

The human milk probiotic *Lactobacillus fermentum* CECT 5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. A Randomised Controlled Trial comparing a prebiotic containing follow-on formula vs the same formula plus probiotic.

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This work has been supported by Puleva Food S.L. own funds.

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This work has been supported by Puleva Food own funds.

Key words: *Lactobacillus fermentum*, Probiotics, Infection, infants, follow-on formula.

ABSTRACT

Objectives: To examine the effects of a follow-on formula containing *Lactobacillus fermentum* CECT5716 (*L.fermentum*) on the incidence of infections in infants between the ages of 6 to 12 months.

Methods: A randomized double blinded controlled study including infants at the age of 6-months was conducted. Infants were assigned randomly to either follow-on formula supplemented with *L.fermentum* plus GOS (experimental group, EG), or the same formula supplemented with only GOS (control group, CG). The main outcome was the incidence of infections for the 6-months duration of the study.

Results: The EG showed a significant 46% reduction on the incidence rate (IR) of gastrointestinal infections, (EG: 0.196 ± 0.51 , CG: 0.363 ± 0.53 , IR ratio = 0.54, 95% CI: 0.307-0.950, $P=0.032$), 27% reduction on the incidence of upper respiratory tract infections (EG: 0.969 ± 0.96 , CG: 1.330 ± 1.23 , IR ratio = 0.729, 95% CI: 0.46-1.38, $P=0.026$), and 30% reduction in the total number of infections (EG: 1.464 ± 1.15 , CG: 2.077 ± 1.59 , IR rate ratio = 0.70, 95% CI: 0.46-1.38, $P=0.003$), at the end of the study period compared with CG.

Conclusions: Administration of a follow-on formula with *L.fermentum* CECT5716 may be useful for the prevention of community-acquired GI and upper respiratory infections.

INTRODUCTION

Infectious diseases are the most common type of illness for infants worldwide. In Spain, data from 2007 show that of all hospital admissions due to infectious diseases, the admission of infants below 1-year of age represent more than 50% of the total, with an average of 2174 cases per 100.000 people (1). Breastfed children have a lower incidence of infections than formula-fed children, which could be probably mediated in part through modulation of the intestinal microflora by breast milk components (2). Indeed, breastfed infants seem to develop a gut microflora richer in lactobacilli and bifidobacteria with reduced pathogenic bacteria compared with formula-fed infants (3). Due to its many benefits, exclusive breastfeeding for the first six months of life is the recommended way of feeding infants (4). However, when breastfeeding is not possible or insufficient, infant formula represents an alternative aimed to partially imitate the effects of breast milk. In the last decade, the manipulation of the intestinal microbiota of infants through the administration of probiotic strains has been recognized as a potential application for the treatment and prevention of infectious diseases. In a recent review by the ESPGHAN (European Society of Pediatric Gastroenterology, Hepatology And Nutrition) Committee of Nutrition, it was stated that a few probiotics supplemented to infant or follow on formulae may be associated to a reduction in the risk of non specific gastrointestinal infections and a reduction of the risk of antibiotic use (5 and references therein). However, the scientific evidence supporting the use of probiotics for the prevention of infectious diseases is only emerging. The efficacy of probiotics in the prevention of community-acquired infections in children has been investigated in several RCTs but the results shown are contradictory, showing that, apart from the variability seen between study populations, designs and methodologies, overall the results suggest that effects observed by one probiotic strain cannot be extrapolated to others (6,7).

Prebiotics such as galactooligosaccharides (GOSs) are indigestible nutrients found in human breast milk, that stimulate the growth and metabolic activity of beneficial bacteria in the gut flora which may also produce a direct immunologic effect (8,9).

Recently, it has been described that human milk is a source of lactic acid bacteria for the infant gut, which may play an important role in infant protection against infectious agents (10, 11). We identified and selected *Lactobacillus fermentum* CECT5716 (*L.fermentum*) from human breast milk and characterized the safety and the probiotic properties of the strain using in vitro, animal models and RCT in human adults (12-15). Since this probiotic strain is naturally present in human breast milk, we hypothesized that its use in a follow-on formula could benefit the bottle-fed infant. In this RCT we evaluate the effect produced by a follow-on formula containing *L.fermentum* and GOS in comparison to a control follow-on formula with only GOS on the incidence of infections in infants between the age of 6 to 12 months.

