



Bifidobacterium longum subsp infantis CECT7210 supplemented formula reduces diarrhoea in healthy infants: a randomized controlled trial

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Running title: Less infant diarrhoea by a new probiotic

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Mireia Morera, Jose Antonio Moreno Muñoz and Montserrat Rivero are employees of ORDESA SL. The other authors affirm that there is no potential conflict of interest and that there were no financial ties to products in the study.

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ABSTRACT

Background: Intestinal microbiota of breast-fed infants is plenty of beneficial bifidobacteria. We aimed to determine whether an infant formula supplemented with probiotic *Bifidobacterium longum* subsp. *infantis* CECT7210 (*B. infantis* IM1®) is effective at reducing diarrhoea incidence in healthy term infants. **Methods:** Double-blinded, randomized, multicentre, controlled clinical trial, where formula-fed infants (<3 months) received an infant formula supplemented (Probiotic) or not (Control) with 10^7 cfu/g of *B. infantis* IM1® over 12 weeks. Diarrhoeas, growth, digestive symptoms, stool bifidobacteria and microbiota were assessed. **Results:** 97 (Control) and 93 (Probiotic) infants were randomized, 78 (Control) and 73 (Probiotic) completed the 12 weeks follow-up. In the overall study period, a median of 0.29 ± 1.07 and 0.05 ± 0.28 diarrhoea events/infant was observed in the Control and Probiotic groups respectively ($p=0.059$). This trend to less diarrhoea episodes in the Probiotic group reached statistical significance at 8 weeks (0.12 ± 0.47 vs. 0.0 ± 0.0 events/infant, $p=0.047$). Constipation incidence was higher (OR 2.67 (1.09-6.50)) and stool frequency lower (2.0 ± 1.0 vs. 2.6 ± 1.3 stools/day, $p=0.038$) in the Control group after 4 weeks. No differences were found at other timepoints nor in other digestive symptoms, growth or formula intake. **Conclusions:** A *B. infantis* IM1®-supplemented infant formula may reduce diarrhoea episodes; being safe, well tolerated and associated with lower constipation prevalence.

INTRODUCTION

There is compelling evidence that infant nutrition has a great impact on the bowel microbiota balance and also on the beneficial effects of *Lactobacillus* and *Bifidobacterium* genera (1,2). The microbiota modulates the development of intestinal immunity, and previous studies demonstrated that an increased presence of both *Bifidobacterium* and *Lactobacillus* is associated with a lower incidence of gastrointestinal infections (3,4).

Breast-fed infants intestinal microbiota includes higher proportions of *Bifidobacterium* and *Lactobacillus* than those of formula-fed, who have a less diverse microbiota and an increase of pathogens, such as *Bacteroides*, *Enterobacteriaceae* and *Clostridium* (5). These differences have been associated with higher rates of infectious and non-infectious disease in the latter, suggesting that breastfed infants are better protected against gastrointestinal infections than formula-fed infants. Consistently, Lara-Villosada *et al.* (6) demonstrated an association between higher amount of *Lactobacillus* and lower incidence of diarrhoeain breast-fed infants.

Formula manufacturers aim to offer infant formulas that partially mimic human milk benefits (7), for example adding probiotics.

Previous studies and a systematic review by the ESPGHAN Committee on Nutrition concluded that probiotics supplementation of infant formula has no adverse effects on infant growth (8,9) and may produce benefits, such as the prevention of infections (10,11), allergic disorders (12) and inflammatory bowel intestinal disease in older children (13). Regarding gastrointestinal infections, some studies have reported the benefits of probiotics in reducing their incidence and severity (11,14). However, as these observations were inconclusive, the ESPGHAN Committee on Nutrition found that further studies were necessary(15). Moreover, the ESPGHAN Working group on Probiotics and Prebiotics

showed that the effects of probiotics were strain-specific, and thus, the efficacy, safety as well as recommendations for use should be established of each microorganism (16).

According to these results, the *Bifidobacterium longum* subsp. *infantis* CECT7210 (*B. infantis* IM1®), a new probiotic strain isolated from breastfed infant faeces, has been shown *in vitro* to inhibit rotavirus replication and to protect against virus infection in animal models (17). This result, in addition to previous data about probiotic use, led us to hypothesize that *B. infantis* IM1® could have an impact on immune function by improving both specific biomarkers and infection incidence. Our aim is to determine whether an infant formula supplemented with this probiotic is effective at reducing the incidence of diarrhoea in healthy term infants.

METHODS

Study design

The study was designed, following the CONSORT statement (www.consort-statement.org), as a multicentre, double-blind, randomized, placebo-controlled parallel group trial, where formula-fed healthy term infants (<3 months) received an infant formula supplemented with 10^7 cfu/g of *B. infantis* IM1® (Probiotic) or a standard formula (Control) during 12 weeks with a 1:1 allocation ratio. Study visits were performed at 4, 8 and 12 weeks of intervention. Research staff was blinded to both treatment allocation and data analysis. In addition, participants and caregivers were blinded throughout the study. No changes to methods after trial commencement were included.

Participants

Subjects were recruited at the Hospital Infantil La Paz de Madrid, the Hospital Universitari de Tarragona Joan XXIII and the Hospital Universitari Sant Joan de Reus (Spain). Seven different investigators contacted families at the Hospitals, before the delivery discharge, to

explain the study only if their infants were exclusively formula-fed. The same investigators performed visits at the Hospitals. The inclusion criteria were: to be healthy-term newborns (≥ 37 weeks), with normal birth weight (3rd to 97th percentiles for gestational age), normal growth curve according to current Spanish references (18) and ≤ 3 months of age at enrolment. Moreover, exclusively formula-fed at enrolment, with ≤ 30 days of breastfeeding (stopped at least 15 days before inclusion), ≤ 48 hours of feeding with pre- or probiotic-supplemented infant formulas (with a washout period of 7 days with control formula). Families agreed to exclude any source of pre- or probiotics during the study. The exclusion criteria were: congenital illness or malformations that may affect infant feeding and/or growth, significant pre- or post-natal diseases, family history of atopy, any pathology related with the immune or gastrointestinal system, suspected or known allergy to cow's milk protein, and receiving pre- or probiotics within less than 15 days prior to the enrolment.

