Efficacy of *Bifidobacterium breve* CECT7263 for infantile colic treatment: an open-label, parallel, randomised, controlled trial

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Abstract

Infantile colic is a prevalent condition characterised by excessive crying with no effective treatment available. We aimed to evaluate the efficacy of *Bifidobacterium breve* CECT7263 and a combination of this and *Lactobacillus fermentum* CECT5716 versus simethicone in reducing the daily time spent crying in colicky infants. A multicentre randomised, open-label, parallel, controlled trial of 28 days was performed in 150 infants who were diagnosed with colic according to the Rome III criteria and who randomly received simethicone (80 mg/day; Simethicone group), *B. breve* CECT7263 (2×10^8 cfu/day, Bb group), or a combination of *L. fermentum* CECT5716 and *B. breve* CECT7263 (1×10^8 cfu/day per strain, Bb+Lf group). The main outcomes were minutes of crying per day and the percentage of reduction in daily crying from baseline. Data were analysed per intention to treat. All treatments significantly decreased the daily crying time at the end of the intervention (P -time < 0.001). However, the infants in the Bb group had significantly decreased crying time from the first week of the study (P < 0.05), whereas the Bb+Lf group and the simethicone group had significantly decreased crying time from the second week (P < 0.05). The percentage of reduction in the minutes of crying from baseline in the Bb group was significantly higher than that in the Simethicone group every week of the intervention (-40.3 vs -27.6% at 1-week; -59.2 vs -43.2% at 2-weeks; -64.5 vs -53.5% at 3-week and -68.5 vs -59.5% at 4-weeks, P < 0.05). Additionally, in the Bb group, infants had better night sleep, and parents reported a more positive mood at the end of the intervention. All the products used in the study were safe and well tolerated. In conclusion, the breastmilk-isolated probiotic strain *B. breve* CECT7263 is a safe and effective treatment for infantile colic, presenting an earlier and more robust effect than the reference prescribed drug, simethicone.

Keywords: probiotics, bifidobacteria, paediatric population, infant colic

1. Introduction

Infantile colic is a common condition characterised by excessive crying, irritability or fussing reported by parents that occur without obvious cause or illness, independently of the type of feeding (breastfeeding or formula feeding) and that cannot be prevented or resolved by caregivers, occurring in infants up to 5 months of age (Lucassen *et al.*, 2001; Benninga *et al.*, 2016). It is usually diagnosed on the basis of the Rome III criteria, which are paroxysms of irritability, fussing, or crying that start and stop without obvious cause, occurring in episodes that last 3 or more hours per day and at least 3 days/week for at least 1 week with no failure to thrive (Hyman *et al.*, 2006). The prevalence of infantile colic varies widely, although recent studies estimated it to affect as many as 20% of infants less than 4 months of age (Vandenplas *et al.*, 2015).

The etiopathogenesis of infantile colic remains unclear, although evidence suggests that it is most likely a multifactorial condition, including behavioural factors and biological causes (Ong *et al.*, 2019). Among them, dysbiosis in the infant intestinal microbiota has been proposed as one of the main causes of infant colic, since it may affect gut motility and gas production, leading to abdominal pain and colicky behaviour (Gupta, 2002). In fact, there is growing evidence that shows that the intestinal microbiota of infants with colic differs from that of healthy infants, with low diversity and stability (Rhoads *et al.*, 2018; Rhoads *et al.*, 2009), higher levels of *Proteobacteria* and gram-negative bacterial genera (Zeevenhoooven *et al.*, 2018) and low levels of *Bifidobacteria* and *Lactobacilli*, including species with anti-inflammatory effects, and fewer butyrate-producing species (Pärtty *et al.*, 2012; Savino *et al.*, 2009).

Although infantile colic is considered to be a self-limiting and benign condition, it can cause considerable distress to parents and caregivers (DTB, 2013), and it was related to higher maternal depression risk in the postpartum period (Radesky *et al.*, 2013; Vik *et al.*, 2009). Different approaches for infant colic management have been proposed, although with limiting evidence of effectiveness, highlighting the need for new therapeutic strategies. In this regard, due to emerging evidence suggesting that the intestinal microbiota has a role in the pathogenesis of infant colic, probiotic bacteria have been proposed as a potential treatment option for colic (Schreck Bird *et al.*, 2017). *Lactobacillus* spp. and *Bifidobacterium* spp. are considered to be safe and well tolerated in healthy infants, and because of that, they are the most commonly used probiotics in infants with colic (Daelemans *et al.*, 2018). However, the efficacy of different strains in reducing daily crying in colicky infants remains controversial, and experts suggest that more evidence is needed to recommend the use of probiotics in the prevention and treatment of infantile colic (Ong *et al.*, 2019; Sung, 2018). In this regard, our research group observed

that the addition of *Lactobacillus fermentum* CECT5716 or *Bifidobacterium breve* CECT7263, two probiotic strains naturally found in breast milk, to infant formulae is safe and induces beneficial effects in healthy infants (Gil-Campos *et al.*, 2012; Maldonado *et al.*, 2012; Maldonado-Lobón, 2015) and presents several characteristics that make them suitable for use as potential treatments for infant colic. Specifically, we observed that infants receiving an infant formula supplemented with *B. breve* CECT7263 had a 1.7 times lower risk of crying than the control group. Additionally, the incidence and duration of diarrhoea in infants receiving the formula supplemented with *L. fermentum* CECT5716 was lower than in infants receiving the control formula (Maldonado *et al.*, 2019).

