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Infant formula enriched with milk fat globule membrane, long-chain polyunsaturated fatty acids, synbiotics, gangliosides, nucleotides and sialic acid reduces infections during the first 18 months of life: The COGNIS study

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ABSTRACT

Functional nutrients like synbiotics or milk-fat globule membrane (MFGM) affect positively host immunity, modifying intestinal microbioma and reducing early childhood infections. We compared effects of an experimental enriched-infant formula with long chain polyunsaturated fatty acids, MFGM, synbiotics, sialic acid, nucleotides and gangliosides to a standard infant formula and breastfeeding regarding infections incidence and evolution in infants until 18 months of age. 170 healthy infants were enrolled in a RCT to receive either a bioactive functional nutrients enriched infant formula (EF, n = 85) or a standard formula (SF, n = 85). 50 breastfed (BF) infants were also enrolled. At age 12 months, EF group compared to SF and BF groups showed a lower incidence of infectious episodes. Moreover, they also presented less incidence of respiratory tract infections and gastrointestinal infections than SF infants, reducing risk by 30.2% and 32.5%, respectively. Infants fed with an EF seem to have reduced incidence of certain infections at 12 months of age.

1. Introduction

Breastfeeding is the gold-standard for infant nutrition containing both nutritive and non-nutritive components. It is well known that BF protects newborns and infants against infections (Victora et al., 2016). Many components of human breast milk, including short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), immunoglobulins, antibacterial enzymes, glycosylated proteins, antiviral lipids, and leukocytes (Sánchez et al., 2021), play an important role in development of infant immune response due to their ability to bind pathogenic bacteria, while promoting growth of protective enteric bacteria (Ballard & Morrow, 2013; Newburg, 2013). Moreover, breast milk also contains maternal pathogen-specific antibodies, which provide infants immune protection during their first months of life, a period in

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which infant́s immune system has not reached yet its full potential (Zhang et al., 2017); as well as other components, such as human milk oligosaccharides (HMOs), which favor development of a beneficial microbiota (Gopalakrishna & Hand, 2020; Lyons et al., 2020). Maternal leucocytes transferred by breast milk also seem to offer protection and enhance development of infants immune system (Laouar, 2020). However, it is not always possible or suitable to maintain breastfeeding along time. In that case, infant formulas have to be offered to satisfy infants' nutritional and energy requirements. In this line, bioactive nutrients such as pre- and probiotic or milk fat globule membrane (MFGM) are currently being added to infant formulas to narrow the functional and nutritional gap between breast milk and infant formulas (Hernell et al., 2016; Ratsika et al., 2021). In fact, there is evidence that supplementation with functional nutrients during early childhood might influence gut microbiota development, interfering on immune response in a positive way (Nauta et al., 2013). Both scGOS and lcFOS have shown to have prebiotic and immunomodulatory effects comparable to HMOs, reducing certain type of infections (Ballard & Morrow, 2013; Doherty et al., 2018; Triantis et al., 2018), particularly upper respiratory tract infections (RTI) (Gopalakrishna & Hand, 2020; Lyons et al., 2020). Prebiotics are capable to enhance growth of beneficial gut microbiota and, hence, may also provide a protective effect against allergic manifestations (Cheng et al., 2019), as well as to diminish infections duration (Neumer et al., 2021; Thai & Gregory, 2020). Long chain polyunsaturated fatty acids (LC-PUFAs) may have also a positive effect on immune maturation (Gottrand, 2008; Miles et al., 2021; Ramiro-Cortijo et al., 2020).

Several probiotics have also shown a beneficial effect on typical childhood infections and immune-related diseases. For instance, *Lactobacillus rhamnosus* GG (LGG) reduces risk of RTI and atopic dermatitis (Nermes et al., 2011; Rautava et al., 2009), while *Bifidobacterium* BB12, *Streptococcus thermophilus* (Phuapradit et al., 1999) and *Bifidobacterium longum* subsp. *infantis* CECT 7210, employed in our study formula, seem to be able to inhibit rotavirus infection (Muñoz et al., 2011). Other Bifidobacteria like *Bifidobacterium longum* BL999 may interfere positively in RTI (Puccio et al., 2007). *Lactobacillus rhamnosus* H001, another probiotic strain also included in our study formula, has shown a good safety profile in infants (Dekker et al., 2009) and beneficial effects in atopic eczema (Wickens et al., 2008). Furthermore, *Bifidobacterium lactis* BB12, *Lactobacillus reuteri* or *Lactobacillus fermentum* CECT5716 have been associated with reduction in both incidence and risk of diarrhea and RTI in infants (Maldonado et al., 2019; Rautava et al., 2009; Weizman et al., 2005). The combination of pre- and probiotics (synbiotics) shows a good evidence in reducing RTI (Chan et al., 2020).

There is evidence suggesting that LC-PUFAs also contribute to a better immune response in infants (Gottrand, 2008; Thai & Gregory, 2020). Several trials found evidence in infants fed with LC-PUFAs supplementation to have a lower incidence regarding RTI, gastrointestinal infections (GII) and allergic manifestations (Lapillonne et al., 2014; Miles et al., 2021; Pastor et al., 2006).

On the other hand, milk fat globule membrane (MFGM), a complex structure present in human and bovine milk (Liao et al., 2011; Reinhardt & Lippolis, 2006), contains a broad variety of integral and peripheral proteins, glycoproteins (butyrophilin, lactadherin, or mucins), enzymes, and lipids with antimicrobial and antiviral effects (Fuller et al., 2013; Peterson et al., 1998; Spitsberg, 2005). MFGM-enriched infant formulas seem to reduce incidence of acute otitis media (AOM) (Timby et al., 2015) and diarrhea (Zavaleta et al., 2011) in young infants. According to a recent meta-analysis, MFGM are safe and data is pointing out to beneficial effects regarding infections (Ambrożej et al., 2021). Therefore, there is broad evidence that infant formula supplemented with functional nutrients, such as MFGM, prebiotic, probiotic or both (synbiotics), each improves gut microbiome development, inducing immune response modulation and eventually reducing infection burden in infants. The goal of the present study was to analyze effects of a novel combination of bioactive nutrients-enriched infant formula containing MFGM components, synbiotics (pre- and probiotics), LC-PUFAs, gangliosides, nucleotides and sialic acid, on number and duration of infections, need for health-care seeking, and infection related medication during the first 18 months of life in healthy infants, compared to those fed with a standard infant formula or breast milk.