Study intervention

Infants received an infant formula supplemented with *B. infantis* IM1® in a dose of 10^7 cfu/g (Probiotic) or without supplementation (Control) (Supplemental Table S1 (online)). Laboratorios Ordesa S.L. (Spain) produced both formulas, in compliance with current EU standards (Commission Directive 2006/141/EC).

Two codes for each study formula were used. The random allocation sequence was generated by ORDESA S.L. (by software in blocs of eight patients) and provided to a study member not involved in recruitment or study visits. Preparation instructions were given to avoid excessive heating of the study formula. Intake of any pre- or probiotic supplemented product or intake of other formulas over more than 10 days was a study discontinuation criteria and were controlled through a food intake questionnaire.

Study outputs

The primary outputs for this RCT were diarrhoea incidence, growth and digestive tolerance. Secondary outputs were other infections, immune system response and microbiota analysis.

Infection incidence

Clinical data were recorded at recruitment, 4, 8 and 12 weeks. A complete examination was performed at recruitment by a paediatrician to detect any physical or system (respiratory, oropharyngeal, ophthalmic and other) anomaly. Infections were assessed in each study visit using a diary record validated by the paediatrician. The record included the presence/absence (yes/no) and number of events of diarrhoea described as 3 or more liquid stools in 24 hours (in infants who do not usually fulfil this deposition pattern), vomiting, respiratory infection (upper and lower tracts), otitis, conjunctivitis and urinary tract infections reported by the parents and confirmed by the paediatrician by assessing the clinical symptoms and prescribed medication. The use any medication was reported in a concomitant medication diary record completed by parents with the physician advice.

Growth and digestive tolerance

Anthropometry was assessed at recruitment, 4, 8 and 12 weeks (± 3 days). The nude weight and length of the infant were determined using a SECA 336 scale (SECA, Hamburg, Germany) (precision: ± 0.05 Kg) and a SECA 232 stadiometer (precision: ± 1 mm), and the body mass index (BMI) was calculated. Head circumference was determined using a SECA non-extendable insertion tape (precision: 1 mm). Z-scores for anthropometric measurements were calculated according to the World Health Organization Standards (WHO) using the WHO software (19). Parents recorded during three days before each visit the presence/absence of digestive symptoms (flatulence, abdominal pain, constipation (defined as less than 1 hard stool/day or less than 1 stool/2 days), and clinical symptoms compatible with the infant colic (defined as incontrollable crying for more than

3 h/day, more than 3 days/week and more than 3 weeks)). The stool frequency (stools/day), stool consistency (according to the Modified Bristol Stool Form Scale for children adapted to types 1-3), vomiting (n/day) and regurgitation (from 0 to 5, from 5 to 15 or >15 events/day) were also recorded. Finally, crying (h/day), sleeping behaviours (sleeping hours and breaks during the night and totals) and the presence of diaper dermatitis were also recorded. Any other adverse event was recorded and classified as mild, moderate or severe.

Feeding data (days of previous breastfeeding, age at formula introduction, type and total volume intake of formula per day (ml/day)) were recorded at recruitment. During follow-up, total volume intake of study formula per day and the use of other formulas were recorded.

Immune system response

Saliva samples were obtained at recruitment and after 12 weeks of intervention. We used specific swabs for saliva collection (Sarstedt, Nümbrecht, Germany). A minimum of 0.5 ml of saliva was needed to perform posterior analyses. When insufficient baseline sample was obtained, the procedure was repeated at posterior follow-up visits. Secreted immunoglobulin A (IgA) (mg/dl) was measured using a commercial enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Bethyl Laboratories; Montgomery). After the ELISA for IgA detection, a second ELISA was performed to confirm that the detected IgA concentration corresponded to the secretory fraction by a monoclonal mouse anti-human secretory component antibody (HyTest Laboratories; Turku, Finland) and the ELISA components used previously.

Rotavirus-specific IgA was also analysed in the saliva samples by an in-house-developed ELISA method as previously described by Colomina *et al* (20).

All the vaccinations received by the infant throughout the study period were recorded.

Microbiota analysis in stool samples

Stool samples were obtained within the two days before study visits, frozen and transported to the study centres with ice pads. The samples were preserved at -80°C until they were shipped to a central laboratory (Biopolis S.L, Valencia, Spain) for their analysis. Total amount of *Bifidobacterium* and *B. infantis* IM1® were determined by Real-Time-PCR using specific primers for genus, (21) and specific primers and probe for *B. infantis* IM1® (with a specificity greater than 95% (data not shown)) (Details are described in Supplemental Table S2 (online)). Total DNA was extracted from faeces using “QIAamp DNA stool mini kit” (Qiagen Inc., Barcelona, Spain) following manufacturers’ manual. A microbiota analysis by massive genome sequencing was performed in a subsample of infants (25 and 26 from Probiotic and Control groups, respectively) using 454 platform (Roche Life Science). DNA was isolated following Yuan et al. (22) with minor modifications by MagnaPure Compact System (Roche Life Science). Libraries were built following Roche recommendations and using Sim and co-workers set of primers (23), specially modified in order to optimize *Bifidobacterium* detection. Sequencing was performed using 454 platform under pattern B flow, following manufacturer’s instructions. The resulting sequences for each patient were treated for quality and the clean sequences were used for taxonomical association using QIIME (24).

Ethics

The study was performed following the Helsinki declaration and guidelines for the ethical conduct of medical research involving children and was approved by the local Ethical Committees involved. All parents received written information and signed informed consent. The trial was registered at Clinicaltrials.gov as NCT02096302 (<https://clinicaltrials.gov/ct2/show/NCT02096302?term=NCT02096302.&rank=1>).

Statistics

Quantitative data were presented as the mean \pm SD. Categorical variables were presented as absolute and relative frequencies (n, %).

