difficult to explain why YCF-A (and YCF-C to a lesser extent) increased GITI incidence compared with YCF-Ref. Further investigations should clarify whether the anti-inflammatory effects of 2'-FL and other HMOs were desirable (mimicking findings in breastfed infants).^{26,28} It is possible that these effects help shape the immune system and prevent allergy in infants, but they might come at the cost of lowering defense against common early-life infections.²⁸

The effects of YCFs on fecal microbiome in our toddlers generally matched those in infants.²⁹ The bifidogenic effects of 2'-FL on gut microbiome of infants and young children still need to be established, and the current study found similar relative abundances of bifidobacteria and other main genera among YCF-C, YCF-A, and YCF-Ref in toddlers (see Supporting Information). It is possible that GOS (4 g/L) contained in all YCFs was sufficient to establish/maintain a bifidobacterium-dominated gut microbiome. Finally, 16S sequencing of the nasal microbiome showed profiles expected for toddlers. Indeed, the main bacterial genera detected from nose swabs were *Dolosigranulum*, *Moraxella*, and *Corynebacterium*, which were also identified from deeper nasopharyngeal swabs in infants.³⁰

In conclusion, three new YCFs with varying combinations of bioactive proteins, 2'-FL, and milk fat were generally ineffective in reducing the incidence and duration of URTIs and GITIs in healthy Chinese toddlers. All new YCFs had similar safety profiles to the reference YCF. Unexpectedly, YCF-C containing 3 g/L 2'-FL was associated with prolonged URTI duration. Further human studies are needed to investigate whether the positive pre-clinical immune-boosting effects of HMOs and bioactive proteins can be extrapolated to infants and toddlers.

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CONFLICT OF INTEREST

Drs Ulfman, Khouw, Delsing, and Bovee-Oudenhoven are employees of the study sponsor FrieslandCampina. Nonetheless, they were not involved in subject screening, recruitment and randomization, data collection and monitoring, and statistical analysis for study outcomes.

AUTHOR CONTRIBUTION

Ting F. Leung: Conceptualization (equal); Formal analysis (lead); Funding acquisition (lead); Investigation (supporting); Methodology (lead); Project administration (lead); Supervision (lead); Validation (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **Laurien H. Ulfman:** Conceptualization (supporting); Methodology (supporting); Project administration (supporting); Resources (supporting); Writing-review & editing (supporting). **Marc K. C. Chong:** Data curation (supporting); Formal analysis (lead); Validation (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). **Kam L. Hon:** Conceptualization (supporting); Data curation (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Supervision (supporting); Validation (supporting); Writing-review & editing (supporting). **Ilse M. S. L. Khouw:** Conceptualization (supporting); Investigation (supporting); Methodology (supporting); Resources (supporting); Writing-review & editing (supporting). **Paul K. S. Chan:** Conceptualization (supporting); Data curation (supporting); Investigation (equal); Methodology (equal); Project administration (supporting); Supervision (supporting); Writing-review & editing (supporting). **Dianne J. Delsing:** Conceptualization (supporting); Investigation (supporting); Methodology (supporting); Resources (supporting); Writing-review & editing (supporting). **Guus A. M. Kortman:** Formal analysis (supporting); Investigation (lead); Methodology (lead); Writing-review & editing (supporting). **Ingeborg**

M. J. Bovee-Oudenhoven: Conceptualization (lead); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (lead); Writing-review & editing (supporting).

ORCID

Ting F. Leung  <https://orcid.org/0000-0002-6469-1926>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ONLINE SUPPLEMENT

Leung TF, Ulfman LH, Chong MKC, et al. A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers.

Members of Study and Supporting Teams

The study team consisted of Frankie Cheng, Ho Chung Yau, Assunta Ho, Ka Yau Lai, Wing Kwan Leung, Wanny Leung, Elsa Chan, Nancy Li, Nancy Cheng, Suk Tak Lee, Po Yin Cheng, Lucia Ko, Clara Yu, Winson Li, Lawrence Cheng, Jack Szeto, Alfred Tang, Tsz Kin Wong and Elaine Wong for subject recruitment and field works; Benny Zee for conducting statistical analysis; Gordon Cheung, Albert Li and Benjamin Yip for serving as DSMB members; and Apple Yeung for performing pathogen analysis of illness samples. This clinical trial was also supported by the following staff of FrieslandCampina: Zwannet Dijkstra, Ingrid Halsema, Margo Jansen, Frank Verouden and many more colleagues for producing the study YCFs; Marjolijn Bragt-van Wijngaarden for nutritional and trial support; Laureen Leung for supporting local clinical trial logistics; and Joost van Neerven and Paula Garcia for boosting the trial initiative. Jos van der Vossen, Tim van den Broek and Margreet Heerikhuisen of TNO and Jos Boekhorst of NIZO were responsible for analyzing nasal and fecal microbiome respectively.