METHODS

Study design and protocol

A randomized double blinded controlled study with two study groups was carried out in collaboration with the Pediatrics Department of three Spanish hospitals: University Hospital San Cecilio (Granada, Spain), University Hospital Virgen de las Nieves (Granada, Spain) and “Poniente” Hospital of El Ejido (Almería, Spain). Families that lived in the proximity of the hospitals whose mothers had delivered their babies at the hospital and/or attended regular visits to the pediatrician were considered for the study and contacted by the research nurse during scheduled visits to the hospital. Before the inclusion, infants received a physical examination and their clinical records were consulted for previous diseases and pharmacological treatments. Healthy, 6 month old infants who were exclusively formula-fed were recruited into the study between May 2008 to July 2009 after informed written consent was obtained from the parents or tutors. The exclusion criteria included gastrointestinal disorders (history of chronic diarrhoea or constipation, gastroesophageal reflux), gastrointestinal surgery, cow’s milk protein allergy, metabolic disorders (diabetes, lactose intolerance), immune deficiency, antibiotic prescription 1-week prior to inclusion and previous use of formula containing prebiotics or probiotics.

Sample size was estimated based upon the effects on GI infections. Based on previous data regarding incidence of diarrhea (29), the study was designed to have sufficient power (85%) to detect a 20%

difference between the groups with a 0.05 significance level. The number of infants necessary in each group was 83 infants. However, we recruited 30% more infants to allow for dropouts.

Two hundred and fifteen infants were selected and distributed into two study groups according with a randomization list generated by a computer program (SIGESMU[®]). The formulas administered were standard powdered follow-on with a nutritional composition in accordance with current EU regulations, supplemented with GOS (0.4 g/100 mL) in the case of control group (CG), and with the same amounts of GOS plus *L.fermentum* CECT5716 (*Lactobacillus fermentum* Hereditum[®], Biosearch Life, Granada, Spain) at an average dose of 2×10^8 cfu per day in the case of the experimental group (EG). The concentration of the probiotic in the formula was analysed and confirmed every two months. Both formulas were consumed during the 6 month intervention period. The follow-on formulas were provided by Puleva Food SL (Granada, Spain) in identical containers labelled in plain white with a code number that referred to the study groups. In order to ensure the blinding of the trial both formulas were submitted to a sensorial test by an expert panel that find both products to be identical. The paediatricians prescribed the amounts of formula per day to be administered to the infants. Parents received general guidelines for complementary feeding according with current ESPGHAN guidelines (16). Infants were scheduled to receive a clinical evaluation at baseline at the age of 6 month (T0), after 3 months at the age of 9 month (T3) and after 6 months at the age of 12 months (T6). Faecal samples were also collected at T0, T3 and T6. The formulas of the study were home-delivered every two months and the emptied containers were also collected. Exclusion criteria during the study were lack of compliance with the study protocol, adverse effects derived from the consumption of any of the formulas of the study and do not attend scheduled visits to the hospital.

This study was carried out according to the Helsinki declaration, and the protocol was approved by the Regional Ethics Committee of the Sistema Andaluz de Salud based in Seville (Spain). The trial is registered in the registry sponsored by the US Library of Medicine (www.clinicaltrial.gov) with the number NCT01215656.

Study Outcomes and data collection

The primary outcome of the trial was the incidence of infections, including gastrointestinal, respiratory, otitis, urinary and other less common infections. Secondary outcomes were evolution of weight, length and head circumference, fever episodes, antibiotic prescriptions, and concentrations of short chain fatty acids (SCFA), IgA and microbiota composition in feces. The incidence of recurrent (defined as three or more events) respiratory infections was also considered a secondary outcome.