We performed a cross-sectional analysis comparing both study groups at individual timepoints (recruitment, 4, 8 and 12 weeks) as well as a longitudinal analysis comparing changes throughout the study. Cross-sectional differences in quantitative variables were analysed by Student's T test or Mann-Whitney U test as appropriate. Frequencies of categorical variables were analysed by Chi square test. When possible, OR (95% CI) for the association between categorical variables were calculated. The longitudinal analysis was performed by two-way ANOVA for repeated measures. In this model, timepoints (recruitment, 4, 8 and 12 weeks) and group (Control vs. Probiotic) were analysed together as the two factors influencing the outcomes. The *p* value for the interaction between both factors showed the probability of a different longitudinal evolution between groups. Multivariate analysis was performed to evaluate the combined effect of *B. infantis* IM1® and vaccinations on the salivary concentration of IgA using a linear regression model. Statistical significance was accepted at the level of 0.05. We used IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM corp). The sample size (93 per group) was calculated, using data reported by Weizman et al. (25) to detect a difference between groups of about 0.033 events/infant in diarrhoea episodes (1/3 of the SD in the control group). We used the Epidat 3.1 program (available at: <http://www.paho.org/spanish/sha/epidat.htm>), an α error of 0.05, a power of 80% and assumed an attrition rate of 20%. R version 3.1.3 was used to assess alpha and beta diversity in the microbiome analysis, as well as to compare different parameters under Wilcoxon non-parametric test.

RESULTS

Study sample and recruitment characteristics

From January 2011 to August 2012, 194 eligible infants were recruited. Among those, four were excluded because of previously undetected exclusion criteria (3 cases) or consent removal. One hundred ninety infants finally started the study (93 and 97 in Probiotic and Control groups, respectively), from which one hundred fifty-one (73 and 78 in Probiotic and Control groups, respectively) completed the 12 weeks of intervention (Figure 1). The distribution of infants into the study groups was similar in all the participating centres ($p=0.764$). The mean age at recruitment was 7.3 ± 13.3 days (with a median and IQR of 3 (2,4) days and a range between 1-56 days or 0-83 days for Probiotic and Control groups, respectively).

Baseline characteristics, such as gender distribution, age at recruitment, anthropometry at birth and at recruitment (Figure 2, a), type of delivery, number of siblings, formula intake, and digestive symptoms were similar among groups (Table 1). Incidence of infant colic was higher in the Control group (9.5% vs. 2.2%, $p=0.034$) (Table 1). There were no differences in the physical and system infants' examinations between groups (data not shown).

Effect of intervention on infections

The incidence of diarrhoea was very low, as only 7 Control and 3 Probiotic infants had events throughout the study period. Although percentage of infants that suffered diarrhoea did not show statistically significant differences between study groups (4.1% in Probiotics vs. 9.0% in Control; $p=0.230$) (OR 0.44 (0.11-1.75)), the number of episodes was significantly higher in the Control group (23 events compared to 4 in Probiotic group). In the overall study period, infants in the Control group showed a median of 0.29 ± 1.07 events/infant whereas those in the Probiotic showed 0.05 ± 0.28 events/infant ($p=0.059$).

This lower prevalence of diarrhoea episodes among the Probiotic group was significant at 8 weeks of intervention (0.12 ± 0.47 events/infant vs. 0 ± 0 events/infant for the Control and Probiotic groups, respectively; $p=0.047$) but did not reach statistical significance at the other timepoints (Figure 3). The incidence of other infections was not significantly different between the study groups. A total of 33.3% of infants in the Control group and 38.5% in the Probiotic had respiratory infections ($p=0.520$) throughout the study period. The other assessed infections had a lower incidence (1.4%, 1.4% and 4.2% for otitis, urinary tract infection and conjunctivitis, respectively) and were equally distributed between the study groups. Concomitant medication diaries showed that 74.6% of the infants did not receive any medication during the study. Oppositely, 7 infants in total received oral antibiotic therapy for any adverse event. Those infants correspond to 3.6% of the sample without differences in distribution between groups (4 in Control group and 3 in Probiotic, $p=0.268$). Moreover, we identified 21 infants whose parents reported the use of medication but we fail to assess the type of medication due to incomplete information in the concomitant medication diary. Those infants correspond to 11% of the sample and were equally distributed between groups (13 in Control group and 8 in Probiotic, $p=0.307$).

Effect of intervention on infant growth, digestive symptoms and formula intake.

There were no significant differences between groups in the z-scores of anthropometric data throughout the study period, and there were no cross-sectional differences upon longitudinal analyses (Figure 2, a). The infants in the Probiotic group had a lower BMI at recruitment compared to those in the Control group but showed similar results at other timepoints (Figure 2).

Deposition frequency was higher in Probiotic group compared with Control (at 4 weeks) ($p=0.038$) (Figure 2, b). Moreover, longitudinal analysis showed that the drop in stool

frequency observed throughout the study follow-up in both groups was lower in the Probiotic group than in the Control ($p=0.046$). Accordingly, the percentage of infants that reported constipation was lower in the Probiotic group (22.6% vs. 9.9% at 4 weeks ($p=0.027$) and 13% vs. 2.8% at 12 weeks of intervention ($p=0.033$) for the Control and Probiotic groups, respectively). The OR for suffering constipation in infants from the Control group compared with the Probiotic was 2.67 (1.09-6.50) and 5.22 (1.10-24.73) at 4 and 12 weeks, respectively. No differences between groups were observed in the usual stool consistency throughout the study (Figure 2, b).

There were no differences between groups in terms of digestive symptoms, behaviour or total daily formula intake throughout the study period (Table 2). We found a significant difference in sleeping hours at night at 8 weeks of follow-up (Table 2). There were no significant differences in the presence of adverse events between study groups nor in the absolute number score of the severity of the adverse event (data not shown).

Effect of intervention on the microbiota in the faeces and immunological parameters

Real-Time PCR results showed no differences in total *Bifidobacterium* levels in the faeces between groups. However, as expected, there was a significant increase of *B. infantis* IM1® counts in the Probiotic group. These differences appeared after 4 weeks of intervention and were maintained throughout the study period (Figure 4, a). This effect was reproduced in the microbiota analysis, where an increase in *B. longum* was detected at final point in the Probiotic group ($p=0.023$), that was not observed in the the Control ($p=0.45$) (Figure 4, b).