Supplementary Methods

Supplementary Results

Table S1 Nutritional composition of different YCFs

Table S2 Detection of respiratory pathogens in nasal swabs from subjects with URTI

Table S3 Detection of pathogens in fecal specimens from subjects with GITI

Table S4 Subgroup analyses for the primary and secondary efficacy outcomes

Figure S1 Hard stools were determined using the following Bristol Stool scale. Stool with type 1 and/or 2 consistency was considered as hard stools.

Figure S2 Changes in anthropometric data among study participants for z-scores of (a) weight-for-age; (b) height-for-age and (c) weight-for-height. Error bars represent means and one standard error.

Figure S3 Principal Component Analysis (PCA) of fecal microbiota relative abundance on the genus level, on all samples.

This supplementary material has been provided by the authors to give readers additional information about their work.

Supplementary Methods

Study exclusion criteria

Subjects with the following criteria were excluded: premature birth (<37 weeks' gestation), underweight, wasting or stunting (<2 SD of z-scores for weight-for-age [WAZ], weight-for-height [WHZ] or height-for-age [HAZ]), chronic or congenital diseases, suspected pulmonary tuberculosis, history of lactose intolerance, cow's milk allergy or severe eczema or asthma, in sick condition within 2 weeks, any breastfeeding within 2 months before the start of the intervention, unwillingness to stop consumption of probiotic, prebiotic or synbiotic supplement or a milk formula containing these ingredients during the study, influenza vaccination within 12 months, intake of antibiotics, anti-viral drugs, montelukast, laxatives or anti-diarrheal drug within 1 month, use of systemic immunosuppressive drugs within 3 months, and participation in another clinical trial within 2 months of study enrollment.

Randomization and blinding

Eight letter codes were used to label the four products (two letters per YCF). To maintain blinding, parents/caregivers, investigators, research nurses and laboratory personnel were unaware of the letter codes. During the 6-month intervention period, milk consumption was restricted to two servings of 200 mL/day for the allocated YCFs. Subjects preferring to drink more than two daily servings were also provided with regular YCF (similar composition to YCF-Ref), to be consumed only after consumption of the allocated YCF. To preserve the integrity and functionality of bioactive proteins and 2'-FL, parents were instructed to prepare each serving by adding milk powder (33 g from a single-use sachet) to lukewarm (40°C) water (180 mL) and to feed subjects the YCF within 1 hour of reconstitution.

Eligible children were randomly allocated to one of the YCF groups and randomization was stratified according to gender and age (1.00-1.50; 1.51-2.00; 2.01-2.50 years). Recruitment targets were 60±5%, 30±5% and 10±5% of children into each age category, respectively. Twin siblings were allocated to the same treatment group to prevent product mix up. Recruitment of a younger sibling of a subject from the same household was allowed providing that the elder subject had completed the study and returned all unused YCF. A randomization table with treatment codes was generated using SAS version 9.1 (SAS Institute, Inc, Cary, NC) by an independent statistician. Study staff and parents were unaware of the intervention until all data analyses were completed and only after the blind review meeting the database was unlocked. An independent data safety monitoring board (DSMB; including a pediatrician, nutritionist and epidemiologist/biostatistician) and an individual at FrieslandCampina kept sealed envelopes allowing unblinding of study product if clinically indicated.

Microbiome analyses of nasal and fecal samples

Stool and nasal samples were collected at final visits for microbiome analyses. Fecal samples collected on diaper or clean disposable potty at home were transferred by caregivers to sterile containers. These samples were kept at 4°C and transported to our laboratory within 24 hours when subjects returned for the visits. Nasal samples were collected during study visits by research staff using FLOQSwabs™ flocked swabs (Copan diagnostics, Murrieta, CA, USA), which were stored at 4°C and transported to laboratory within 8 hours. These stool and nasal samples were stored at -80°C in our laboratory until analysis in one batch.