2. Methods

2.1. Study design and subjects

The COGNIS study (*A Neurocognitive and Immunological Study of a New Formula for Healthy Infants*) was designed as a prospective, randomized double-blind, nutritional intervention study based on bioactive compounds-enriched infant formula (registered at www.ClinicalTrials.gov; Identifier: NCT02094547). Detailed information has been published elsewhere (Nieto-Ruiz et al., 2019, 2020). Briefly, 220 healthy full-term babies were involved in the study; from these, 170 infants aged between 0 and 2 months old were randomized using a mathematical statistical method (ratio 1:1) to receive, during their first 18 months of life, either a standard infant formula (SF n = 85), or an experimental infant formula (EF n = 85) Additionally, 50 breast-fed (BF) infants were evaluated along 18 months of age as reference group. A detailed participant flowchart from baseline visit to 18 months is presented in Fig. 1.

The current analysis included a total of 171 infants who attended the 6 months visit (SF = 60; EF = 69; BF = 42), 152 at 12 months visit (SF = 51; EF = 63; BF = 38) and 141 infants at 18 months visit (SF = 48; EF = 56; BF = 37).

Both standard (SF) and experimental (EF) infant formulas follow the guidelines of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee of Nutrition and European Food Safety Authority (EFSA) (Koletzko et al., 2005; Lázaro-Almarza & Benjamín, 2010), as well as international and national recommendations for the composition of infant formulas. A full description of the nutritional composition is detailed in Table 1. Briefly, EF was designed to contain functional nutrients, including MFGM components [10% of total protein content (wt:wt)]; synbiotics [mix of FOS and inulin (ratio 1:1), *Bifidobacterium infantis* IM1 and *Lactobacillus rhamnosus* LCS-742]; LC-PUFAs arachidonic acid (ARA) and docosahexaenoic acid (DHA); gangliosides; nucleotides and sialic acid. Both standard and experimental initiation infant formulas were offered until 6 months of life; then, infants received its corresponding follow-on formula from 6 to 18 months of age. The pediatrician recommended a pattern of complementary feeding introduction and content according to Spanish Association of Pediatrics (Lázaro-Almarza & Benjamín, 2010).

2.2. Ethics, consent, and permissions

This study was carried out in accordance with ethical standards established by the Declaration of Helsinki 2004 (World Medical Association General Assembly, 2004), the EEC Good Clinical Practice recommendations (document 111/3976/88 July 1990) and the Spanish legislation governing clinical research in humans (Royal Decree 561/1993 on clinical trials). All procedures were also approved by the Ethical Committee of the University of Granada, the Bioethical Committees for Clinical Research of the Clinical University Hospital San Cecilio and the Mother-Infant University Hospital of Granada, as well as respective Ethical Committees of the Health District of Granada, Spain. All families were informed about all procedures during the follow-up and a signed informed consent was obtained from each parent or legal guardian before child enrolment in the study.

2.3. Data collection

Baseline characteristics of parents and their offspring were obtained using questionnaires and medical records, including parental age,