Microbiota analysis pinpoint a non-significant decrease in the presence of pathogens (*Escherichia*, *Clostridium*, *Salmonella*, *Campilobacter* and *Yersinia*) in the Probiotic group at 12 weeks compared with those taking the Control formula, which did show equal values

than at baseline. We also observed in the Control group a significant increase of *E. coli* at final timepoint with respect to baseline whereas the presence of the pathogen did not change along the follow-up in the Probiotic group (Supplemental Figure S3 (online)).

An 86.3% of the infants in our sample were vaccinated at least once during the study period, with no differences in distribution between groups. A 34.2% of the participants were vaccinated against rotavirus (1 or 2 doses), also equally distributed between study groups.

Regarding immune markers, both groups showed a non-significant tendency towards an increase in IgA concentrations throughout the study. We also found similar concentrations of IgA among groups (1.24 ± 1.99 vs. 0.78 ± 1.10 mg/dl, $p=0.739$ at recruitment and 2.26 ± 2.34 vs. 2.66 ± 3.85 mg/dl, $p=0.803$ at 12 weeks of intervention; Control and Probiotic groups, respectively). However, using a linear regression model (adjusted by the number of vaccinations received by the infant (range between 0 and 15) to avoid the effect of this confounding factor) we observed that the presence of *B. infantis* IM1® in the faeces modulated IgA concentration at the end of the intervention. The results showed that each \log_{10} cfu/g of *B. infantis* IM1® at 4 weeks of intervention increased IgA concentration by 0.185 mg/dl (95%IC: 0.032, 0.338; $p=0.019$) at the end of the study. The mean difference of *B. infantis* IM1® in the faeces of the Probiotic group infants compared to the Control was approximately 3 \log_{10} , suggesting a possible effect of a 0.555 mg/dl of increase in IgAs.

The results of the rotavirus-specific IgA analysis showed a similar percentage of infants with a positive titre of IgAs in both groups (22.0% vs. 18.5%, for the Control and Probiotic groups at 12 weeks of intervention, respectively; $p=0.648$). We also compared the absolute titres, and we did not find differences between the study groups (nor among all infant samples, neither for subgroups of infants (positive titres or vaccinated against rotavirus)

(data not shown)). The geometric mean titres at 12 weeks of follow-up were 71.2 and 78.8 for the Control and Probiotic groups, respectively.

DISCUSSION

During the last decade, it is known that the human intestinal microbiota can play a major role in modulating health and disease along lifespan. Thus, efforts have been made to modulate this microbiota through different products such as probiotics, aimed to enrich or selectively stimulate the growth and/or activity of beneficial intestinal bacteria.

This is the first multicentre, double-blind, RCT to demonstrate the safety and efficacy of a *B. infantis* IM-1[®]-supplemented infant formula in healthy infants. Although previous studies evaluated the effect of different probiotics on infants (15), the effect of this specific strain has not been assessed yet.

Efficacy

The efficacy of the *B. infantis* IM-1[®]-supplemented formula was demonstrated by the higher presence of the probiotic in stools samples among supplemented infants. In addition, microbiota analysis showed that supplemented formula was associated with constant levels in the Proteobacteria such as *E.coli* in contrast with the increase observed in the Control group. Similar results were observed in breastfed infants, as breastmilk prevents the gut colonization by Proteobacteria; potentially reducing the infection risk (5).

The expected increase of the *B. infantis* IM-1[®] in the faecal microbiota in the Probiotic group compared to the Control one was not associated with a change in *Bifidobacterium* concentration. Others have previously observed that the major bacterial groups proportions were not influenced by probiotic treatment (26–28). Our results coincide with those

previous findings and demonstrate the ability of the supplemented formula to modify the microbiota.

Despite a lower diarrhoea episodes trend among the Probiotic group, results did not show a sustained effect along the study period, probably due to the small diarrhoea incidence among the whole sample. This small incidence of diarrhoea was probably due to the infants' young age (<3 months) and the geographic area incidence of gastrointestinal infections. Early in life, infants are under the protective effect of the placenta transferred maternal antibodies that can protect against the most common germs, and have an effect beyond the first six months of life. Moreover, our infants did not attend childcare, what further reduces infections risk. Even in those protected conditions, we found that infants fed probiotic formula had less diarrhoea episodes compared to control infants at one of the timepoints, suggesting a possible effect of probiotics decreasing the incidence of gastrointestinal infections. However, we did not find a significant effect in terms of OR. Previous studies have reported the effect of specific strains of *Bifidobacterium* on recovery from diarrhoea, especially reducing the duration and severity (29). Prior to our study, Muñoz *et al.* (17) had already demonstrated the ability of the *B. infantis* IM-1[®] to inhibit virus infection in an *in vitro* study as well as to protect against rotavirus infection in a *in vivo* mice model. Our results are consistent with those findings but further studies should find out if *B. infantis* IM-1[®] may also inhibit rotavirus infection in healthy infants.

Regarding other infections, the incidence was similar between groups. The lower incidence of those infections throughout the study period may probably support these results. Consistent with our findings, Gibson *et al.* (30) also found no differences in respiratory infections among supplemented-formula-fed infants.

In parallel to the lower incidence of diarrhoea episodes in supplemented infants, we found a relationship between the *B. longum* IM-1[®] levels in the faeces and the secretory IgA concentration measured at the end of the follow-up. A clear effect of probiotic supplementation was not observed for this immunoglobulin, as concentrations were similar in both groups. However, as the different vaccination patterns in each infant could influence these results, we adjusted the relationship between the *B. infantis* IM-1[®] content and the secretory IgA concentration by the number of vaccines received. Results showed that an increase of the probiotic count in the faeces was associated with higher IgA concentrations, suggesting that the increase in *B. infantis* IM-1[®] is related with the stimulation of the immunologic system function.