The diagnosis of infectious diseases was made by the paediatrician in every visit based on specific symptoms and standardized definitions. Gastrointestinal infection was defined as loose or watery stools at least four times per day with or without fever or vomiting. Upper respiratory tract infections were defined as the presence of abundant mucosity and/or cough during two or more consecutive days with or without fever, including common cold, laryngitis, pharyngitis/tonsillitis, laringotracheitis, acute rhinitis and acute rhinosinusitis. Lower respiratory tract infections were defined as mucosity and/or cough during two or more consecutive days with or without fever and presence of wheezing and/or crepitations, including acute bronchitis, bronchiolitis and pneumonia. Otitis was defined by the following criteria: otalgia, expressed in the infant as unexplained irritability or rude awakening and recent otorrhea or a bulging eardrum with or without strong redness. Urinary tract infections were diagnosed by fever $\geq 38^{\circ}\text{C}$, pyuria (2 concordant consecutive test results with white cell counts $\geq 25/\text{mL}$), and urine culture (2 concordant consecutive tests with growth of only 1 microorganism ≥ 100000 colony-forming units/mL).

Under other infections we included chickenpox, conjunctivitis, oral candidiasis, Epstein Barr virus, Herpes virus, and fever episodes of unknown origin.

Three types of questionnaires were used in the study: 1) scheduled visit questionnaire, completed by the paediatricians during the scheduled visits (T0, T3, T6), included study parameters and events related to the health behaviour of the infant; 2) parents diary and 15-day questionnaires, completed by the parents, where information regarding daily number of depositions, respiratory symptoms, unscheduled visits to the doctor, diagnosed infections, sleeping and crying habits, changes in sleeping pattern, fever episodes, gastrointestinal discomfort and prescription of antibiotics were recorded. The diaries were used as a

reference to fill the 15-day questionnaires in which the relevant information from the dairies was summarised. The parents were given instructions on completing the questionnaire and were encouraged to contact the research staff if necessary; 3) occasional questionnaires, completed by the paediatricians during unscheduled visits due to a suspected infection or a health problem. In the case of an emergency room visit a copy of the patient's report was sent to the pediatrics for inclusion in the research file.

Faecal bacteria quantitation

Faecal samples were homogenized individually in a peptone-saline solution (100 mg/mL). To estimate the concentration of bacterial groups, appropriate dilutions were spread in quadruplicate onto plates of MRS agar for lactic acid bacteria, MRS agar supplemented with 0.5mg/L dicloxacilin, 1 g/L LiCl and 0.5 g/L L-cysteine hydrochloride for bifidobacteria, Reinforced Clostridial Agar containing 20 µg/mL of polymyxina for clostridia and Bile Aesculin Agar for bacteroides. All media were obtained from Oxoid (Basingstoke, UK) whereas antibiotics and other supplements were obtained from Sigma Chemical Co. (St Louis, Mo). Culture plates were incubated in anaerobiosis at 37 °C for 24 to 48 h. Similarly, 1mL of each suitable dilution was spread onto specific Count Plates Petrifilm (3M St Paul, MN) for total aerobes. Petrifilms were incubated in aerobiosis at 37°C for 24 h. After incubation, colonies grown on the selective culture media were counted and logarithm of the numbers of viable microorganisms per gram of feces (cfu/g) were calculated and represented as the average ± SEM.

Short chain fatty acids quantitation

Faecal samples were homogenized with 150 mM NaHCO₃ (pH 7.8) (1:5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37°C and stored at -80°C until the extraction. To extract the SCFAs, 50 µL of 100 mM 2-methylvaleric acid (internal standard), 10 µL of sulfuric acid and 0.3 mL of ethyl acetate were added to 1 mL of the homogenate. The mix was centrifuged at 10,000 × g for 5 min at 4°C. The supernatants were dehydrated with sodium sulphate (anhydrous) and centrifuged 10,000 × g for 5 min at

4°C. Later, the sample (0.5 mL) was splitless inoculated into a gas chromatograph (mod. CP-3800, Varian, Lake Forest, CA) equipped with an ID (CPWAX 52CB 60 m × 0.25 mm), and connected to a FID detector (Varian). Helium was used as the carrier and the make-up gas, with a flow rate of 1.5 mL/min. The injection temperature was 250°C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of the resulting peaks using the Star Chromatography WorkStation program (version 5.5), which was connected on-line to the FID detector.

Faecal IgA quantitation

Faecal samples were homogenized individually in a peptone-saline solution (100 mg/mL) and centrifuged at 10,000xg during 5 minutes at 4°C. IgA concentration was measured in the supernatants by ELISA quantitation kits, following manufacturer's instructions (Bethyl, Montgomery, TX).