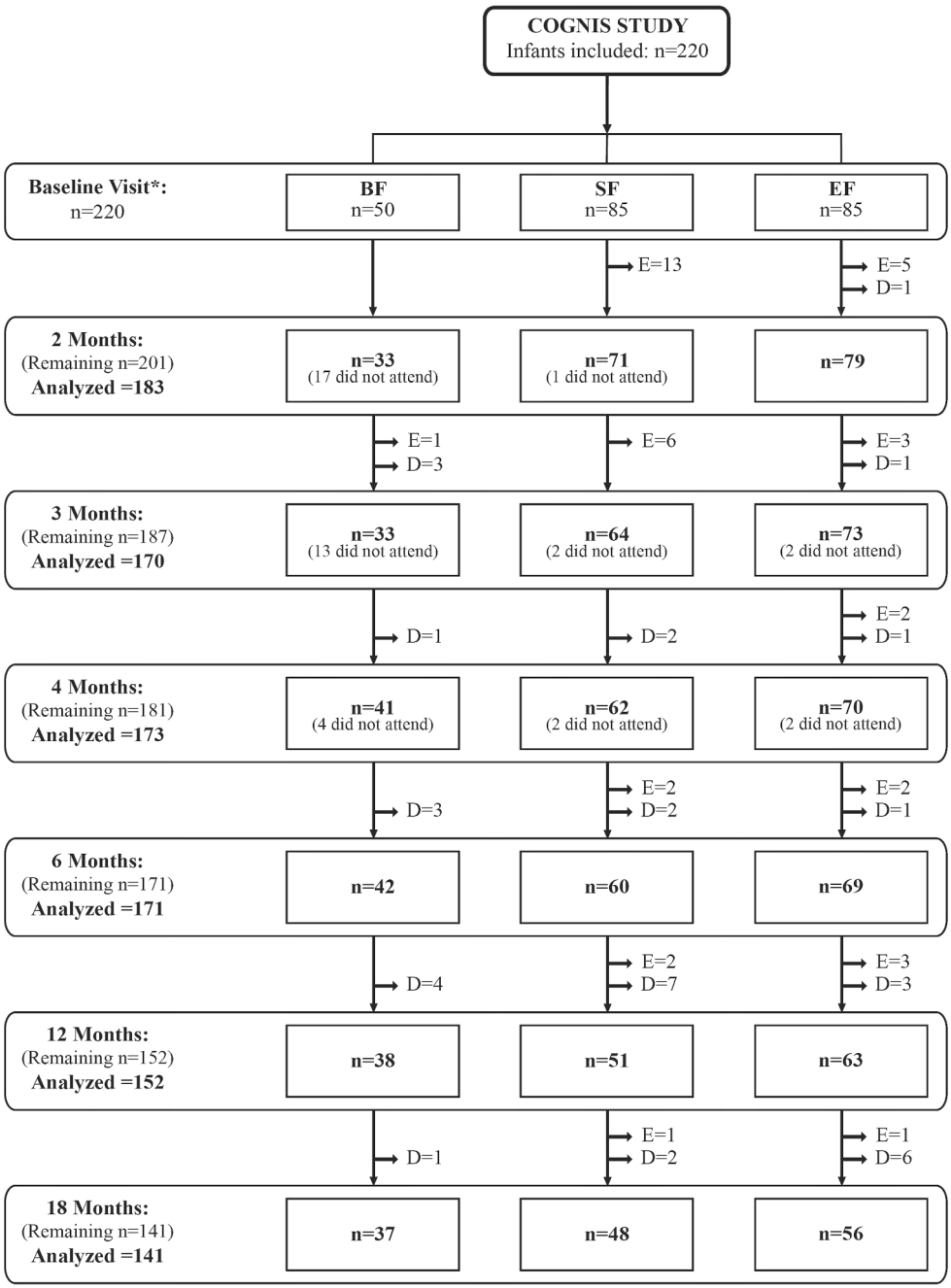

Fig. 1. Study profile from baseline visit to 18 months old. *Participant flow-chart from baseline visit to 18 months.* BF: Breastfed infants; D: Dropouts; E: Exclusions; EF: Experimental infant formula; n: Sample size; SF: Standard infant formula. Up to 18 months of life, a total of 40 infants were excluded in the SF and EF groups as follows: 24 were excluded in the SF group (1 infant due to perinatal hypoxia, 1 infant had growth deficiency, 15 infants did not take the infant formula, 2 had colic of the infant, 3 were excluded due to lactose intolerance, 1 infant due to digestive surgical intervention, and 1 infant suffered hydrocephalus); 16 infants were excluded in the EF group (2 infants presented growth deficiency, 2 infants lactose intolerance, 11 infants did not take the infant formula, and 1 was excluded due to epileptic seizure). Furthermore, 1 infant was excluded in the BF group due to she/he was not exclusively breastfed.

educational level and intelligence quotient (IQ) (Cattell's G-Factor intelligence test) (Cattell, 1963; Cattell & Cattell, 1994), smoking habit during pregnancy, preconceptional BMI, gestational weight gain, gestational age and type of delivery, number of siblings, birth weight, length and head circumference, infant sex, days of breastfeeding and attendance to kindergarten.

2.4. Infection related follow-up and further outcome measures

Infants were followed up at 3, 4, 6, 12 and 18 months of age, and underwent a physical examination by an expert pediatrician. Parents were asked to report infectious episodes, medication, and clinic visits, also submitting reports and medical documents regarding infectious episodes and other relevant health related events on each visit. Medical history was completed by face-to-face interviews.

Regarding infectious episodes, primary outcome measures were as follows: 1) number and duration of infectious episodes, disclosed in RTI, AOM, GII, and urinary tract infections (UTI), as well as other infectious episodes; 2) days of fever, 3) days of antibiotic treatment; 4) need for health care assistance; and 5) days of hospitalization.

Longitudinal prevalence (LP) of symptoms and medication were calculated by dividing number of days with reported symptoms/medication by total number of registered months (Morris et al., 1996).

2.5. Statistical analysis

All statistical analyses were performed using IBM® SPSS Statistics® program, version 22.0 (SPSS Inc. Chicago, USA). Shapiro-Wilks and Kolmogorov-Smirnov test, as well as visual inspection of histograms, were applied for all variables in the COGNIS study groups to check for normality. Normally distributed variables were presented as mean and standard deviation (SD), and non-normal variables as median and

Table 1
Nutritional composition of the Standard (SF) and Experimental (EF) Infant Formulas used in the COGNIS study.

	Standard Formula (SF)		Experimental Formula (EF)	
	Initiation	Follow-on	Initiation	Follow-on
	100 ml (13.5%)	100 ml (14.5%)	100 ml (13.5%)	100 ml (14.5%)
Energy (kcal/kJ)	69/288	70/294	68/285	69/290
Proteins (g)	1.35	1.8	1.35	1.8
Casein/whey (%)	40/60	50/50	40/60	50/50
Carbohydrates (g)	7.97	8.5	7.56	8.1
Lactose (g)	7.17	7.2	6.82	7.3
Maltodextrin (g)	0.8	1.3	0.7	0.8
Fat (g)	3.5	3.2	3.5	3.2
LA (mg)	579	517	569	517
ALA (mg)	49	45	49	45
ARA (mg)	–	–	15.8	10.2
DHA (mg)	–	–	11.2	10.2
Gangliosides (mg/L)	1.5	1.5	9	9
Sialic acid (mg/L)	82	80	105	105
MFGM-10 (wt/wt)	–	–	10%	10%
Nucleotides (mg)	–	–	2.92	2.94
Cytidine-5′-Monophosphate (mg)	–	–	1.09	1.12
Uridine-5′-Monophosphate (mg)	–	–	0.88	0.9
Adenosine-5′-Monophosphate (mg)	–	–	0.41	0.41
Guanosine-5′-Monophosphate (mg)	–	–	0.27	0.26
Inosine-5′-Monophosphate (mg)	–	–	0.27	0.26
Prebiotics				
FOS: Inulin (ratio 1:1)	–	–	0.4	0.4
Probiotics				
Bifidobacterium L. infantis CECT7210 *(Bifidobacterium infantis* IM1*)*	–	–	1×10^7 cfu/g	1×10^7 cfu/g
Lactobacillus rhamnosus LCS-742	–	–	1×10^7 cfu/g	1×10^7 cfu/g

Initiation formula: Up to 6 months of age. Follow-on formula: between 6 and 18 months of age. ARA: Arachidonic acid; cfu: colony forming unit; DHA: Docosahexaenoic acid; FOS: Fructooligosaccharides; LA: Linoleic acid; MFGM: milk fat globule membrane; ALA: α-Linolenic acid. Power diluted 13.5% (13.5 g infant formula made up to 100 ml with water); Power diluted 14.5% (14.5 g infant formula made up to 100 ml with water).