Previous studies have observed no changes in faecal secretory IgA after *Bifidobacterium* supplementation (27,31). Holscher *et al.* (31) found that probiotic supplementation induced a significant increase in secretory IgA only among those infants born by vaginal delivery. Accordingly, we performed the same analyses in vaginally delivered infants (71.3% of our sample), and we observed that the increase in secretory IgA during the study tended to be higher among probiotic-supplemented infants ($p=0.057$, data not shown). Those previous studies analysed the secretory IgA in the faeces, which has a higher concentration of IgA than the saliva (32), and this feature could influence our results.

Safety and tolerance

The safety and tolerance of the *B. infantis* IM-1[®]-supplemented formula was demonstrated as the growth parameters, digestive symptoms, crying behaviour, tolerance and compliance with the study formula were similar compared to the non-supplemented group.

All infants showed similar growth patterns throughout the follow-up. Infants fed probiotic formula showed similar weight, length and head circumference z-scores at any timepoint, as well as similar BMI z-scores except at recruitment. Moreover, as observed in previous studies (30,33,34), the evolution of these parameters throughout the study was also similar. Consistent with these results, a systematic review by Mugambi *et al.* (15) reported no differences in weight, length and head circumference gain in infants supplemented with different probiotics. Therefore, an ESPGHAN Committee on Nutrition commentary concluded that the supplementation of infant formula with some probiotic strains has no adverse effects on growth in healthy term infants (35). Our results confirm that the *B. infantis* IM-1[®] strain is also safe in terms of growth.

Digestive symptoms such as regurgitation, vomiting and flatulence were similar between both groups during the follow-up period coinciding with Gibson *et al.*, who showed no significant differences in these parameters of digestive tolerance. However, other studies found a lower frequency of regurgitation in probiotic-supplemented formula-fed infants compared to the control ones (34,36).

Constipation is a common adverse effect of feeding infants with a standard formula rather than breastfeeding (37). In our study, infants in the Probiotic group had a higher stool frequency than the Control at 4 weeks of supplementation. Moreover, longitudinal analysis showed that the expected reduction of the stool frequency during the study was lower in the Probiotic group compared to those observed in the Control. These differences, even that small, had a clinical relevance, as the percentage of infants with constipation at 4 and 12 weeks was lower in the Probiotic group than in the Control. These results are consistent with those from Vendt *et al.* (38), who observed a higher stool frequency in probiotic-supplemented infants, although most of the studies found no significant differences in the

number of bowel movements (15,30,33). Thus far, the evidence-based recommendations developed by ESPGHAN and NASPGHAN in 2014 do not support the use of probiotics in the treatment of childhood constipation (39) and the efficacy must be tested for each specific strain. Regarding the effect of supplemented formula in the stool consistency, we observed no differences between both formula groups similarly to Petschow *et al.* (40) and Weizman *et al.* (33). Consistent with this, the most recent systematic review and commentary by the ESPGHAN Committee on Nutrition concluded that the supplementation of infant formula with probiotics has an unclear effect on the consistency of the stool (35).

To observe the tolerance and compliance with the supplemented formula, we assessed the overall intake of formula. We found similar formula intake in both groups, and thus, the study results suggest that the *B. infantis* IM-1[®]-supplemented formula is well accepted and safe in terms of infant compliance. Some authors have reported a similar intake between probiotic and control groups (25), whereas a recent meta-analysis showed that infants in the probiotic group had significantly higher formula intake than the control (15).

Some limitations of our study may be that some recruited infants had been fed breastmilk (11.2%) or infant formula with pre- or probiotics (33.9%). These infants underwent a washout period (7 days for formula-fed infants and 15 days for breastfed infants) and were equally distributed between the study groups, but we cannot reject that this previous feeding pattern may have influenced our results. Another potential source of bias is the use of antibiotics observed in 3.6% of the infants in our sample. Unfortunately, we cannot discard antibiotic use in some more infants due to incomplete data in the medical. In total, an 85.8% of the infants did not receive any antibiotic during the study without differences in distribution between study groups ($p=0.255$).

In summary, this study suggest that *B. infantis* IM-1[®] supplementation may prevent from diarrhoea episodes and is associated with a lower incidence of constipation as well as higher stool frequency compared to the control group. In addition, growth rate and digestive tolerance of the supplemented formula is adequate and similar to those of the non-supplemented formula. We also found a relationship between the total IgA concentrations and the *B. Infantis* IM-1[®] content in the faecal microbiota. Thus, we conclude that supplemented infant formula containing 10⁷ cfu/g of *Bifidobacterium longum* subsp. *infantis* CECT7210 (*B. infantis* IM-1[®]) is safe, effective and well tolerated in healthy term infants.

STATEMENT OF FINALTIAL SUPPORT

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DISCLOSURE

Mireia Morera, Jose Antonio Moreno Muñoz and Montserrat Rivero are employees of ORDESA SL. Their role was to critical review the manuscript and were not involved in the statistical analysis or manuscript redaction.

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FIGURE LEGENDS

Fig. 1. Flowchart of recruitment, randomization, follow-up and withdrawals at each study time point.

Fig. 2. Anthropometric characteristics (a) and stool frequency and consistency (b) of infants by feeding group at each study time point. W: weeks. $*p < 0.05$.

Fig. 3. Infants with diarrhoea episodes and number of episodes throughout the study period by feeding group.

Fig. 4. Total Bifidobacterium and *B. infantis* IM1® detected by RT-PCR in stool samples (a) and *B. longum* presence detected in the whole microbiota analysis (b). $*p < 0.05$ with respect to the baseline.