To determine the fecal microbiome composition, DNA was isolated and quantified from a random 30% subset of all subjects. 16S sequences were analyzed using a workflow based on Qiime 1.8.^{S1} OTU clustering, taxonomic assignment and reference alignment were done with

the pick_open_reference_otus.py workflow script of Qiime, using uclust as clustering method (97% identity) and GreenGenes v13.8 as reference database for taxonomic assignment. Reference-based chimera removal was done with Uchime.^{S2} Samples with less than 5000 reads after removal of chimera's and singletons (reads in OTUs with only a single sequence) were removed.

To determine the nasal microbiota composition at baseline and endline of the study, DNA was isolated and quantified from a random 30% subset of all subjects as described before by Bosch et al.^{S3} 16S rDNA amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene was performed according to Kozich et al.,^{S4} using F515/R806 primers.^{S1} Pair-end sequencing of amplicons was conducted on the Illumina MiSeq platform (Illumina, Eindhoven, The Netherlands) and the sequence data were processed with Mothur v.1.36.1.

Pathogen identification in illness samples

Nasal samples were collected from subjects with diagnosed URTI by FLOQSwabs™ flocked swabs (COPAN Diagnostics, Murrieta, CA, USA), which were immediately placed in viral transport medium (2 mL), transported to the laboratory at 4°C and stored at -80°C until analysis. A final volume of total nucleic acids (60 µL) was extracted from 200 µL samples using QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). RespiFinder RG Panel (Qiagen) was then performed, which detected 16 RNA viruses, two DNA viruses and three atypical bacteria (Table S2). For GITI episodes, fresh stool samples were collected from diapers or potty, kept at 4°C during transport to the laboratory where they were frozen at -80°C until analysis. A final volume of total nucleic acids (60 µL) was extracted from around 0.2 g of fecal specimens using AllPrep PowerFecal DNA/RNA kit (Qiagen). Briefly, host and microbial cells in homogenized stool were lysed by mechanical collisions between beads and chemical disruption of cell membranes. Following inhibitor removal, the samples were

analyzed by GastroFinder 2SMART which detected nine species/groups of bacteria, four parasites and five viruses (Table S3). Pathogen nucleic acids were detected in both platforms using Rotor-Gene Q MDx instrument (Qiagen).

Statistical analysis

Intention-to-treat (ITT) analyses were performed for outcomes in all children allocated randomly to treatment and consuming the products at least once. Per-protocol (PP) analyses were also conducted for subjects who provided $\geq 80\%$ of primary outcome data in the study diaries and consumed $\geq 70\%$ of supplied YCF during the study period. Chi-squared or Fisher exact tests were used to compare between-group categorical variables, and Student's *t* test was used to analyze normally-distributed numerical variables. Mann-Whitney U test was used for data not distributed normally. Generalized estimating equation (GEE) was used to analyze factors identified by univariate analysis, adjusted for age, weight and height as confounders. Kaplan-Meier survival plots were used to analyze the time to first URTI or GITI episodes. Statistical Package for Social Science version 23.0 for Windows (SPSS Inc, Chicago, IL) was used for all analyses.

Biostatistical analysis of the fecal microbiome was performed by univariate analyses of differences between contrasts with regard to taxonomy relative abundances was tested by the non-parametric Mann-Whitney U, corrected with Bonferroni for multiple testing. Statistical tests were performed by custom, in-house R-scripts (version 3.1.2; <https://www.r-project.org/>) or as implemented in SciPy (<https://www.scipy.org/>), downstream of Qiime analyses as described above. Principal component analysis and multivariate redundancy analysis (RDA) were done in Canoco^{S5} using default settings of the analysis type “unconstrained” and “constrained” respectively. Relative abundance values for either taxa were used as response data and YCF-group as explanatory variable. RDA calculates p-values using a Monte Carlo

permutation.

Biostatistical analysis of nasal swab microbiota data was performed to determine possible effects related to the different interventions. The step-wise analysis included PERMANOVA, hierarchical clustering with complete linkage on a Bray-Curtis distance matrix, generalized linear models with a Gaussian link, and K-means clustering.