Therefore, the main objective of the present study was to evaluate the efficacy of *B. breve* CECT7263 and a combination of *L. fermentum* CECT5716 and *B. breve* CECT7263 versus a standard prescribed drug (simethicone) in reducing the daily time spent crying in infants diagnosed with infantile colic. The secondary aims were to evaluate the tolerability and acceptability of the treatments.

2. Materials and methods

Study design and subjects

This was a prospective, multicentre, open label, randomised, controlled trial with three study groups and a duration of 28 days. The study was carried out in the paediatrics departments of two Spanish hospitals (Hospital Virgen de las Nieves (Granada) and Jaen City University Hospital (Jaén)), three Spanish private paediatric clinics (Pediatric Clinic Roquetas de Mar (Almería), Vithas Málaga (Málaga) and 9 of October (Valencia)), and eight public paediatric health centres belonging to the Andalusian Health Service (Spain). The study was conducted according to the Declaration of Helsinki, and the protocol was approved by the Regional Ethics Committee of the Sistema Andaluz de Salud based in Seville (Spain). Infants were included in the study after informed written consent was obtained from the parents or caregivers. The trial adhered to CONSORT guidelines and was retrospectively registered in the US Library of Medicine (<http://www.clinicaltrials.gov>) as NCT03467334.

The main outcome for the calculation of the sample size was the reduction in the daily crying time observed in other studies. It was calculated that a minimum of 40 subjects would be required in each group to demonstrate a difference of at least 40 min in daily average crying time among the three groups with a power of 80% and a confidence level of 95%. Assuming a drop-out rate of 10%, 44 infants per group were needed.

Study participants met the following inclusion criteria: to have a diagnosis of infantile colic according to the Rome

III criteria (crying episodes lasting 3 or more hours per day occurring for at least 3 days/week for at least 1 week), age from 3 to 12 weeks; ≥ 34 weeks gestation at birth and a birth weight $\geq 2,000$ g. Exclusion criteria included a major medical problem or acute illness, growth failure or history of antibiotic treatment or probiotic supplementation before or during the study.

After enrolment, all children were randomly assigned to receive simethicone, given at a dose of 20 mg, 4 times per day in drops, half an hour before meals (Simethicone Group), *B. breve* CECT7263 at a dose of 2×10^8 cfu/day, (Bb Group), or a combination of *L. fermentum* CECT5716 at a dose of 1×10^8 cfu/day and *B. breve* CECT7263 at a dose of 1×10^8 cfu/day (Bb+Lf Group). Probiotic treatments were provided as capsules containing the daily dose to be opened by parents and added to human milk, infant milk or water. The probiotics *B. breve* CECT7263 and *L. fermentum* CECT5716 were produced by Biosearch Life at its industrial facility in Granada. Capsules containing the probiotics were prepared by the Department of Pharmacy and Pharmaceutical Technology of the Faculty of Pharmacy of the University of Granada (Granada, Spain). Simethicone was commercialised by Uriach (Barcelona, Spain). Treatments were given to subjects for 28 days. Capsules were kept at 4 °C during the intervention study.

Study outcomes and data and sample collection

The study’s primary outcomes were the changes in the mean of the minutes of crying per day for each week of the intervention and the percentage of reduction in the daily crying from baseline. Secondary outcomes were gastrointestinal parameters, sleep behaviour of the children, infant faecal microbiota, adverse effects, and satisfaction of the parents with the treatment.

At the baseline visit, the parents of the infants who met the inclusion criteria were informed about the study details and were asked to sign the informed consent form. Baseline data were collected, and the corresponding treatment was dispensed for the total duration of the study. Additionally, parents were instructed to complete a data-collection diary to record the daily crying time (in minutes), fussing behaviour, type of feeding, stool frequency and characteristics, gastrointestinal disorders, time of sleeping, parents’ mood and any adverse events experienced (Table 1). The follow-up visits to the paediatrician were conducted on days 7, 14, 21 and 28 of the study, at which data collected in the diary were reviewed. Infant faecal samples were taken at baseline and at the end of the study. Parents collected infant faecal samples in sterilised tubes 24 h before the visits, preserved at -20 °C and took them to the corresponding study centre. The researchers received the faecal samples and kept them at -20 °C. Faecal samples

were cold transported to Biosearch Life facilities in Granada (Spain), where were processed and analysed within 1 month from its collection.