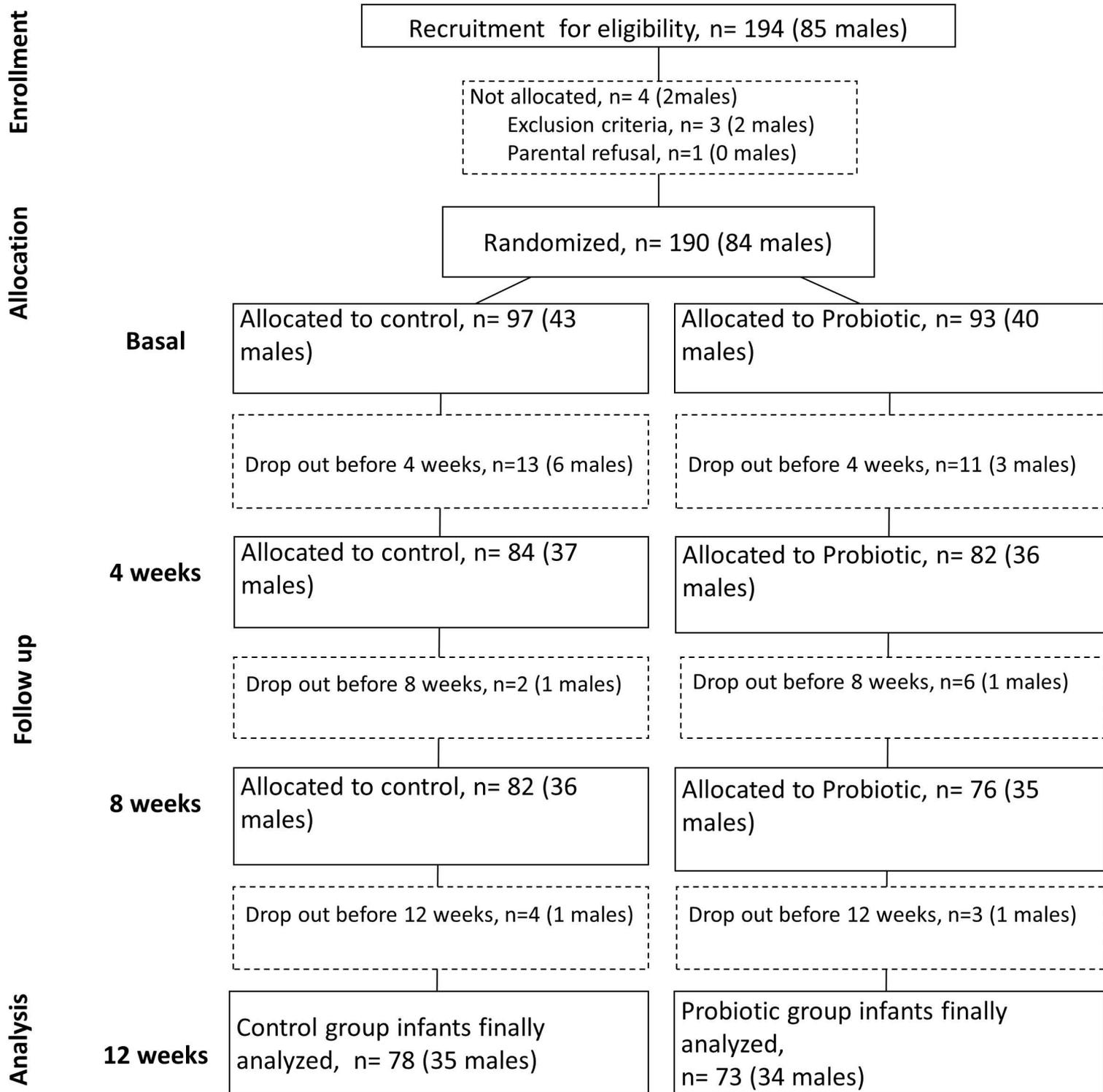
Accepted manuscript

Table 1. Baseline characteristics of infants.

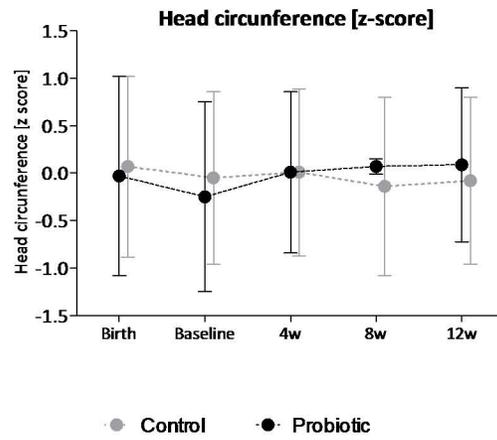
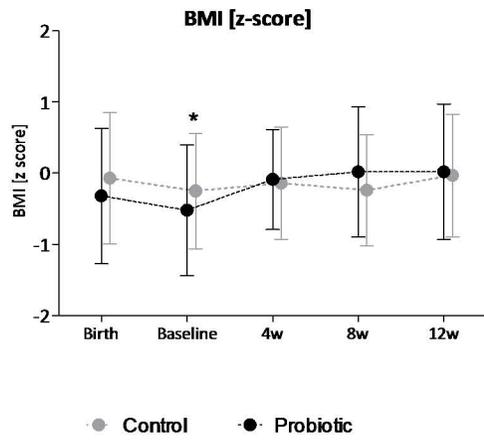
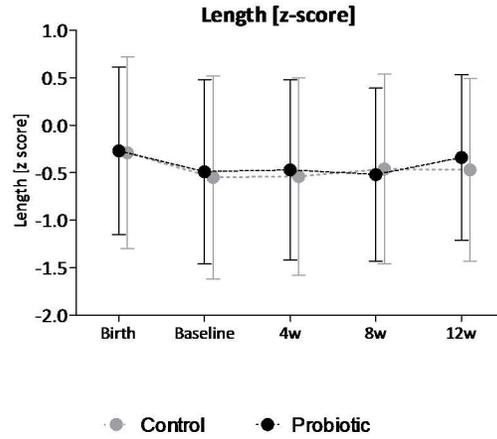
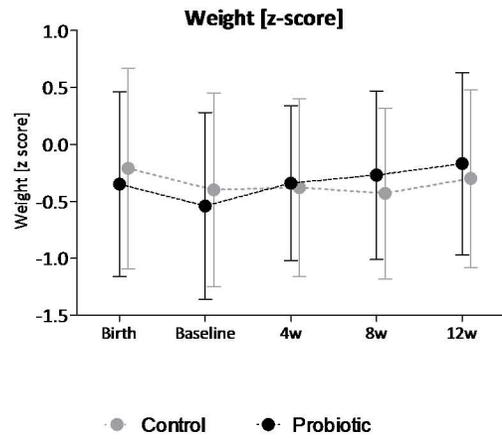
	Control group (Mean \pm sd) or (%) n=97	Probiotic group (Mean \pm sd) or (%) n=93	P value
Age at recruitment (days)	8.6 \pm 16.3	5.9 \pm 9.2	0.500
Gender (% males)	44.3	43.5	0.906
Gestational age (weeks)	39.2 \pm 1.1	39.3 \pm 1.2	0.802
Mother age (years)	31.9 \pm 4.7	32.6 \pm 5.1	0.288
Perinatal complications (% yes)	4.2	5.4	0.684
Delivery type (% caesarean)	31.2	26.1	0.209
Siblings (n)	0.7 \pm 0.7	0.8 \pm 1.0	0.930
Mean intake volume (ml/day)	210.0 \pm 237.7	190.7 \pm 207.8	0.500
Stool frequency (n/day)	3.3 \pm 2.0	2.9 \pm 1.6	0.183
Vomits (n/day)	0.1 \pm 0.4	0.2 \pm 0.6	0.411
Regurgitation (n/day)	1.1 \pm 0.4	1.1 \pm 0.3	0.653
Irritability (% yes)	12.8	13.0	1.000
Flatulence (% yes)	22.3	18.5	0.587
Abdominal pain (% yes)	17.9	8.7	0.085
Colics (% yes)	9.5	2.2	0.034
Constipation (% yes)	6.3	6.5	1.000