Supplementary Results

Pathogen analysis of nasal and fecal samples

Respiratory pathogens were detected in 255 (85.0%) of the analyzed nasal samples (n=300) and gastrointestinal pathogens were confirmed in 62 (91.2%) of the fecal samples (n=68). The most common URTI pathogens were rhinovirus/enterovirus, bocavirus and parainfluenza viruses (Table S2). Co-infections occurred in 11-24% of samples. The interventions were not significantly associated with specific URTI pathogen profiles. GITI samples showed high percentages of co-infections (Table S3). The most common GITI pathogens were enteropathogenic *E. coli*, *Clostridium difficile* toxin A (generally as co-infection) and noroviruses. The interventions were not significantly associated with any pathogen in stool.

Effects of YCFs on fecal and nasal microbiome

Sequencing data obtained from fecal samples found that the most dominant microbes were classes Clostridia (44%), Actinobacteria (35%; mostly represented by *Bifidobacterium*), Coriobacteria (5.6%), Bacteroidia (5.3%) and Bacilli (4.8%). Principal component analysis of fecal microbiota relative abundance at the genus level identified distinct *Collinsella*-rich and *Collinsella*-poor sample clusters (Fig. S3) that were independent of the time point of sample

collection or treatment. On the other hand, there was no significant effect of YCF-A, YCF-B or YCF-C on fecal microbiota compared with YCF-Ref. Phylogenetic diversity analysis indicated that within-sample diversity in older toddlers was significantly higher than in younger toddlers ($P < 0.0001$). Available baseline subject meta-data minimally explained the variance in fecal microbiome: age 2.1% ($P = 0.001$), habitual dietary intake 1.0% (energy and all nutrients combined), having a sibling 0.7% ($P = 0.016$), stool consistency 0.5% ($P = 0.014$), daycare attendance 0.4% ($P = 0.062$) and breastfeeding at infancy 0% ($P = 0.611$). Dominant genera detected by nasal swab sequencing were *Corynebacterium*, *Dolosigranulum*, *Moraxella*, *Streptococcus*, *Staphylococcus* and *Haemophilus*. Inter-individual variance was high and no significant treatment effect was detected.

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219 **Table S1** Nutritional composition of different YCFs

Description	Unit	YCF-Ref	YCF-A	YCF-B	YCF-C
		Per 100 mL	Per 100 mL	Per 100 mL	Per 100 mL
Protein (Nx6.25)	g	2.7	2.7	2.7	2.7
Immunoglobulins	g	0	0.1	0.01	0
Lactoferrin	g	<0.002	0.17	0.01	<0.002
TGF- β	μ g	0.99	1.5	1.5	0.99
Fat	g	2.5	2.5	2.5	2.5
Milk fat	g	0.05	1.7	0.05	0.05
Linoleic acid	mg	337	281	337	337
Available carbohydrates	g	10	9.7	10	9.7
Fiber	g	0.4	0.69	0.4	0.69
Galacto-oligosaccharides	g	0.4	0.4	0.4	0.4
2' Fucosyl lactose	g	0	0.3	0	0.3
Energy	kcal	73	72	73	72

220 All YCF were produced to the highest quality and packed in The Netherlands. Despite
 221 composition differences, all YCFs were compliant with CODEX and local legislation for this
 222 product type and age group. To preserve milk protein bioactivity, YCF-A and YCF-B
 223 underwent mild processing in the factory. Bioactivity of the immunoglobulins, lactoferrin and
 224 TGF- β , and 2'-FL concentrations were analyzed in the YCFs immediately after production,
 225 and at regular intervals until trial end to confirm product stability.

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230 **Table S2** Detection of respiratory pathogens in nasal swabs from subjects with URTI