Faecal bacteria quantification

Lactobacillus spp., *Bifidobacterium* spp., *Clostridium* spp., *Bacteroides* spp. and *Escherichia coli* counts were measured by quantitative polymerase chain reaction. The ATP™ Genomic DNA Mini Kit (Tissue) AGT300 (ATP Biotech Inc., Taipei City, Taiwan) was used for bacterial DNA isolation. In brief, the faecal samples were homogenised in peptone water at a concentration of 100 mg/ml under sterile conditions, and 200 µl of the previous suspension was added to an Eppendorf tube (20 mg/ml) for DNA extraction following the instructions of the ATP Genomic DNA Mini Kit (Tissue) AGT300 protocol. DNA quantification was performed by qPCR using SYBR® green as a fluorophore (Qiagen, Hilden, Germany), and specific primers were used for each group as previously described by Maldonado-Lobon *et al.* (2015).

Statistical analysis

The distributions of all measured variables were assessed for normality by normal probability plots and the Shapiro-Wilk test. Data were also tested for homogeneity by the Levene’s test in order to meet the criteria for applying later the ANOVA test. Data are presented as the mean (standard deviation) for continuous variables and as n (%) for categorical variables. The baseline characteristics of

Table 1. Parameters recorded in the data-collection diary.

During the last week the number of fecal depositions per week was	1 = ≤ 7 times, 2 = 8-21 times, 3 = > 21 times
During the last week the consistency of feces was	1 = hard lumps or sausage with cracks, 2 = soft sausage, 3 = mushy (like porridge) or watery
During the last week the infant suffered flatulency	1 = never/rarely/sometimes, 2 = frequently, 3 = very frequently
During the last week the infant suffered stomachache	1 = never/rarely/sometimes, 2 = frequently, 3 = very frequently
During the past week the total sum of sleeping hours in 24 h was on average	1 = < 11 h/day, 2 = 11-14 h/day, 3 = > 14 h/day
During the past week the sleeping hours during night was on average	1 = < 6 h/day, 2 = 6-8 h/day, 3 = > 8 h/day
During the past week the parents’ mood was	1 = disappointed, 2 = normal, 3 = satisfied

participants were compared between groups using ANOVA or the non-parametric Kruskal-Wallis test for quantitative measures and the chi-square test for qualitative measures.

The minutes crying outcome was measured daily during the time of the study. To analyse the full data among days and compare results between treatments, a general additive mixed model (GAMM) was fitted with a zero-inflated gamma distribution. Adjustment for intra-patient random effects and curves of time during the study was considered, as were the intraweek effects during the study. Fixed effects to check treatments at the different weeks of the study were fitted, and tests on the estimated marginal results were performed. Several clinically relevant variables were included in the statistical model to test for their association with the change in minutes crying. The adjusted minutes of crying and effects along with their 95% confidence intervals are presented. The estimated marginal probabilities and effects were estimates, and the *p*-values for testing differences are provided. The derived endpoint percentage difference in crying with respect to baseline was obtained. For the previous variable, a GAMM model was applied; in this case, the family distribution considered was Student's *t*, to fit the skewness of the data to the 100% value. Estimate parameters, along with the marginal means of percentages by group and time, are provided.

Secondary outcomes recorded at days 7, 14, 21, and 28 were analysed depending on the type of data with different methods. Variables recorded as categories with more than 2 classifications were analysed using multinomial mixed regression models. The effect of the treatment over time for the different classifications was analysed. The estimated effect and probabilities of each classification per group are provided. Statistical tests were performed to check the association of clinically relevant variables with the change in probabilities of each classification. Variables collected as categories with two-classification, dichotomic values, were analysed using a multivariate logistic mixed model. The estimated probabilities and odds ratios were obtained for comparing treatments over time. In the variables recorded on an ordinal scale, a cumulative logit mixed model using the adaptive Gauss-Hermite quadrature method was used to estimate how likely a subject under each treatment would have a response category. Continuous variables were analysed using linear mixed regression models. Additionally, linear mixed regression models were applied to test for the effect of treatment on the microbial loads.

A general alpha level of 0.05 was used as the cut-off point for statistical significance. Statistical analysis was carried out using R version 3.1.

3. Results

Population

A group of 150 infants were included in the study and randomised between May 2017 and May 2019. The intervention ended in June 2019. A flow chart of the participants is shown in Figure 1. Eighteen percent of the infants did not complete the intervention and withdrew from the study. Causes of withdrawal were the perception by the parents that the infant was not getting better, antibiotic use, constipation, stomach pain, diaper rash and upper respiratory tract infection, although most of the withdrawals were voluntary and parents stopped attending the visits without explanation. No differences among the groups were detected between the number and causes of withdrawal. The data for all the infants included in the study that had attended to at least one of the follow-up visits were analysed (analysis per intention to treat).

The baseline characteristics of the 150 infants included in the statistical analyses are presented in Table 2. Of those, 78 were males (52%), and 72 were females (48%). The mean age of the subjects when entering the study was 5.6 ± 2.7 weeks old, and they presented an entry mean weight of $4,451.4 \pm 1,047.8$ g. At the outset of the study, all infants presented a diagnosis of infantile colic according to the Rome III criteria, with a mean daily crying time of 4.5 ± 2.6 h/day. No significant differences were detected between subjects in the study groups in the baseline characteristics.