Statistical analysis

Data were analysed with statistical package STATA 11.1 (Stata Corp LP, College Station, Texas) by a blinded statistician. Poisson multilevel regression analysis used the scheme of fixed effects/random intercept considering two levels of data organization: the fixed effects factors were the group (control and experimental) and the period (0-3months, 3-6 months) and the random effect factor were the child nested in the group and the measures of the period nested in each child. The incidence rate ratios were computed as measures of the effect when used number of events as the dependent variable. All the data about infections occurred during the period 0-6 months were pooled and analysed by classical Poisson regression and classical logistic regression were computed for the corresponding dependent variables. Incidence rates were computed as the number of diagnoses (numerator) divided by the person-time contributed. The Incidence Rate Ratios (IRRs) with 95% confidence intervals and *p* values were the outputs of the analysis.

When the variables were numeric the multilevel linear regression model was used as in the case of previous one. Results coming from the different centers were also treated separately and compared and no statistically significant differences were found (data not shown).

RESULTS

Population

Of the 215 infants randomized in two study groups, 27 were excluded from the trial: 7 in the control group and 20 in the experimental group for the following reasons: three infants moved out of the study area (1 in CG and 2 in EG), seven infants did not receive the probiotic experimental formula and the mistake was detected after they did not attend first medical visit, 7 infants were excluded after study termination due to incomplete data collection (2 in group CG and 5 in group EG) and 10 infants did not attend baseline medical visits (4 in group CG and 6 in group EG). Thus, the total number of volunteers analyzed (per protocol) was 188, of whom 91 were in the group CG and 97 in the experimental group EG. A flow chart of participants in the study is shown in Figure 1. Before inclusion, none of the recruited children have consumed a probiotic formula, since at the moment of recruitment there were no infant formula (from 0 to 6 months) supplemented with probiotics in the Spanish market. No statistically significant differences were observed between the groups in any of the baseline parameters analyzed (Table 1).

Five paediatricians participated in the recruitment and monitoring of the children (JM, FC, FV, ARS, EN) in representation of three centers, Hospital Virgen de las Nieves (Granada, Spain), Hospital Clínico (Granada, Spain) and Hospital de Poniente (Almería, Spain). The number of infants recruited and followed by each center was respectively 68, 64 and 56.

Formula intake and tolerance and growth.

Both study formulas were well tolerated and compliance was good. No adverse effects related with the consumption of the formulas (including upper gastrointestinal symptoms, such as spitting up) were reported and none of the volunteers abandoned the trial during the intervention period. No significant differences were found between the study groups regarding daily intake of formula (CG: 741 ± 146 mL/day vs. EG: 732 ± 150 mL/day, Table 1). Baseline values showed significant differences between groups for length ($P=0.047$) and head circumference ($P=0.029$) as a result of the randomization. However,

no differences were found for weight, length, head circumference and growth rate between the study groups at times T3 and T6, indicating that the consumption of the study formula was safe (Table 2).

Infants' health.

During the 6 months intervention, 72.5% of the infants suffered from respiratory infections, 15.7% from GI infections, 5.7% otitis, 1.8% urinary infections and 4.2% from other infections as defined in the methods section (Table 3).

The experimental group showed a significant 46% reduction on the incidence rate (IR) of GI infections (IR: 0.196 ± 0.51) compared to control (IR: 0.363 ± 0.53) at the end of the study period (IR ratio = 0.54, 95% CI: 0.307-0.950, $P=0.032$). Regarding total respiratory infections, the experimental group showed a significant 26% reduction ($P=0.022$) on the incidence rate (IR: 1.093 ± 1.09) compared with the control (IR: 1.473 ± 1.31) at the end of the period (IR ratio = 0.74, 95% CI: 0.580-0.957, $P=0.022$). 90% of the total respiratory infections were infections of the upper respiratory tract. We observed a 27% reduction on the incidence of upper respiratory infections in the experimental group compared to controls (IR ratio = 0.729, 95% CI: 0.46-1.38, $P=0.026$), with no significant difference regarding the rate of lower respiratory infections. The experimental group also reduced the incidence of recurrent respiratory infections by 72%.