Pathogen	YCF-Ref (n=84)	YCF-A (n=66)	YCF-B (n=72)	YCF-C (n=78)
Co-infection	10 (11.9)	12 (18.2)	17 (23.6)	12 (15.4)
Influenza A	1 (1.2)	3 (4.5)	3 (4.2)	2 (2.6)
Influenza B	1 (1.2)	1 (1.5)	1 (1.4)	0
Any influenza	2 (2.4)	4 (6.1)	4 (5.6)	2 (2.6)
RSV-A	2 (2.4)	3 (4.5)	5 (6.9)	8 (10.3)
RSV-B	7 (8.3)	1 (1.5)	2 (2.8)	1 (1.3)
Any RSV	9 (10.7)	4 (6.1)	7 (9.7)	9 (11.5)
Rhinovirus/enterovirus	45 (53.6)	37 (56.1)	42 (58.3)	30 (38.5)
Parainfluenza type 1	2 (2.4)	2 (3.0)	1 (1.4)	4 (5.1)
Parainfluenza type 2	1 (1.2)	0	2 (2.8)	1 (1.3)
Parainfluenza type 3	10 (11.9)	2 (3.0)	6 (8.3)	2 (2.6)
Parainfluenza type 4	1 (1.2)	1 (1.5)	0	1 (1.3)
Any parainfluenza virus	14 (16.7)	5 (7.6)	9 (12.5)	8 (10.3)
Adenovirus	0	1 (1.5)	3 (4.2)	4 (5.1)
HCoV-229E	1 (1.2)	1 (1.5)	0	0
HCoV-HKU1	0	0	1 (1.4)	0
HCoV-NL63	0	1 (1.5)	2 (2.8)	0
HCoV-OC43	4 (4.8)	3 (4.5)	0	1 (1.3)
Any HCoV	5 (6.0)	5 (7.6)	3 (4.2)	1 (1.3)
Human metapneumovirus	5 (6.0)	2 (3.0)	2 (2.8)	6 (7.7)
Bocavirus	10 (11.9)	11 (16.7)	9 (12.5)	12 (15.4)
<i>Mycoplasma pneumoniae</i>	1 (1.2)	0	0	1 (1.3)
<i>Chlamydia pneumoniae</i>	0	0	0	0
<i>Legionella pneumophila</i>	0	0	0	0
<i>Bordetella pertussis</i>	0	0	0	0

231 HCoV, human coronavirus; RSV, respiratory syncytial virus; URTI, upper respiratory tract
 232 infection. Results are expressed in number (percentage).

233 **Table S3** Detection of pathogens in fecal specimens from subjects with GITI

Pathogen	YCF-Ref (n=12)	YCF-A (n=20)	YCF-B (n=10)	YCF-C (n=26)
Co-infection	6 (50.0)	13 (65.0)	8 (80.0)	14 (53.8)
Norovirus	2 (16.7)	4 (20.0)	0	2 (7.7)
Rotavirus	1 (8.3)	0	0	1 (3.8)
Adenovirus	0	1 (5.0)	0	0
Astrovirus	0	1 (5.0)	1 (10.0)	3 (11.5)
Sapovirus	0	0	0	1 (3.8)
<i>Campylobacter</i> spp.*	1 (8.3)	0	1 (10.0)	1 (3.8)
<i>Salmonella</i> spp.**	2 (16.7)	4 (20.0)	2 (20.0)	3 (11.5)
Enteropathogenic <i>E. coli</i> (EPEC)	7 (58.3)	14 (70.0)	9 (90.0)	17 (65.4)
Enterotoxigenic <i>E. coli</i> (ETEC)	0	4 (20.0)	3 (30.0)	0
Shigella/Enteroinvasive <i>E. coli</i> (EIEC)	0	0	0	0
Shiga toxin producing <i>E. coli</i> (STEC)	0	0	0	1 (3.8)
<i>Clostridium difficile</i> toxin A	4 (33.3)	8 (40.0)	4 (40.0)	10 (38.5)
<i>Clostridium difficile</i> toxin B	1 (8.3)	4 (20.0)	1 (10.0)	6 (23.1)
<i>Yersinia enterocolitica</i>	0	0	0	0
<i>Entamoeba histolytica</i>	0	0	0	0
<i>Giardia lamblia</i>	0	2 (10.0)	0	0
<i>Cryptosporidium</i> spp.***	0	0	0	0
<i>Dientamoeba fragilis</i>	0	1 (5.0)	1 (10.0)	1 (3.8)

234 GITI, gastrointestinal tract infection.

235 Results are expressed in number (percentage).

236 * Includes *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*.

237 ** Includes *S. enterica* and *S. bongori*.

238 *** Includes *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis* and *C. canis*.