interquartile range (IQR). Categorical variables were showed as frequencies and percentages. Differences in prevalence of symptoms and infection episodes among the three COGNIS groups were tested using analysis of variance (ANOVA), T-test, Kruskal-Wallis rank-sum test for non-normal continuous variables, and Chi-square (χ^2) or Fisher test for categorical variables. When significant differences were found between groups, *post-hoc* Bonferroni correction was applied to identify significant pairwise group differences. Furthermore, those variables statistically different between study groups (maternal IQ and educational level, and paternal educational level) were included as confounders in analysis of covariance (ANCOVA). A logistic regression model (Wald method) was performed to calculate Odds Ratio (OR) and 95% Confidence Intervals (CI) to establish influence of the study groups or confounder variables on incidence of infections. As confounder variables, we selected maternal and paternal educational level and breastfeeding after delivery as these items showed significant differences between study groups, and kindergarten attendance as a known risk factor for common childhood infections (Ball et al., 2002; Côté et al., 2010). P-values < 0.05 were considered statistically significant.

3. Results

Background and baseline characteristics of parents and children are shown in Table 2. BF group showed a significantly higher maternal IQ ($P < 0.001$) and educational level ($P < 0.001$), as well as higher paternal educational level ($P = 0.003$), compared to the formula groups; however, no differences in these parameters mentioned above were found between both formula groups. Mothers of infants participating in the EF group were slightly younger than those included in SF or BF groups ($P = 0.068$). Regarding maternal smoking during pregnancy, mothers of BF groups smoked in less proportion (4.70%) compared to those of both SF and EF groups, who were more prone to smoking habit during pregnancy (18.80% and 13.0%, respectively) ($P = 0.098$). Besides, due to the COGNIS study design, a statistically significant difference was detected in days of breastfeeding in BF group compared to formula groups ($P < 0.001$). It is important to note that no significant differences were found among study groups with respect to attendance to kindergarten at 6 months of life ($P = 0.930$).

Incidence of infectious episodes between study groups in each follow-up visit (6, 12 and 18 months of life) is showed in Table 3. Analysis revealed that need for health care seeking (pediatric visits) up to 18 months was lower in the EF group, reporting the lowest need for pediatrician consulting ($P = 0.026$) at 6 months and a trend to less hospitalization ($P = 0.064$) at 12 months of age, compared to the SF and BF groups. At 12 months of life, EF fed infants presented lower episodes of unspecific fever compared to BF ones ($P = 0.005$). Interestingly, BF infants reported the highest incidence of conjunctivitis compared to those who fed either the SF at 12 months, or the EF at 18 months ($P = 0.042$; $P = 0.013$, respectively) (Table 3).

Regarding direct comparison between EF and SF, results showed that SF infants presented a trend to higher incidence of infections ($P = 0.068$), and more unspecific fever episodes ($P = 0.049$) at 6 months. At 12 months there were significantly less infections in the EF group ($P = 0.044$), particularly RTI ($P = 0.031$) and GII ($P = 0.030$), than in SF fed infants. No significant differences between formula fed study groups were detected at 18 months of life (Table 3).

Evaluation of disease evolution, longitudinal prevalence (LP) of days with fever, days of infection, kindergarten absenteeism and antibiotic administration in infants participating at 6, 12 and 18 months of age are shown in Table 4. When comparing between three COGNIS groups, in unadjusted analysis, we found that mean of infection days was lower in the EF fed infant group at 12 months ($P = 0.048$) compared to SF and BF groups. A lower number of fever days in the EF group was also found at 12 months, although not in a statistically significant manner ($P = 0.083$). However, after adjustment for selected confounding variables including maternal IQ and educational level, and paternal educational level, no significant differences were found in those outcomes mentioned above (Table 4).

After analysis comparing EF and SF, we observed that EF group presented less days with fever ($P = 0.031$) and infections ($P = 0.048$) compared to SF group at 12 months of life. Regarding these outcomes, after adjustment for above confounding variables, those differences did not remain statistically significant (Table 4).

Overall register of infections and symptoms between baseline visit and 18 months showed no significant differences between study groups. Direct comparisons between formula groups showed, in the unadjusted analysis, a significant lower number of fever days ($P = 0.044$) and a slight tendency to less infectious diseases ($P = 0.093$) in EF infants' group. However, after adjustment for confounding variables, those differences disappeared (Table 4). Surprisingly, days of kindergarten absenteeism were higher in EF infants compared to SF infants ($P = 0.019$) after adjustment.

Finally, to evaluate association of confounder variables on RTI and GII in the formula fed groups, logistic regression model (Wald method), adjusted by confounders (kindergarten attendance, maternal and paternal educational level and breastfeeding after delivery), was

Table 2
Baseline characteristics of the COGNIS study participants.