No significant differences were found for the incidence rates of otitis, urinary tract and other infections possibly due to the low number of events obtained in both study groups. When the total number of infections were analyzed, the experimental group showed a significant 30% reduction on the incidence rate (IR: 1.464 ± 1.15) compared with the control (IR: 2.077 ± 1.59) at T6 (IR rate ratio = 0.70, 95% CI: 0.46-1.38, $P=0.003$).

Regarding antibiotic treatment and the number of fever episodes, no significant differences between study groups were observed. Events of diarrhea associated with antibiotic treatments were detected in 17.5% of control infants vs. 9.6% of infants in the experimental group (not significant, $P=0.234$).

Faecal parameters.

Faecal samples from only 145 infants (80%) at T0, T3 and T6 reached the laboratory in good conditions and were analyzed. Regarding the intestinal microbiota (Table 4), bacterial counts of both lactobacilli and bifidobacteria were significantly higher ($P=0.008$ and $P=0.022$, respectively) in the experimental group compared to controls at the end of the study. However, within-group comparisons T0 vs. T6 only showed increasing trends in the experimental group for both lactobacilli and bifidobacteria and decreasing trends in the control group. No significant differences were observed for the other bacterial groups analyzed, and, also, no significant differences were observed between both groups in faecal SCFAs such as butyric, propionic and acetic acids. Finally, the concentration of IgA in faecal samples did not change throughout the study in either group.

DISCUSSION

We have shown that infants receiving a follow on formula enriched with *L.fermentum* had a reduction of gastrointestinal and respiratory infections. The study of the effects of *L.fermentum* administered to infants has been carried out for the first time. *L.fermentum* was chosen due to its natural presence in human breast milk (10). This strain is also able to colonise the mammary gland when administered to lactating mothers in a capsule, compared with controls (15). Apart from the origin of *L.fermentum* and its ability to colonise the human gut (14), this strain was selected due to its safety (17,18), anti-infectious (19) and immunomodulatory (20) properties. In addition, two RCTs in adults have shown the ability of *L.fermentum* to reduce the incidence of episodes associated with influenza when administered prior and after vaccination (14), and to be an efficient alternative to the use of antibiotics for the treatment of infectious mastitis during lactation (15). To investigate the health benefits of *L.fermentum* in healthy children we designed this trial. A standard follow-on formula containing moderate amounts of GOS for both groups were used, aiming to theoretically enhance the effects of the probiotic strain, knowing that GOS has been described to stimulate the growth and activity of beneficial gut flora (21, 22). Results indicate that the infants receiving the experimental formula containing *L.fermentum* had a reduced incidence of gastrointestinal infections,

respiratory infections including upper respiratory infections and fewer infectious diseases overall at the completion of the study, compared with the infants fed the control formula.

The recruitment lasted 13 months thus including those seasons during which the rates of respiratory and GI infections are high. The definition used to qualify for a GI infection (loose or watery stools ≥ 4 times/day) was stricter but in line with the WHO definition of diarrhea (23) or the ESPGHAN definition of acute gastroenteritis (24) and has been extensively used in clinical trials investigating prevention of diarrhea (25). Using this definition, the GI infection rates obtained in both study groups are in agreement with the incidence rates reported for infants of this age group in the south of Spain which vary between 0.4-0.6, with an average of 0.47 episodes/year (26). The rates obtained in the trial for respiratory infections were also in agreement with reported values for infants of this age (1).

The rate of reductions in GI infection observed in the experimental group is comparable to other trials that reported a successful prevention of community-acquired GI infections or diarrhea episodes using a probiotic infant formula. For example, the administration of a formula containing either 10^7 cfu/g of *Bifidobacterium lactis* or 10^7 cfu/g of *Lactobacillus reuteri* to children of 4-10 months of age over a 3-month period reduced the numbers of diarrhea episodes compared to the control by 58% and 92%, respectively (7). In another study using a formula containing 2×10^7 cfu/g of *Bifidobacterium lactis* and 14 mg/g of a prebiotic blend, the rate of diarrhea was reduced by 20% compared with a control (27). A recent large trial showed that the administration of 10^7 cfu/g of *Bifidobacterium longum*, 10^6 cfu/g *Streptococcus thermophilus* and 28 mg/g FOS over a period of 3 months reduced the incidence of GI infections by 50% (28).