Table 2. Frequency of digestive symptoms (vomiting, regurgitation, flatulence and abdominal pain) and behaviour as well as formula intake throughout the study period.

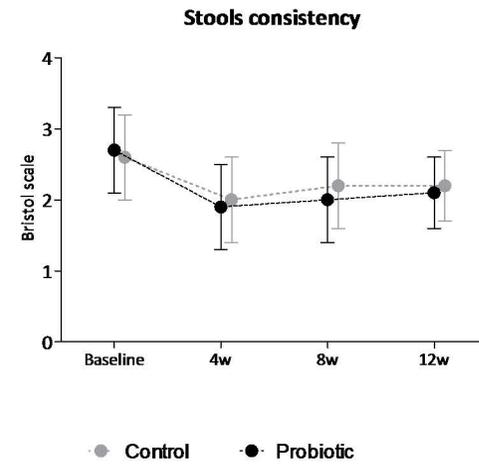
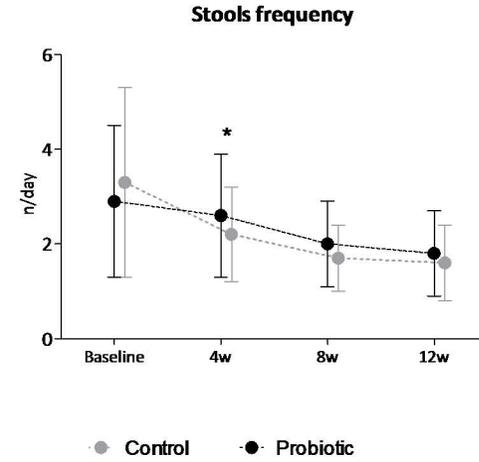
	Control group n=95	Probiotic group n=93	P value
Vomits (% children with 1 or more than 1 vomit/day)			
Baseline evaluation	9.5	11.9	0.492
At 4 weeks	12.3	11.3	0.587
At 8 weeks	8.2	6.1	1.000
At 12 weeks	6.3	6.0	0.854
Regurgitation (% children with more than 5 regurgitation/day)			
Baseline evaluation	10.7	12.9	0.570
At 4 weeks	13.1	14.8	0.908
At 8 weeks	11.0	9.3	0.734
At 12 weeks	5.2	9.7	0.291
Flatulence (% children with flatulence)			
Baseline evaluation	22.3	18.5	0.514
At 4 weeks	54.3	50.0	0.588
At 8 weeks	47.2	47.9	0.937
At 12 weeks	35.1	28.2	0.368
Abdominal pain (% children with pain)			
Baseline evaluation	17.9	8.7	0.085
Sleeping breaks at night (% of ves)			
Baseline evaluation	12.6	13.2	1.000
At 4 weeks	18.3	20.3	0.842
At 8 weeks	9.1	19.4	0.098
At 12 weeks	6.7	6.9	1.000
Sleeping hours at night (mean \pm SD)			
Baseline evaluation	7.4 \pm 1.9	7.5 \pm 1.6	0.858
At 4 weeks	7.1 \pm 1.6	6.8 \pm 1.4	0.271
At 8 weeks	8.3 \pm 1.6	7.4 \pm 1.5	0.012
At 12 weeks	8.3 \pm 1.4	8.0 \pm 1.5	0.260
Formula intake (ml/day, median \pm SD)			
At 4 weeks	816 \pm 153	801 \pm 149	0.513
At 8 weeks	856 \pm 152	825 \pm 189	0.255
At 12 weeks	890 \pm 169	851 \pm 145	0.133