239

240 **Table S4** Subgroup analyses for the primary and secondary efficacy outcomes

Outcome	YCF-Ref	YCF-A	YCF-B	YCF-C
<i>Breastfeeding < 2 months</i>	<i>n=58</i>	<i>n=52</i>	<i>n=42</i>	<i>n=62</i>
GITI incidence (Agustina) per subject	0.3 ± 0.7	0.7 ± 1.1 [†]	0.3 ± 0.5	0.5 ± 0.9
GITI incidence (WHO) per subject	0.2 ± 0.6	0.4 ± 0.7	0.2 ± 0.4	0.3 ± 0.5
Duration of GITI (WHO), days	3.9 ± 1.5	3.6 ± 2.1	3.9 ± 1.4	2.9 ± 1.4
<i>Breastfeeding ≥ 2 months</i>	<i>n=42</i>	<i>n=52</i>	<i>n=62</i>	<i>n=41</i>
GITI incidence (Agustina) per subject	0.3 ± 0.7	0.4 ± 0.7	0.4 ± 0.9	0.5 ± 0.9
GITI incidence (WHO) per subject	0.1 ± 0.3	0.3 ± 0.4	0.2 ± 0.5	0.2 ± 0.5
Duration of GITI (WHO), days	4.4 ± 2.6	2.7 ± 1.5	4.8 ± 4.3	4.7 ± 3.0
<i>Exposed to household tobacco smoke</i>	<i>n=36</i>	<i>n=34</i>	<i>n=37</i>	<i>n=34</i>
URTI incidence per subject	1.8 ± 1.3	1.9 ± 1.8	1.7 ± 1.6	2.3 ± 1.6
Duration of URTI, days	7.0 ± 4.2	6.2 ± 3.8	7.7 ± 4.5	9.5 ± 5.6 [§]
<i>Not exposed to household tobacco smoke</i>	<i>n=78</i>	<i>n=80</i>	<i>n=77</i>	<i>n=80</i>
URTI incidence per subject	1.9 ± 1.6	1.9 ± 1.4	1.7 ± 1.4	1.9 ± 1.6
Duration of URTI, days	7.5 ± 4.8	8.8 ± 8.3	9.0 ± 6.5	8.6 ± 5.6

241 GITI, gastrointestinal tract infection; URTI, upper respiratory tract infection.

242 Results are expressed in mean ± standard deviation.

243 [†] *P* = 0.009 and [§] *P* = 0.055 when compared with YCF-Ref.

244

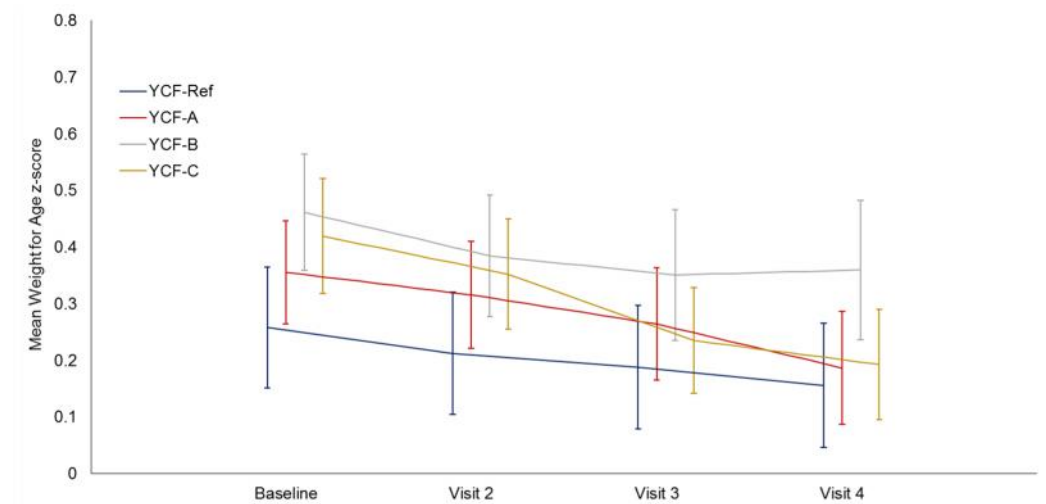
Figure S1 Hard stools were determined using the following Bristol Stool scale. Stool with type 1 and/or 2 consistency was considered as hard stools.