Primary outcomes

Table 3 shows the estimated means (95% confidence interval (CI)) of the minutes of crying per day at each week of the intervention by treatment, adjusted by recruiting centre and time. The three treatments significantly decreased the daily crying time at the end of the intervention compared to baseline (*P* time <0.001). However, in the infants from the Bb group, the decrease in the time spent crying was observed earlier, from the first week of the study compared to baseline (*P*<0.05), whereas the Bb+Lf group and the simethicone group had significantly decreased time spent crying from the second week compared to baseline (*P*<0.05). There were no differences between the mean daily minutes of crying, except at week 2, where infants that received the combination Bb+Lf presented a significantly lower time spent crying than infants receiving the Bb and simethicone (*P*<0.05).

Figure 2 represents the estimated means of the percentages of reduction in the minutes of crying with respect to baseline adjusted by hospital and time. At each time point, the percentage of reduction from baseline in the Bb group was significantly greater than the percentage of reduction

Figure 1. Diagram of the consolidated standards of reporting trials.

Table 2. Baseline characteristics of the subjects participating in the study.^{1,2}

		Simethicone Group (n=44)	Bb + Lf Group (n=57)	Bb Group (n=49)	P-between groups
Age (weeks)	Mean (SD)	5.3 (2.4)	5.9 (2.6)	5.7 (3.2)	0.469
Entry length (cm)	Mean (SD)	54.3 (3.2)	54.5 (3.4)	54.8 (3.5)	0.868
Entry weight (g)	Mean (SD)	4,461.8 (972.5)	4,424.9 (860.8)	4,467.5 (1310.3)	0.863
Entry head circumference (cm)	Mean (SD)	37.8 (3.2)	37.4 (2.1)	37.4 (1.6)	0.844
Mean daily crying (hours)	Mean (SD)	4.4 (2.9)	4.6 (2.3)	4.6 (2.8)	0.831
Sex					0.554
Female	n (%)	18 (40.9)	30 (52.6)	24 (48.9)	
Male	n (%)	26 (59.1)	27 (47.3)	25 (51.1)	
Type of delivery					0.557
Vaginal	n (%)	33 (75.0)	43 (75.5)	40 (81.6)	
C-section	n (%)	11 (25.0)	14 (24.5)	9 (18.4)	
Antibiotics in delivery	n (%)	23 (52.3)	25 (43.8)	15 (30.6)	0.160
Type of feeding					
Exclusively breastfeeding	n (%)	20 (45.5)	25 (43.9)	27 (55.1)	0.602
Exclusively formula-fed	n (%)	10 (22.7)	10 (17.5)	8 (16.4)	
Partial breastfeeding	n (%)	14 (31.8)	22 (38.6)	14 (28.5)	
Pacifier users	n (%)	16 (36.4)	28 (49.1)	26 (53.1)	0.161

¹ Values are means (standard deviation (SD)) for continuous variables and n (%) for categorical variables. P indicates differences between treatment groups. Mean daily crying is referring to the mean daily crying during the previous week of the intervention.

² Lf = *Lactobacillus fermentum* CECT5716; Bb = *Bifidobacterium breve* CECT7263.

Table 3. Minutes crying per week by treatment group.

	Simethicone Group	Bb + Lf Group	Bb Group
Baseline	251.1 (207.8-294.4)	261.4 (221.7-301.1)	286.0 (232.7-339.3)
1-week	210.8 (197.4-224.2)	230.2 (217.2-243.2)	216.2 (199.9-232.6) *
2-week	178.5 (167.3-189.8) **a	133.4 (125.3-141.5) **b	157.7 (145.5-169.9) **a
3-week	116.8 (109.2-124.5) **#	114.5 (107.2-121.7) **	132.3 (122.0-142.5) **#
4-week	99.2 (92.3-106.0) **##§	101.7 (95.1-108.4) **#	86.3 (80.9-91.7) **##§

¹ Data are mean (95% confidence interval) adjusted by hospital and time. In the same intervention group, * $P<0.05$ respect to baseline, ¥ $p<0.05$ respect to 1-week, # $P<0.05$ respect to 2-week, § $P<0.05$ respect to 3-week. In different intervention groups, different letters indicated significant differences ($P<0.05$) in the same time point.

² Lf = *Lactobacillus fermentum* CECT5716; Bb = *Bifidobacterium breve* CECT7263.

in the Simethicone group (-40.3 vs -27.6% at week 1; -59.2 vs -43.2% at week 2; -64.5 vs -53.5% at week 3 and -68.5 vs -59.5% at week 4). Moreover, at week 1, the Bb group also presented a higher percentage of reduction than the Bb+Lf group (-40.3 vs -30.6%), whereas at week 2, the Bb+Lf group presented a higher percentage of reduction than the Simethicone group (-56.5 vs -43.2%).