This trial showed a modest 26% reduction in the total number of respiratory infections. This finding applies only to upper respiratory tract infections, perhaps due to the low number of infections affecting the lower respiratory tract (12 in experimental group and 13 in control group).

Three studies have also shown reductions of respiratory infections with the administration of probiotic strains to infants. A RCT with 571 children between 1 to 6 years of age, administration of *Lactobacillus GG* over a period of 7-months resulted in a 17% reduction in the number of children suffering from

respiratory tract infections with complications (29). Another RCT with 281 children (age range 1-7 years), administration of *Lactobacillus GG* for 3-months resulted in a 33% risk reduction in upper respiratory tract infections compared with the control (30). Results of a recent RCT with 69 newborn infants indicate that the administration of *Bifidobacterium animalis* BB-12 reduced the numbers of respiratory infections by approximately 30% compared with the control (6). Additional studies reported either trends (31) or no effects at all (7, 28, 29).

Feces samples were analyzed to investigate the possible mechanism responsible for the reduction in GI infections. Although the production of SCFA and IgA did not change during the study, the intake of the experimental formula resulted in a 78% increase of lactobacillus and 70% in bifidobacteria at the end of the study. These changes in the gut flora could at least in part explain the reductions in the number of GI episodes observed in this group.

Interestingly, the population of Bifidobacteria significantly increased in the experimental group in spite of the supplementation with Lactobacilli only. This phenomenon has been observed for other probiotic strains (32, 33). It has been suggested that an increase in Bifidobacteria may be the result of the metabolic activity, nutrient competition and gut cell adhesion rates of Lactobacilli, which may favour the growth of Bifidobacteria. Using in vitro and in vivo studies we have previously shown that *L.fermentum* presents a high cellular adhesion rate, produces a number of antimicrobial substrates, releases significant amounts of the antioxidant glutathione, and is able to synthesise bifidogenic carbohydrates under certain conditions (11, 19). In addition, in vitro studies have shown that *L.fermentum* inhibit the adhesion of certain pathogens bacteria to intestinal mucus and increase the expression of mucines, which could also be involved in the antiinfective effect shown by this probiotic strain (19). Although previous studies have demonstrated that *L.fermentum* is able to colonise the gut (14), *L.fermentum* was not quantified during this trial, thus limiting the interpretation of the results. Another limitation was that the feces of the infants diagnosed for GI infections were not analyzed for single pathogens.

Regarding the mechanism for the effects on respiratory infections, a previous RCT in adults carried out with *L.fermentum* in combination with a flu vaccine showed a significant reduction of influenza-like illness

(including respiratory symptoms) in the experimental group. The reduction was explained by the significant increases in the proportions of Natural Killer cells, T-helper and T-cytotoxic lymphocytes measured in the experimental group compared with the control (14). In the present study we did not observe differences in the fecal concentration of IgA. As no blood samples were obtained from the infants of the study, we can only speculate a stimulation of the immune response as a possible mechanism responsible for the reductions of the respiratory infections. Furthermore, another limitation of this study was the absence of a group exclusively breast fed for comparison.

It has been reported that human milk contains oligosaccharides having anti-inflammatory and anti-infectious properties (34). Since both formulas contained GOS (0.5g/100Kcal), the effects observed in this study cannot be attributed to its GOS content. However, *L.fermentum* and GOS together may have a synergic effect that could be superior to the health benefits of the individual components. It was, however, outside the scope of this study to investigate the potential synergistic effects of the combination of *L.fermentum* and GOS which should be investigated in future studies.

In conclusion, considering the significant decrease in the number of infections, the administration of a follow-on formula enriched with *L.fermentum* may be useful for the prevention of community-acquired GI and upper respiratory infections in infants. Additional controlled clinical studies investigating the effects of *L.fermentum*, alone or in combination with other strains, in different settings, using biomarkers and infants of different age groups are needed before recommendations can be made.

Acknowledgements

Authors are very grateful to Juan de Dios Luna (PhD) from the Biostatistics Department of the Universidad de Granada for his helpful contribution to the statistical analysis of data and to randomization of the infants to the different interventions groups.