		BF (n = 50)	SF(n = 85)	EF (n = 85)	P^1	P^2
Parents characteristics						
Maternal age (years)		32 (30–36.25)	32 (24.75–35.25)	30.50 (26.25–34.75)	0.068	0.651
Maternal educational level	Primary	1 (2.00)[b]	19 (22.40)[a]	19 (22.40)[a]	**<0.001**	0.518
	Secondary	5 (10.00)[b]	28 (32.90)[a]	29 (34.10)[a]		
	VT	16 (32.00)	15 (17.60)	21 (24.70)		
	University	28 (56.00)[b]	23 (27.10)[a]	16 (18.80)[a]		
Maternal IQ (points)		111 (99–118)[b]	102 (92–111)[a]	100 (86–108)[a]	**<0.001**	0.241
Smoking during pregnancy		2 (4.70)	13 (18.80)	10 (13.00)	0.098	0.332
Preconceptional BMI (kg/m^2)		24.18 (21.75–27.61)	24.18 (21.75–27.61)	23.68 (21.14–27.30)	0.842	0.616
GWG (kg)		6 (4–9)	7 (3.5–10)	6 (3–9.5)	0.781	0.549
Gestational Age (weeks)		39.5 (38–40.25)	40 (38–40)	40 (39–40)	0.925	0.732
Type of delivery	Vaginal	37 (74.00)	62 (73.00)	60 (70.60)	0.899	0.865
	Caesarean	13 (26.00)	23 (27.00)	25 (29.40)		
Siblings	0	28 (56.00)	33 (38.80)	42 (49.40)	0.128	0.164
	≥1	22 (44.00)	52 (61.20)	43 (50.60)		
Paternal age (years)		35.07 ± 5.01	32.68 ± 6.89	33.31 ± 7.03	0.134	0.576
Paternal educational level	Primary	6 (12.80)[b]	28 (35.00)[a]	36 (46.20)[a]	**0.003**	0.393
	Secondary	11 (23.40)	25 (31.30)	16 (20.50)		
	VT	12 (25.50)	13 (16.30)	12 (15.40)		
	University	18 (38.30)[b]	14 (17.40)[a]	14 (17.90)[a]		
Paternal IQ (points)		108 (99–117)	108 (96–117)	102 (92–111)	0.062	0.084
Newborn and child characteristics						
Infant sex	Boy	21 (42.0)	49 (57.6)	51 (60.00)	0.105	0.755
	Girl	29 (58.0)	36 (42.4)	34 (40.00)		
Birth weight (g)		3321.20 ± 431.73	3266.25 ± 459.08	3347.76 ± 486.41	0.513	0.264
Birth length (cm)		51 (49–51)	50 (49–52)	51 (49–52)	0.431	0.262
Birth HC (cm)		35 (33.25–35)	35 (34–35.5)	34.25 (34–35)	0.481	0.265
Breastfeeding (days)		420 (270–540)	8 (0–22)	7 (1–28)	**<0.001**	0.772
Attendance to kindergarten at 6 mo.		4 (9.5)	6 (10.0)	8 (11.6)	0.930	1.000
Attendance to kindergarten at 12 mo.		15 (39.5)	20 (39.2)	26 (41.3)	0.971	0.850
Attendance to kindergarten at 18 mo.		26 (70.3)	26 (54.2)	33 (58.9)	0.311	0.693

Data are presented as n (%) for categorical data, mean ± SD for parametrically distributed variables, and median (IQRs) for non-parametrically distributed data.
BF: Breastfed infants; SF: Standard infant formula; EF: Experimental infant formula; BMI: Body Mass Index; IQ: Intelligence quotient; mo: months of age; VT: Vocational training; GWG: Gestational weight gain; HC: Head circumference.

[1] *p*-values for overall differences between three COGNIS-groups. ANOVA for normally distributed, Kruskal-Wallis rank-sum test for non-normal continuous, and Chi-square or Fisher test for categorical variables. Values not sharing same suffix (a,b) are significantly different in a Bonferroni *post hoc* test.

[2] *p*-values for differences between SF and EF COGNIS-groups. T-Test for normally distributed, U Mann-Whitney test for non-normal continuous, and Chi-square or Fisher test for categorical variables. P-values < 0.05 are highlighted in bold.

performed (Table 5). Type of early feeding was positively associated with lower incidence of RTI at 12 months of life. In fact, infants who fed EF showed a decreased risk of suffering RTI [OR: 0.366 (95% CI: 0.145–0.918), P = 0.030] and GII [OR: 0.325 (95% CI: 0.130–0.811), P = 0.016] compared to SF infants. Moreover, attendance to kindergarten increases the risk of suffering RTI at 6 months of life [OR: 14.812 (95% CI: 3.137–69,947), P = 0.001], as well as RTI and GII infections at 12 months [OR: 3.447 (95% CI: 1.281–9.117), P = 0.014; OR: 2.561 (95% CI: 1.036–6.331), P = 0.042; respectively], and RTI risk at 18 months [OR: 2.565 (95% CI: 1.120–5.876), P = 0.026].

4. Discussion

Results of the present study suggest differences in infections incidence and infection-related parameters in infants at 6 months of age, and especially at 12 months, with better outcomes in those infants fed with bioactive nutrients EF compared to the SF group. In fact, there was a tendency in the EF group of more infants free from infections at 6 months, as well as significant lower proportion of infants with more than 2 infections at 12 months, compared to the SF group. Moreover, incidence of GII and RTI was significantly lower in the EF group at 12 months. Regarding BF infants, it is remarkable that there were no significant differences compared to the formula fed infants on number of overall infections, fever days or antibiotic use, after adjusting for baseline confounders. Thus, our results might suggest that infant formula supplemented with bioactive nutrients, including MFGM, synbiotics, LC-PUFAs, nucleotides, gangliosides and sialic acid, could have beneficial effects on defense against infections and immune system development in infants during their first 18 months of life.

Breastfeeding is the best nutritional option to prevent infant morbidity and mortality; it is considered the gold standard for infant nutrition due to its health benefits for mother and child, as well as environmental, economic and psychosocial aspects (Ball & Wright, 1999; Lassi et al., 2014; Ratsika et al., 2021). Breastfeeding has been associated with lowered incidence of all-cause and infection-related mortality in infants (Sankar et al., 2015). Breast milk includes a variety of functional nutrients with diverse beneficial roles, promoting optimal development and maturation of infant immune system. Among others, HMOs present in human breast milk have shown bifidogenic effects, leading to a persistent finger print on gut microbiota (Beghetti et al., 2019; Wiciński et al., 2020). Consequently, efforts are made to transfer nutritional and immunological benefits of breast milk to infant formulas in order to guarantee an optimal nutrition, in case breast feeding is not possible. Several functional nutrients, especially pre- and probiotics, as well as MFGM, are under investigation and have shown individually or in different combination, to interfere in a protective way on risk of common infant infections (Arslanoglu et al., 2007; Maldonado et al., 2012, 2019; Sierra et al., 2015; Timby et al., 2015).

Results from the current analyses are consistent with other studies concerning infections prevention by nutritional intervention with probiotics (Liu et al., 2018). Weizman et al. reported beneficial effect of infant formula supplemented with probiotics [*B. lactis* BB12 and *L. reuteri* (American Type Culture Collection 55730)] on diarrhea, but not on RTI, in a 12 week follow-up in 4–10 months old infants (Weizman et al., 2005). Another study showed a lower incidence of GII, upper RTI and total incidence of infections, in a 6 month follow-up of infants 6 months old feeding a formula supplemented with probiotics (*L. fermentum* CECT5716) compared to standard formula (Maldonado

Table 3
Incidence of different types of infections at 6, 12 and 18 months.