The model adjusted for relevant clinical variables indicated a shorter crying time in children who started the study at a later age (β -0.038 per unit increase in age; $P<0.0001$). Babies born by C-section cried more minutes than those born by natural birth (difference in effect on logarithmic

scale $\beta=0.098$ or equivalently cried 10.3% more on average than those born by natural birth; $P<0.0001$). In cases where antibiotics were administered during delivery, babies cried significantly more than in cases where antibiotics were not administered (difference in effect on logarithmic scale $\beta=0.144$ or equivalently cried 15.4% more on average than births without antibiotics; $P<0.0001$). These results occurred in infants regardless of the treatment group assigned because the interaction between these factors and treatment was not significant ($P>0.05$).

Moreover, we observed an interaction between the type of feeding (breastfeeding, infant formula or mixed feeding)

Figure 2. Percentage of reduction of the minutes of crying respect to baseline in each intervention group. Bars represented the estimated means $\pm$ 95% CI of the percentages of reduction adjusted by hospital and time. For each week, symbols represent differences between groups: ¥ $P<0.05$ Simethicone vs Bb, ¶ $P<0.05$ Bb+Lf vs Bb, * $P<0.05$ Simethicone vs Bb+Lf. Lf = *Lactobacillus fermentum* CECT5716; Bb = *Bifidobacterium breve* CECT7263.

and the treatment group ($P < 0.05$). Figure 3 represents the estimated means of the percentages of reduction in the minutes of crying with respect to baseline by type of feeding, adjusted by recruiting centre, time, age of the infants, type of delivery and antibiotics at delivery. In all types of feeding, the three treatments significantly reduced the time of crying, so at week 4, the percentages of reduction in the minutes of crying with respect to baseline were significantly different from week 1 ($P < 0.05$). However, in infants that were breastfed, at week 1, week 2 and week 3, the percentage of reduction from baseline in the Bb group was significantly greater than the percentage of reduction in the Simethicone group and the Bb+Lf group (-49.7 vs -23 and -26.2% at week 1; -66.4 vs -42.9 and 52.5% at week 2; -66.9 vs -52.9 and 56.7% at week 3) and was also greater at week 4 compared to the Bb+Lf group (-69.4 vs 57.3% at week 4). In infants that were exclusively formula-fed, also at week 1, the percentage of reduction from baseline in the Bb group was significantly greater than the percentage of reduction in the Simethicone group (-50.8 vs -32.5%), and at week 2, the percentage of reduction from baseline in the Bb group and in the Bb+Lf group was significantly greater than the percentage of reduction in the Simethicone group (-67.9 and -66.8 vs -49.5%). At weeks 3 and 4, there were no differences between groups. Finally, in infants who received mixed feedings, at weeks 2 and 3, the percentage of reduction from baseline in the Bb+Lf group was significantly greater than the percentage of reduction in the Simethicone group (-60.7 vs -38.3% at week 2, and -60.9 vs -44.7% at week 3), with no differences between groups at weeks 1 and 4.

Secondary outcomes

Parameters related to tolerance and intestinal function were also evaluated based on the questionnaire explained in the methods section (Supplementary Table S1). Regarding faecal depositions, most of the infants showed a stool frequency of 8-21 depositions per week on average (41.6% in the Simethicone group, 40.6% in the Bb+Lf group and 40.7% in the Bb group). No significant changes were observed between baseline and the end of the intervention or between groups. However, when we measured the frequency of improvement (to have a greater number of faecal depositions from baseline), the Bb+Lf group presented a significant improvement compared to the Simethicone group at the end of the study. At week 4, 32.6% of the infants in the Bb+Lf group had increased stool frequency from baseline versus 13.5% of the infants in the Simethicone group ($P = 0.049$). Stool consistency increased with time ($P < 0.001$), although there was no interaction between treatment group and time ($P = 0.389$). However, an interaction with the type of feeding was observed ($p = 0.020$), with infants who were breastfed and took the Bb+Lf being more likely to have soft stools than those children who were breastfed and took the simethicone, which had a greater probability of presenting watery stools ($P = 0.007$). The frequency of the infants suffering flatulency decreased with time ($P < 0.001$) in all groups (P interaction group $\times$ time = 0.403), and the same was observed with respect to the frequency of suffering stomach ache (P time < 0.001 ; P group $\times$ time = 0.224).

Figure 3. Percentage of reduction of the minutes of crying respect to baseline in each intervention group and by type of feeding. Bars represented the estimated means $\pm$ 95% CI of the percentages of reduction adjusted by hospital, time, age of the infants, type of delivery and antibiotics at delivery. For each week and type of feeding, symbols represent differences between groups: ‡ $P < 0.05$ Simethicone vs Bb, ¶ $P < 0.05$ Bb+Lf vs Bb, * $P < 0.05$ Simethicone vs Bb+Lf. Lf = *Lactobacillus fermentum* CECT5716; Bb = *Bifidobacterium breve* CECT7263.