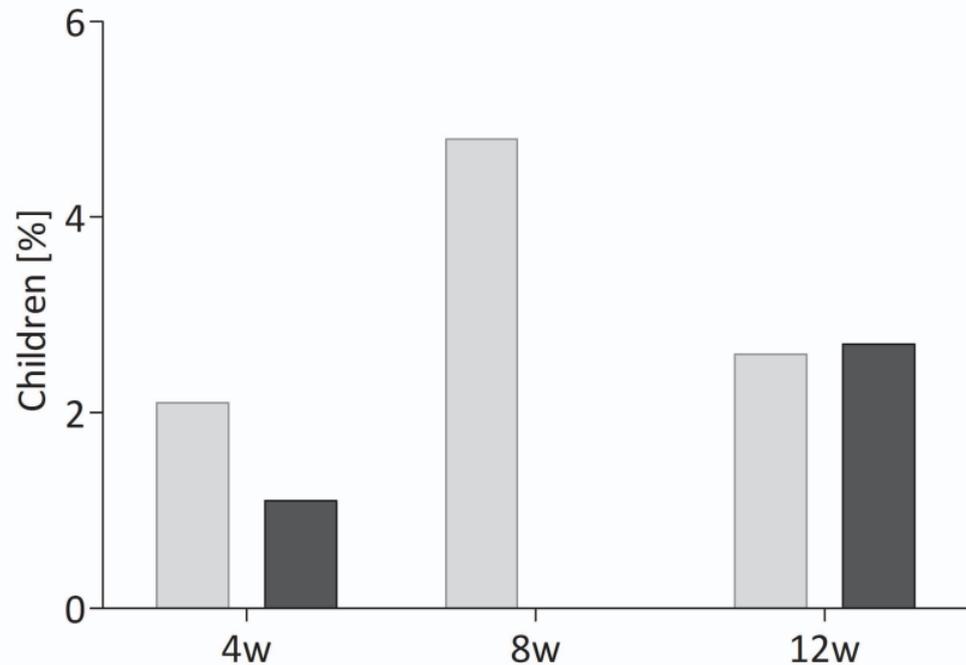
a.



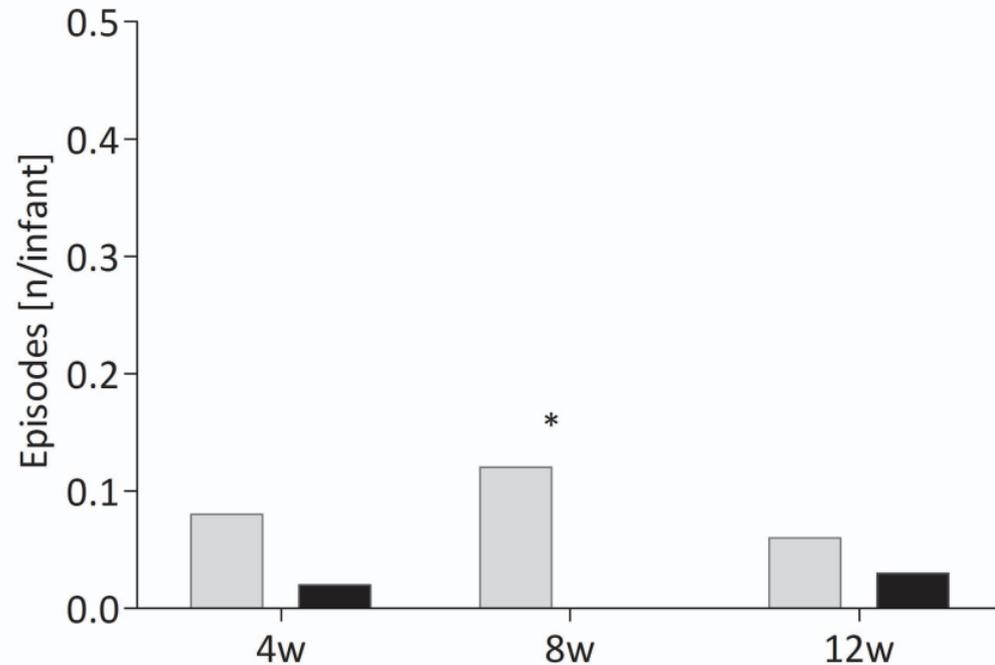
b.



Diarrhoea incidence (%)



Episodes of diarrhoea (n/infant)



Control

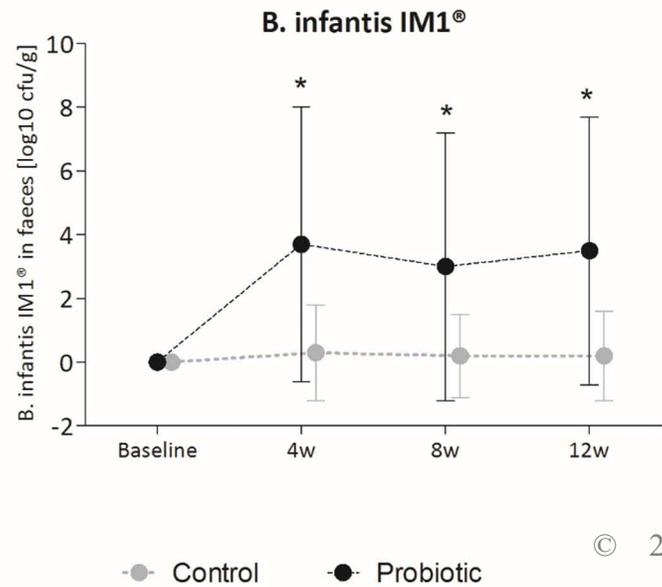
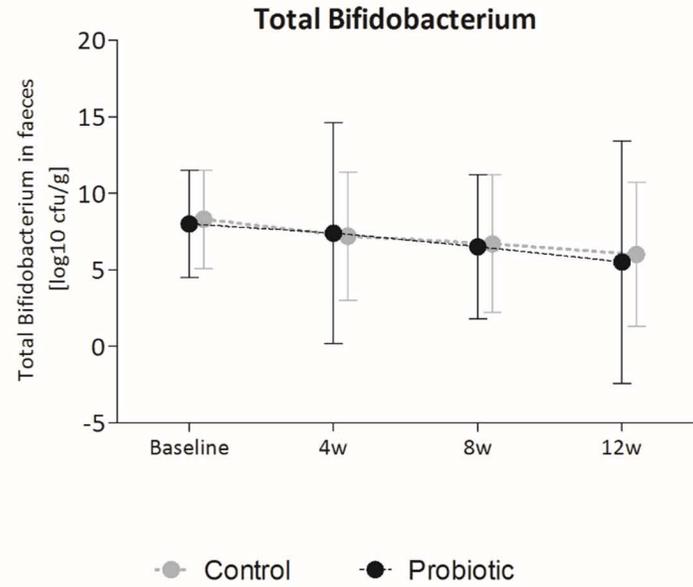
Probiotic

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Control

Probiotic

a.



b.