		6 months					12 months					18 months				
		BF(n = 42)	SF (n = 60)	EF (n = 69)	P^1	P^2	BF (n = 38)	SF (n = 51)	EF (n = 63)	P^1	P^2	BF (n = 37)	SF (n = 48)	EF (n = 56)	P^1	P^2
Number of Infections	0	17(40.5)	26(43.3)	41(59.4)	0.083	0.068	7(18.4)	6(11.8)	15(23.9)	0.184	**0.044**	6(16.2)	12(25.0)	14(25.0)	0.749	0.959
	1–2	25(59.5)	34(56.7)	28(40.6)			26(68.4)	35(68.6)	44(69.8)			28(75.7)	30(62.5)	36(64.3)		
	≥3	0(0.0)	0(0.0)	0(0.0)			5(13.2)	10(19.6)[a]	4(6.3)[b]			3(8.1)	6(12.5)	6(10.7)		
RTI		21(50.0)	24(40)	22(31.9)	0.164	0.337	27(71.1)	39(76.5)[a]	36(57.1)[b]	0.077	**0.031**	26(70.3)	32(66.7)	34(60.7)	0.618	0.530
AOM		0(0.0)	5(8.3)	4(5.8)	0.168	0.733	5(13.2)	6(11.8)	10(15.9)	0.811	0.530	7(18.9)	6(12.5)	7(12.5)	0.630	1.000
Conjunctivitis		2(4.8)	2(3.3)	0(0.0)	0.172	0.214	6(15.8)[b]	1(2.0)[a]	3(4.8)[a,b]	**0.042**	0.627	9(24.3)[a]	4(8.3)[a,b]	3(5.4)[b]	**0.013**	0.701
GII		3(7.1)	8(13.3)	6(8.7)	0.533	0.398	10(26.3)	18(35.3)[a]	11(17.5)[b]	0.095	**0.030**	12(32.4)	17(35.4)	15(26.8)	0.627	0.342
UTI		1(2.4)	1(1.7)	0(0.0)	0.515	0.465	2(5.3)	3(5.9)	0(0.0)	0.086	0.087	0(0.0)	1(2.1)	0(0.0)	0.609	0.462
Allergic reaction		0(0.0)	0(0.0)	1(1.4)	1.000	1.000	2(5.3)	2(3.9)	0(0.0)	0.170	0.198	2(5.4)	2(4.2)	1(1.8)	0.622	0.594
Unspecific fever		1(2.4)	6(10.0)[a]	1(1.4)[b]	0.084	**0.049**	11(28.9)[a]	6(11.8)[a,b]	4(6.3)[b]	**0.005**	0.309	5(13.5)	3(6.3)	10(17.9)	0.137	0.074
Pediatric visit		25(59.5)[a]	31(51.7)[a,b]	24(34.8)[b]	**0.026**	0.053	30(78.9)	45(88.2)	47(74.6)	0.186	0.067	31(83.8)	35(72.9)	41(73.2)	0.425	0.973
Hospitalization		0(0.0)	0(0.0)	1(1.4)	1.000	1.000	3(7.9)	3(5.9)	0(0.0)	0.064	0.087	0(0.0)	0(0.0)	1(1.8)	1.000	1.000
Antibiotics		2(4.8)	9(15.0)	4(5.8)	0.105	0.083	13(34.2)	22(43.1)	23(36.5)	0.651	0.472	15(40.5)	19(39.6)	19(33.9)	0.764	0.551

Data are presented as n (%).
Values not sharing same suffix (ab) are significantly different in a Bonferroni *post hoc* test. *P*-values < 0.05 are highlighted in bold.
BF: Breastfed infants; SF: Standard infant formula; EF: Experimental infant formula; RTI: Respiratory tract infections; AOM: Acute Otitis Media; GII: Gastrointestinal infections; UTI: Urinary infections.
[1] *P*-values are comparisons between COGNIS-groups. Chi-square or Fisher test for categorical variables.
[2] *P*-values are comparisons between SF and EF COGNIS-groups. Fisher test for categorical variables.

et al., 2012). Similar results were also reported with prebiotic supplementation as the one employed in the current study. Arlsanoglou et al. showed beneficial effects of a mixture of neutral scGOS/lcFOS during first 6 months of life in infants, showing a reduction in respiratory infections and antibiotic prescription compared to SF fed infants (Arslanoglu et al., 2007). Similar findings of protective effect of prebiotics have been also reported (Bruzzese et al., 2009). A review on prebiotics supplemented infant formula pointed out to their ability to modify gut flora enhancing Bifidobacteria growth in a dose-related way, and lowering GII and upper RTI incidence (Moreno Villares, 2008).

Recently, there is a focus on beneficial effects of bovine MFGM components to lower the incidence of diarrhea, otitis media and fever episodes (Hernell et al., 2016). Timby et al. showed a protective effect of MFGM components on AOM in infants at 6 months of age (Timby et al., 2015). In our study, there were no significant differences regarding AOM incidence, neither significant difference on antibiotic use, similarly as previously reported (Cohen et al., 2013). Taking into account that use of antibiotics may correlate to bacterial infections, results from the current study might suggest a higher prevention of viral infections in EF infants' group.

However, there is a debate about if early nutritional intervention is able to induce a priming effect on infant intestinal microbiome, which might be the basis of an improved immune response after ceasing supplementation. On one hand, there is evidence of prebiotic supplementation bifidogenic effects (Knol et al., 2005; Scholtens et al., 2006); some authors claim to attribute a reduction of antibiotic prescription, fever and RTI after 2 years follow-up to early nutritional intervention (Arslanoglu et al., 2008). On the other hand, other studies did not find a long lasting difference after early postnatal probiotic supplementation at 6 years follow-up (Rutten et al., 2015), or an on-going change of host microbiota after a probiotic intervention (Laursen et al., 2017). Results of the current study could not detect, however, a significant on-going effect between 12 and 18 months of life. This may be attributed to a gradual weaning of infant formula at this age. Although changes in children's microbiome are possibly induced by nutritional intervention, these effects seem to be vanishing over time, and external influences overwhelm functional nutrients effects. Consequently, a priming effect from early nutrition cannot be established from current results either. Future studies on this topic should be carried out to confirm this hypothesis.