Authors are also grateful to Puleva Food as supplier of the follow on formula used in the trial and to Biosearch Life S.A. (former Puleva Biotech S.A.) as supplier of the probiotics.

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Figure 1 legend. Flow chart of participants.

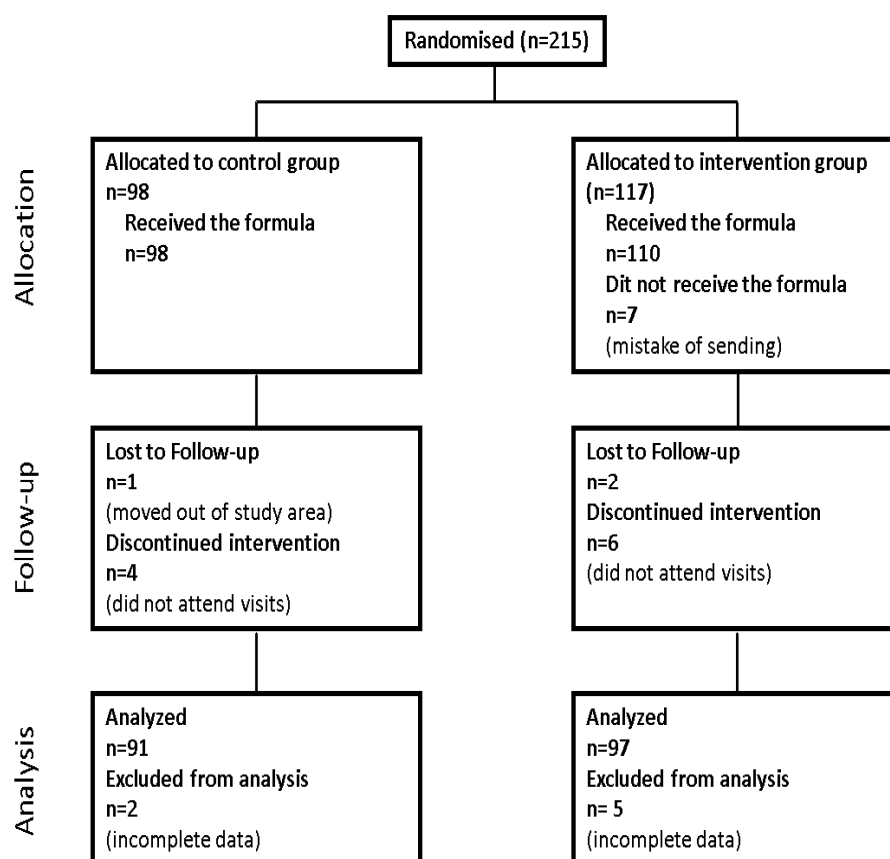


Table 1: Baseline characteristics of the subjects participating in the study.

	<i>Control group</i> (n=91)	<i>Experimental group</i> (n=97)
Male/female, n (%)	40/51 (44/56)	54/43 (56/44)
Age at enrolment (months), mean±SD	6.5±1.3	6.5±1.2
Delivery by cesarean (%)	45	45
Gestational age (weeks) mean±SD	38.9±1.4	38.8±1.4
Breastfed before the study period (%)	71%	69
Total duration exclusive breastfeeding (months-range)	2.8 (0.5-6.5)	3.0 (0.5-6.5)
Daily volume of follow-on formula prescribed (mL) mean±SD	741±146	732±150
Complementary feeding (%)	100	100
Rotavirus vaccination (%)	75	74
Day care or child minder (%)	25	27
Pets at home (%)	13	16
Smoking during pregnancy or lactation (%)	21	21
Smoking in the household (%)	37	38

Table 2: Anthropometric measurements at baseline, T3 and T6 months and gain T0-T6 of infants receiving either control or probiotic formula. Values are means \pm SEM. *, $p < 0.05$ versus control