Some variables related to the sleep behaviour of infants were analysed. The hours spent night sleeping increased with time ($P<0.001$) and were similar in all groups at the end of the study. However, an interaction between time and treatment group was found ($p=0.004$), and a larger number of infants in the Bb group slept more than 8 hours at week 3 compared to that in the Bb+Lf group (48.8 vs 27.1%, $P=0.001$, data not shown). When the total time of sleep was considered, hours of total sleeping increased in all groups ($P<0.001$), with no interaction between time and group ($P=0.321$).

Finally, a questionnaire about parents' mood was recorded (Supplementary Table S2). The mood of the parents positively changed ($P<0.001$) from disappointed at baseline (70.7% on average) to satisfied at the end of the study (74.2% on average). There was no interaction between time and group ($P=0.591$); however, when we measured the frequency of improvement (to have a better mood from baseline), the Bb group presented a significant improvement compared to the Bb+Lf group in the first week. After 7 days of treatment, 72.7% of the parents whose infants took Bb had positively changed their mood compared to 52.8% of the parents whose infants took Bb+Lf ($P=0.047$). Moreover, at the end of the study, 100% of the parents whose infants took the Bb recorded having a more positive mood than at baseline vs 85.1% of the parents whose infants took the Bb+Lf and 92.7% of the parents whose infants took the simethicone ($P=0.031$).

Faecal microbiota

Some of the most representative bacterial genera were studied in the infants' faeces at baseline and after 4 weeks of treatment (Table 4). At day 28 of the study, only the infants in the Bb+Lf group presented significantly higher levels of *Lactobacillus* in their faeces compared to baseline ($P=0.009$), although no significant differences were observed between the Simethicone group and the Bb group at the end of the study ($P=0.08$ and 0.910). Regarding *Bifidobacterium*, a general increase over time was observed in the three groups ($P=0.005$ for the Simethicone group, $P=0.017$ for the Bb+Lf group and $P=0.038$ for the Bb group), with no differences between groups at day 28. It should be noted that *Bifidobacterium* counts were higher in infants who entered the study at a later age ($\beta=0.091$, $P=0.035$) and when antibiotics were not administered during delivery ($\beta=-0.451$, $P=0.048$), independent of the treatment group. *Clostridium* load did not change during treatment in any of the groups, and there were no differences between groups at the end of the study. With respect to *Bacteroides*, a higher count was found in children born by natural birth than in children born by C-section ($\beta=-2.821$, $P<0.001$), with no interaction with the treatment group. Indeed, no significant changes in *Bacteroides* load were observed between baseline and day 28 in any group. *E. coli* levels were also higher in infants born by natural birth ($\beta=-1.647$, $P=0.001$) and significantly increased from baseline to day 28 in the Simethicone group ($P=0.004$) and in the Bb group ($P=0.006$), whereas they did not change in the Bb+Lf group ($P=0.074$).

Table 4. Intestinal microbiota counts in fecal samples of infants (as log10 of cfu/g), at baseline and after 4 weeks of intervention.^{1,2}

		Baseline	Week 4	P time
<i>Lactobacillus</i> spp.	Simethicone Group	6.41 (0.28)	6.34 (0.29)	0.784
	Bb + Lf Group	6.43 (0.25)	7.03 (0.26)	0.009
	Bb Group	6.68 (0.29)	6.99 (0.29)	0.238
<i>Bifidobacterium</i> spp.	Simethicone Group	9.01 (0.23)	9.74 (0.24)	0.005
	Bb + Lf Group	9.55 (0.21)	10.1 (0.27)	0.017
	Bb Group	9.32 (0.24)	9.86 (0.24)	0.038
<i>Clostridium</i> spp.	Simethicone Group	4.01 (0.39)	4.4 (0.41)	0.181
	Bb + Lf Group	4.72 (0.35)	4.88 (0.36)	0.222
	Bb Group	4.45 (0.41)	4.53 (0.41)	0.383
<i>Bacteriodes</i> spp.	Simethicone Group	3.96 (0.49)	3.99 (0.5)	0.925
	Bb + Lf Group	3.2 (0.45)	3.58 (0.45)	0.196
	Bb Group	3.41 (0.52)	3.26 (0.52)	0.747
<i>Escherichia coli</i>	Simethicone Group	7.74 (0.42)	8.87 (0.43)	0.004
	Bb + Lf Group	7.94 (0.37)	8.54 (0.385)	0.074
	Bb Group	7.72 (0.43)	8.79 (0.44)	0.006

¹ Data are mean (standard error) adjusted by hospital, age of the infants, type of delivery, antibiotics at delivery and type of feeding. P time is referring to the differences between baseline and week 4 for each strain in each treatment group.

² Lf = *Lactobacillus fermentum* CECT5716; Bb = *Bifidobacterium breve* CECT7263.

4. Discussion

The present study shows that the breastmilk-isolated probiotic strain *B. breve* CECT7263, alone or in combination with the strain *L. fermentum* CECT5716, seems to be effective in reducing the daily time spent crying in infants diagnosed with infantile colic. After 28 days of treatment, the efficacy of those strains was similar to the medical treatment of reference for infantile colic, simethicone. However, the *B. breve* CECT7263 alone seems to have an earlier and more robust effect than the combination of strains and the simethicone, decreasing at a higher percentage and from the first week of consumption the minutes of daily crying in colicky infants feeding with breastmilk or infant formula.