As shown above, there is evidence of beneficial effects of different bioactive components on infections prevention. Our study employs a novel combination of functional nutrients simultaneously introduced in EF group. Thus, this offers the possibility to study their synergic effects, and would demonstrate a reduction of RTI and GII infection risk by roughly a third in a regression model at 12 months of life. However, it did not allow to determine which specific functional nutrient may be responsible for those changes that lead to modulation of host immunity, and subsequent appearance of infections.

It is important to note that BF group at 12 months showed higher report of symptoms related to infections. In this regard, Timby et al. remarked that interpretation of the comparison between formula fed and breastfed infants have to be made with caution (Timby et al., 2015), as randomization is not possible due to ethical considerations. In our study, baseline differences, especially in social background, were found in the BF group in terms of higher educational level of both parents and higher maternal IQ. This may lead to a better and more accurate infections report in BF group, particularly when there are several months between infection episodes and may also suggest an underestimation of infections incidence in the formula fed groups, where social and intellectual indicators were lower compared to BF group. Moreover, BF mothers are probably more prone to let their children at home during or after mild infections to continue BF, which might explain higher overall rate of kindergarten absenteeism, especially significant after adjusting for confounders. High incidence of conjunctivitis at 12 and 18 months in the BF group might be due to epidemiological factors, as an unusual

Table 4
Analysis of the number of diseases, days with fever, days of infection, days of kindergarten absenteeism and duration of antibiotic administration of the infants participating in the COGNIS project during the first 18 months of life, depending on the study group.

		6 months*	P^1	P^1_{adj}	P^2	P^2_{adj}	12 months†	P^1	P^1_{adj}	P^2	P^2_{adj}	18 months‡	P^1	P^1_{adj}	P^2	P^2_{adj}	Overall**	P^1	P^1_{adj}	P^2	P^2_{adj}
Number of Infectious diseases	BF	0.69 ± 0.69	0.602	0.387	0.169	0.343	1.75 ± 1.54	0.526	0.805	0.378	0.496	1.92 ± 1.40	0.619	0.415	0.962	0.991	4.75 ± 3.56	0.204	0.426	0.093	0.168
	SF	0.70 ± 0.74					1.67 ± 1.21					1.64 ± 1.45					4.92 ± 4.04				
	EF	0.52 ± 0.72					1.4 ± 1.18					1.63 ± 1.59					3.93 ± 3.11				
Days with fever	BF	0.52 ± 1.29	0.880	0.843	0.627	0.796	3.16 ± 3.34	0.083	0.180	**0.031**	0.070	3.14 ± 5.24	0.862	0.834	0.673	0.746	6.82 ± 8.53	0.703	0.589	**0.044**	0.262
	SF	0.80 ± 1.30					3.49 ± 3.81					2.98 ± 3.68					6.54 ± 6.51				
	EF	0.68 ± 1.45					2.17 ± 2.61					2.70 ± 3.12					5.83 ± 6.30				
Days of infection	BF	3.09 ± 4.19	0.480	0.941	0.939	0.897	11.81 ± 11.0[a]	**0.048**	0.098	**0.048**	0.052	7.97 ± 8.39	0.820	0.743	0.561	0.653	23.8 ± 20.8	0.076	0.442	0.496	0.357
	SF	2.84 ± 4.17					10.27 ± 8.43[a]					8.09 ± 8.59					26.1 ± 35.8				
	EF	2.78 ± 5.23					7.00 ± 8.14[b]					7.12 ± 7.97					16.6 ± 18.3				
Days of kindergarten absenteeism	BF	0.10 ± 0.43	0.321	0.449	0.773	0.941	2.22 ± 5.28	0.809	0.977	0.768	0.806	2.86 ± 4.60	0.370	0.372	0.179	0.180	5.03 ± 8.54[a]	0.250	**0.045**	0.372	**0.019**
	SF	0.17 ± 0.74					1.84 ± 4.56					1.73 ± 3.65					2.61 ± 6.16[a]				
	EF	0.13 ± 0.68					1.60 ± 4.12					3.04 ± 6.04					3.69 ± 8.42[a]				
Days of antibiotic administration	BF	0.33 ± 1.51	0.602	0.280	0.123	0.183	3.55 ± 6.27	0.321	0.297	0.235	0.326	3.97 ± 5.66	0.996	0.988	0.948	0.958	7.62 ± 9.19	0.256	0.116	0.145	0.075
	SF	0.97 ± 2.34					5.94 ± 11.56					4.08 ± 5.85					10.6 ± 18.1				
	EF	0.41 ± 1.65					3.90 ± 6.20					4.00 ± 7.07					7.09 ± 10.7				

Data are presented as mean ± SD. P^1: *P*-values for overall differences between COGNIS-groups. ANOVA for normally distributed variables. P^1_{adj} are univariate analysis of covariance (ANCOVA) adjusted by maternal IQ and educational level, and paternal educational level. Values not sharing same suffix (ab) are significantly different in a Bonferroni *post hoc* test.
P^2: *P*-values for differences between SF and EF groups. T-test for normally distributed variables. P^2_{adj} are univariate analysis of covariance (ANCOVA) between SF and EF groups, adjusted by maternal IQ and educational level, and paternal educational level. P-values < 0.05 are highlighted in bold.
* BF n = 42; SF n = 60; EF n = 69; † BF n = 38; SF n = 51; EF n = 63; ‡ BF n = 37; SF n = 48; EF n = 56. BF: Breastfed infants; SF: Standard infant formula; EF: Experimental infant formula.
** Overall uses Longitudinal prevalence (LP) calculated by dividing the number of days with reported symptoms/medication by the total number of registered months (3, 4, 6, 12 and 18 months of life).