Bristol Stool Chart

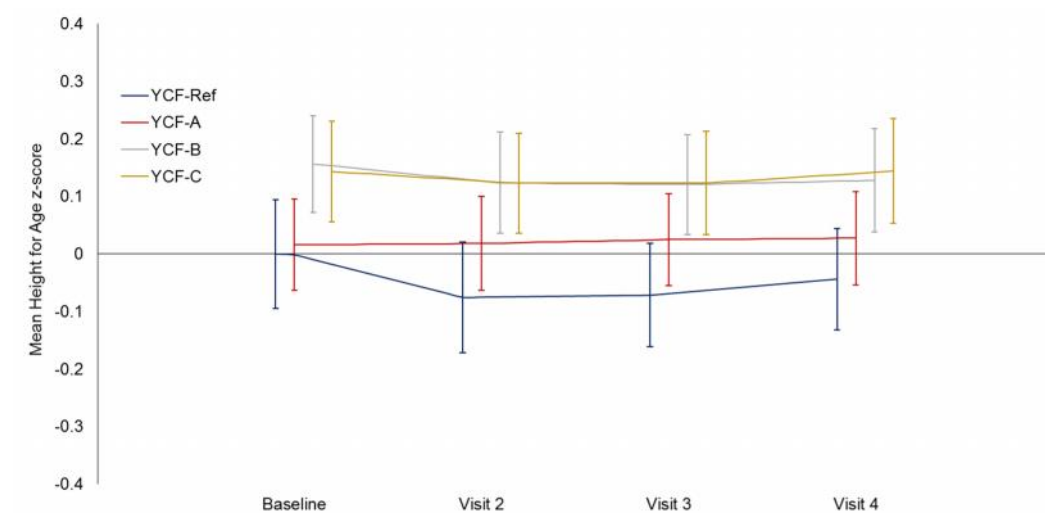
Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. Entirely Liquid

Figure S2 Changes in anthropometric data among study participants for z-scores of (a) weight-for-age; (b) height-for-age and (c) weight-for-height. Error bars represent means and one standard error. Z-scores were calculated using growth charts of Hong Kong children with following references: weight-for-age and weight-for-height^{S6} and height-for-age^{S7}.

(a)



(b)



(c)

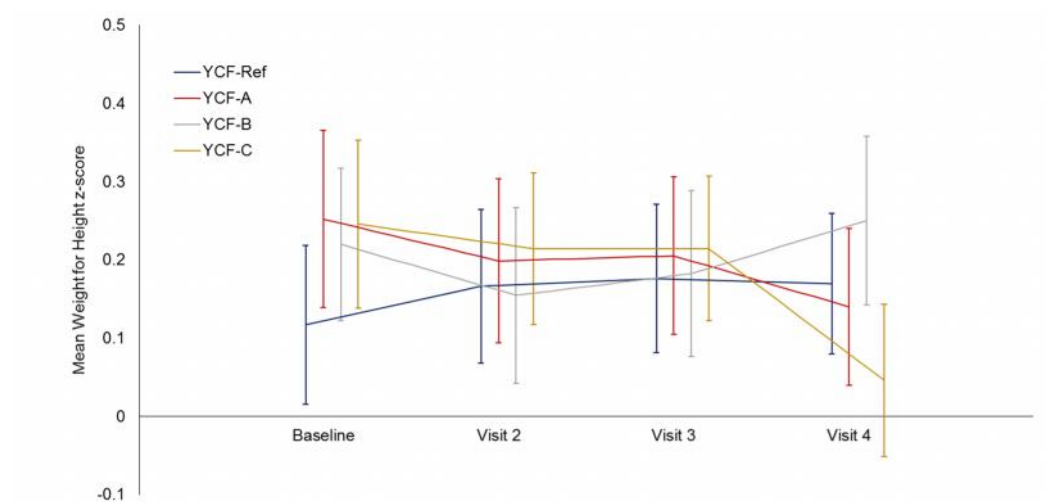


Figure S3 Principal component analysis (PCA) of fecal microbiota relative abundance at the genus level on all samples. Distinct *Collinsella*-rich and *Collinsella*-poor sample clusters were identified, independent of the time point of sample collection or treatment. The most important genera associated with the samples have been plotted as arrows, longer arrows indicate stronger associations. Baseline samples are presented as green circles and endline samples as blue squares. Variation explained on axis 1 and 2 was 16% and 9.3%, respectively.