The use of probiotics for infant colic treatment is spreading, and several clinical trials have been performed in the last decade. In healthy infants, the safety and tolerability of *Lactobacillus* spp. and *Bifidobacterium* spp. are proven, although controversial results have been obtained regarding the efficacy of different strains in reducing daily crying in colicky infants (Ong *et al.*, 2019; Sung *et al.*, 2018). Several randomised clinical trials showed that *Lactobacillus reuteri* DSM17938 reduced crying time in infants with colic compared with a placebo (Ashraf *et al.*, 2015; Chau *et al.*, 2015; Savino *et al.*, 2018; Szajewska *et al.*, 2013). In contrast, two studies concluded that the same strain was ineffective in infantile colic treatment (Fatheree *et al.*, 2017; Sung *et al.*, 2014). The results from different meta-analyses concluded that *L. reuteri* appears to be safe and effective for the management of infantile colic in breastfed infants, but further studies are needed to determine its role in formula-fed infants (Schreck Bird *et al.*, 2017; Sung *et al.*, 2018). *Lactobacillus rhamnosus* GG did not provide additional treatment effect for diary-verified colic crying, although parental report of crying suggested the probiotic intervention effective (Pärty *et al.*, 2015). When this strain was administered in combination with others no effect on infant colic was observed (Kukkonen *et al.*, 2008), whereas a symbiotic product containing this strain plus others and fructo-oligosaccharides showed efficacy at day 7 of the treatment (Kianifar *et al.*, 2014). *Bifidobacterium animalis* subsp. *lactis* BB-12 reduced mean daily crying in colicky infants when provided alone (Nocerino *et al.*, 2020; Xinias *et al.*, 2017) but had no effect in combination with other strains (Vlieger *et al.*, 2009). The results achieved in this trial showed that *B. breve* CECT7263 exhibited efficacy in decreasing the time of daily crying in both infants feeding with breastmilk and formula. However, although the administration of *B. breve* CECT7263 in combination with *L. fermentum* CECT5716 was also effective in reducing crying time, even more than simethicone in some points, the efficacy of the probiotic combination was lower than when *B. breve* CECT7263 was administered alone. Negative interactions between

both strains as competition phenomena have not been observed in *in vitro* assays previously performed (data non shown). Considering that the dose of *B. breve* CECT7263 in the probiotic combination (1×10^8 cfu/day) was half that of the *Bifidobacterium* strain administered alone (2×10^8 cfu/day), the difference in the efficacy might be due to this difference in the concentration. Although only few studies have evaluated the dose response effect of probiotics, some authors have found a relationship between the administered dose and the observed effect (Lee *et al.* 2019; Zhang *et al.* 2016). These results are in agreement with those obtained in a previous study performed with healthy infants who received an infant formula supplemented with *Lactobacillus* or *Bifidobacterium* strains. In this study, it was observed that after 6 weeks of intervention, the percentage of infants that cried more than 3 h/day was 12% in the group that received the infant formula supplemented with *B. breve* CECT7263, compared to the 21% in the group that received the *L. fermentum* CECT5716 supplementation and the 29% that received the non-supplemented infant formula (Maldonado *et al.*, 2019). More studies should be performed to evaluate the dose-response effect of *B. breve* CECT7263 on infantile colic.

Interestingly, the relationship we found between the age of the infants, the method of delivery and maternal intrapartum antibiotic use and crying time reinforces the hypothesis that intestinal microbiota may be one of the main causes for infant colic (Dubois and Gregory, 2016), since all these factors are well known to affect intestinal colonisation in infants (Rodríguez *et al.*, 2015). However, it is important to note that *B. breve* CECT7263 efficacy in reducing mean daily crying was not affected by these infant colic-related factors. Therefore, our results support the role of *B. breve* CECT7263 as a safe, robust and effective treatment option for infant colic.

Indicators of tolerance and intestinal function showed that all the products used in the study were safe and well tolerated. Differences in increased faecal depositions between the combination of the probiotics and the simethicone were expected, according to observations in previous studies (Maldonado *et al.*, 2019). Interestingly, infants that took the *B. breve* CECT7263 had better night sleep, which supports the results regarding the improvement of the colic, since some data suggest that colicky infants have a higher rate of night waking and less nocturnal sleep (Sadeh and Sivan, 2009). Finally, it should be pointed out that parents whose infants ingested the *B. breve* CECT7263 reported an earlier and massive change to a better mood during the study, which also agreed with the reduction of the mean daily crying observed in these infants. Although this variable cannot be considered as an empirical measure, another RCT with probiotics in infants with colic also reported parent satisfaction as a result due to the major burden that this disorder posed to families (Mi *et al.*, 2015).