<i>Growth parameters</i>	<i>Control group</i>				<i>Experimental group</i>			
	T0	T3 months	T6 months	Gain	T0	T3 months	T6 months	Gain
<i>Weight (kg)</i>	7.8 \pm 1.0	9.2 \pm 1.58	9.9 \pm 1.5	2.3 \pm 0.7	7.4 \pm 1.0	8.8 \pm 1.1	9.7 \pm 1.5	2.4 \pm 0.7
<i>Length (cm)</i>	66.6 \pm 3.2	71.5 \pm 2.8	75.2 \pm 3.0	8.8 \pm 2.4	65.6 \pm 3.3*	70.2 \pm 4.0	74.3 \pm 3.8	8.7 \pm 2.3
<i>Head circumference (cm)</i>	43.7 \pm 1.4	45.7 \pm 1.4	47.1 \pm 1.4	3.4 \pm 0.9	43.2 \pm 1.4*	45.4 \pm 1.4	46.6 \pm 1.6	3.5 \pm 1.0

Table 3: Incidence of infectious disease, febrile episodes and antibiotic treatment during the intervention period.

	<i>Control group(n=91)</i>		<i>Experimental group(n=97)</i>		Incidence Rate Ratio (95% CI)	Incidence rate decrease (%)	p value
	N° events	Incidence Rate (SD)	N° events	Incidence Rate (SD)			
<i>Gastrointestinal infections</i>	33	0.363 (0.53)	19	0.196 (0.51)*	0.54 (0.31-0.95)	46	0.032
<i>Respiratory infection</i>	134	1.470 (1.31)	106	1.093 (1.00)*	0.74 (0.58-0.96)	26	0.022
<i>Upper Respiratory</i>	121	1.330 (1.23)	94	0.969 (0.96)*	0.73 (0.56-0.95)	27	0.021
<i>Lower Respiratory</i>	13	0.143 (0.35)	12	0.124 (0.33)	0.87 (0.40-1.90)	13	0.719
<i>Otitis</i>	12	0.132 (0.34)	7	0.072 (0.26)	0.55 (0.22-1.32)	45	0.177
<i>Urinary tract infections</i>	5	0.055 (0.22)	1	0.010 (0.10)	0.19 (0.02-1.56)	81	0.083
<i>Other infections^a</i>	5	0.055 (0.22)	9	0.093 (0.29)	1.69 (0.50-1.85)	-69	0.326
<i>Total infections</i>	189	2.08 (1.59)	142	1.46 (1.16)*	0.70 (0.57-0.88)	30	0.002
<i>Febrile episodes</i>	78	0.857 (0.90)	67	0.690 (0.88)	0.81 (0.68-0.94)	19	0.203
<i>Antibiotic treatments</i>	57	0.626 (0.90)	52	0.536 (0.70)	0.86 (0.59-1.24)	14	0.445

^a Other infections include chickenpox, Epstein Barr, Herpes virus, oral candidiasis, conjunctivitis and febrile episodes of unknown origin

Table 4. Intestinal microbiota counts in faecal samples of infants (as logarithm of cfu/g),
faecal concentration of short chain fatty acids (SCFA, as mg/g feces) and IgA (as µg/g feces).

Values are means ± SEM. *, P<0.05 vs Control.

Bacterial group	<i>Control group (n=70)</i>			<i>Experimental group (n=75)</i>		
	T0	T3 month	T6 month	T0	T3 month	T6 month
<i>Lactobacillus spp.</i>	7.85±0.1	7.72±0.1	7.68±0.1	7.81±0.1	7.86±0.1	8.06±0.1*
<i>Bifidobacterium spp.</i>	8.07±0.1	7.84±0.1	7.81±0.1	7.93±0.1	8.06±0.1	8.16±0.1*
<i>Clostridium spp.</i>	7.77±0.1	7.57±0.1	7.54±0.1	7.74±0.1	7.64±0.1	7.61±0.1
<i>Bacteroides spp.</i>	7.64±0.1	7.65±0.1	7.61±0.1	7.86±0.1	7.86±0.1	7.65±0.1
SCFA (mg/g feces)						
<i>Acetate</i>	10.7±0.8	10.0±1.3	10.1±0.8	9.9±1.03	9.6±0.5	11.3±1.1
<i>Propionate</i>	1.85±0.1	2.17±0.2	2.17±0.2	2.20±0.4	2.30±0.4	2.35±0.3
<i>Butyrate</i>	2.15±0.2	2.76±0.4	2.94±0.2	2.53±0.5	3.05±0.3	2.92±0.3
IgA (µg/g feces)	328±244	ND	322±212	329±170	ND	316±242