Table 5
Association of type of early feeding, and attendance to kindergarten and risk of suffering respiratory tract infections (RTI) and gastrointestinal infections (GII) at 6, 12 and 18 months of life.

	RTI		GII	
	OR (95% CI)	P^1	OR (95% CI)	P^1
6 months of life				
Attendance to kindergarten	14.812 (3.137–69.947)	**0.001**	N/A	N/A
Maternal educational level (Primary)	0.735 (0.157–3.446)	0.696	6.499 (0.700–60.355)	0.100
Paternal educational level (Primary)	2.476 (0.645–9.509)	0.187	1.814 (0.151–21.804)	0.639
Breastfeeding after delivery (No)	0.951 (0.335–2.693)	0.924	0.279 (0.035–2.240)	0.230
EF group*	0.765 (0.326–1.795)	0.539	0.497 (0.154–1.600)	0.241
12 months of life				
Attendance to kindergarten	3.417 (1.281–9.117)	**0.014**	2.561 (1.036–6.331)	**0.042**
Maternal educational level (Primary)	0.387 (0.114–1.319)	0.129	1.088 (0.214–5.532)	0.919
Paternal educational level (Primary)	0.435 (0.090–2.088)	0.298	1.798 (0.444–7.289)	0.411
Breastfeeding after delivery (No)	2.652 (0.757–9.289)	0.127	0.484 (0.149–1.568)	0.226
EF group*	0.366 (0.145–0.918)	**0.032**	0.325 (0.130–0.811)	**0.016**
18 months of life				
Attendance to kindergarten	2.565 (1.120–5.876)	**0.026**	2.246 (0.909–5.550)	0.080
Maternal educational level (Primary)	1.074 (0.217–5.315)	0.129	1.400 (0.262–7.490)	0.694
Paternal educational level (Primary)	1.644 (0.490–5.517)	0.421	1.617 (0.464–5.631)	0.450
Breastfeeding after delivery (No)	0.824 (0.288–2.360)	0.719	0.552 (0.190–1.599)	0.273
EF group*	0.581 (0.242–1.393)	0.233	0.673 (0.282–1.608)	0.373

CI: Confidence Interval; EF: Experimental infant formula; GII: Gastrointestinal infections; N/A: Not available; OR: Odds Ratio; RTI: Respiratory tract infections.
[1] *P*-values were obtained by logistic regression analysis (Wald method). Variables included in the model were: maternal and paternal educational level, breastfeeding after delivery, COGNIS study groups and attendance to kindergarten. P-values < 0.05 are highlighted in bold.
[*] SF group as reference.

accumulation of cases happened within a 3 months period of follow-up where mainly breastfed infants were recalled.

Beside nutritional intervention, other environmental factors shall influence on infections incidence in infants. In this sense, we found a strong relation between the presence of RTI and GI and kindergarten attendance, a factor that obviously lead to an increased exposition to common infant infections (Nicolai et al., 2017).

The COGNIS study is a RCT with nutritional intervention in infants and long-term follow-up up to 18 months, thus giving intrinsic strength to this study. Our investigation employed a novel formula enriched with diverse functional nutrients, with each of them individually being able to contribute to beneficial effects observed on infections incidence, and now investigated together. Another strength of our study was an extensive baseline data collection, which permitted adjusting for different confounder factors. However, further studies are needed to understand better dose and duration of probiotic supplementation (Hao et al., 2015), specific function of prebiotics or complex structures like MFGM (Fewtrell, 2015), as well as specific contribution of individual nutrients and their synergic effects.

Nevertheless, there are some limitations of study design and implementation which have to be considered when interpreting results. As mentioned above, beside nutritional intervention, other factors do interfere with immunity development for which we could not account, as for instance family lifestyle, hygienic conditions or complementary feeding, although a common pattern of complementary feeding based on Spanish Association of Pediatrics recommendations was handed out to all study participants. On the other hand, especially common childhood infections may be taken more or less seriously, which biases parents' accuracy reports. Although follow-up visits were carried out by a specialized pediatrician, infections record was based on caregiver's collaboration and accuracy. Analysis of biological biomarkers like changes in stool microbiota, or saliva levels of secretory immunoglobulin A may serve to overcome this limitation. Regarding dropout rate, it could compromise capability identifying subtle differences, a hazel of many clinical trials.

5. Conclusion

In summary, the unique combination of functional nutrients tested in the current study seem to prevent typical infection episodes in infants during their first 12 months of life, probably through modulation of host immune response in a positive way. Nevertheless, effects derived from early nutritional intervention could not be detectable later on.

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Ethical approval

This study was carried out in accordance with ethical standards established by the Declaration of Helsinki 2004 (World Medical Association General Assembly, 2004), the EEC Good Clinical Practice recommendations (document 111/3976/88 July 1990) and the Spanish legislation governing clinical research in humans (Royal Decree 561/1993 on clinical trials). All procedures were also approved by the Ethical Committee of the University of Granada, the Bioethical Committees for Clinical Research of the Clinical University Hospital San Cecilio and the Mother-Infant University Hospital of Granada, as well as respective Ethical Committees of the Health District of Granada, Spain. All families were informed about all procedures during the follow-up and a signed informed consent was obtained from each parent or legal guardian before child enrolment in the study.

CRediT authorship contribution statement

Florian Herrmann: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Ana Nieto-Ruiz:** Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Natalia Sepúlveda-Valbuena:** Methodology, Investigation, Writing - review & editing. **M. Teresa Miranda:** Formal analysis, Writing - review & editing. **Estefanía Diéguez:** Writing - review & editing. **Jesús Jiménez:** Funding acquisition. **Roser De-Castellar:** Funding acquisition. **María García-Ricobaraza:** Writing - review &

editing. **José Antonio García-Santos:** Writing - original draft, Writing - review & editing. **Mercedes G. Bermúdez:** Writing - original draft, Writing - review & editing. **Cristina Campoy:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Jesús Jiménez and Dr. Roser De-Castellar are employees of Laboratorios Ordesa, S.L. Barcelona, Spain. All the other authors declare that they do not have any conflict of interest.

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