The potential mechanisms for the effect exerted by *B. breve* CECT7263 may be related to the anti-inflammatory properties of this strain. This probiotic showed intestinal anti-inflammatory activity in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced rat colitis, evidenced by a significant reduction in colonic tumour necrosis factor alpha and interleukin 1 β levels, increased glutathione content and reduced inducible nitric oxide synthase expression (Algieri *et al.*, 2013). This is of particular interest since a study performed in colicky infants demonstrated that colic crying was associated with low-grade systemic inflammation, suggesting the importance of using probiotics that have anti-inflammatory properties that may help modulate microbiota and alleviate colic-related inflammation (Pärtty *et al.*, 2017). Moreover, a recent animal study carried out with *B. breve* CECT7263 showed that this strain increased butyrate-producing bacteria (Robles-Vera *et al.*, 2020), and the presence of which has been correlated with the absence of crying in infants, which is attributed to the fact that butyrate may reduce pain sensation (Weerth *et al.*, 2013). Unfortunately, cytokine or butyrate levels were not analysed in the samples collected in the present study, so further studies are needed to determine the mechanisms of action of *B. breve* CECT7263.

Several studies have compared the gut microbiome in colicky versus healthy infants. Remarkably, although these studies were performed in infants of different ages, most of them highlighted the reduced bacterial diversity and the decreased abundance of the genera *Lactobacillus* and *Bifidobacterium* in infants with colic compared to healthy infants (Korpela *et al.*, 2020; Savino *et al.*, 2009; Weerth *et al.*, 2013). However, we observed minor changes in faecal microbiota associated with the probiotic treatments. As previously reported, the administration of *L. fermentum* CECT5716 was associated with an increase in the faecal load of lactobacilli (Maldonado *et al.*, 2019, 2012). However, although the content of *Bifidobacterium* spp. in faeces increased in the three groups according to the normal evolution of this strain in infants, (Nagpal *et al.*, 2017; Turroni *et al.*, 2018) we did not find a higher total load of *Bifidobacterium* in the faeces of the groups treated with the probiotics at the end of the study. These results are in agreement with those observed in a previous study (Maldonado *et al.*, 2019) and may be related to the fact that daily ingestion of *B. breve* CECT7263 was much lower than the *Bifidobacterium* total faeces count, and therefore, it would be difficult to detect significant changes between groups. Indeed, another intervention study with the probiotic *B. animalis* subsp. *lactis* BB-12 reported the presence of this specific strain in faeces but not an increment in the *Bifidobacterium* spp. total load (Baglatzi *et al.*, 2016). Moreover, there is evidence of the ability of various strains of *B. breve* to colonise the gastrointestinal tract of infants (Quigley, 2017). Therefore, although *B. breve* CECT7263 did not induce a significant difference in

the faecal *Bifidobacterium* population, it seems to exert its functionality, according to the observed effects in this study and in a previous one (Maldonado *et al.*, 2019). However, further studies that include a more specific microbiota analysis should be performed to elucidate how this probiotic strain may influence the infant gut microbiota.

Some limitations of the current study should be addressed. First, this was an open trial due to the differences in the appearance and posology between the simethicone and the probiotics. This open-label design may have influenced the obtained results, although both probiotic products have the same appearance, and the parents and researchers did not know if the infants received *B. breve* CECT7263 alone or in combination with *L. fermentum* CECT5716. Second, due to ethical reasons, the intervention with probiotics had to be compared with an intervention with simethicone, the most common medical treatment for infant colic, rather than a placebo, which might have affected the outcomes of the study. A third limitation is that the effect of probiotic treatments on microbiota was restricted to a few genera. A more sophisticated analysis of the microbiota that includes the analysis of all the main genera present in the microbiota would be necessary to give a more complete picture of the effects of probiotic treatment on the microbiota and its interactions on the symptoms of colic. Also, another intervention group that would have received the *L. fermentum* CECT5716 alone would have been of interest to discern the role of this probiotic in infant colic. Finally, as happened in other studies on infant colic, the assessment of the time spent crying relied on parent reports, which cannot be considered an objective measure.

5. Conclusions

The breastmilk-isolated probiotic strain *B. breve* CECT7263 seems to have an earlier and more robust effect on infant colic than simethicone, since the consumption of *B. breve* CECT7263 reduced the daily time of crying in infants diagnosed with infantile colic from the first week of the intervention and at a higher percentage. These results were observed in infants feeding with breastmilk or infant formula and were accompanied by the observation of better infants' night sleep and an improvement in the parents' mood. All of this, together with the fact that the study subjects did not suffer from adverse effects and that the probiotic was well tolerated, supports the role of *B. breve* CECT7263 as a safe and effective treatment option for infant colic, presenting an earlier and more robust effect than the reference prescribed drug for infantile colic, simethicone.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0105>.

Table S1. Parameters related with intestinal function and behavior of infants.

Table S2. Parameters related with the parents' mood during intervention.

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Conflict of interest

JAML, RBR, AS, CR, OB and MO are employees of Biosearch Life. JF has been employee of Biosearch Life. The authors declare no other conflict of interest.

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