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The therapeutic efficacy of *Bifidobacterium animalis* subsp. *lactis* BB-12® in infant colic: A randomised, double blind, placebo-controlled trial

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Summary

Background: The pathogenesis of infant colic is poorly defined. Gut microbiota seems to be involved, supporting the potential therapeutic role of probiotics.

Aims: To assess the rate of infants with a reduction of ≥50% of mean daily crying duration after 28 days of intervention with the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12® (BB-12). Secondary outcomes were daily number of crying episodes, sleeping time, number of bowel movements and stool consistency.

Methods: Randomized controlled trial (RCT) on otherwise healthy exclusively breast-fed infants with infant colic randomly allocated to receive BB-12 (1×10^9 CFU/day) or placebo for 28 days. Gut microbiota structure and butyrate, beta-defensin-2 (HBD-2), cathelicidin (LL-37), secretory IgA (sIgA) and faecal calprotectin levels were assessed.

Results: Eighty infants were randomised, 40/group. The rate of infants with reduction of ≥50% of mean daily crying duration was higher in infants treated with BB-12, starting from the end of 2nd week. No infant relapsed when treatment was stopped. The mean number of crying episodes decreased in both groups, but with a higher effect in BB-12 group (−4.7 ± 3.4 vs −2.3 ± 2.2, $P < 0.05$). Mean daily stool frequency decreased in both groups but the effect was significantly higher in the BB-12 group; stool consistency was similar between the two groups. An increase in *Bifidobacterium* abundance (with significant correlation with crying time reduction), butyrate and HBD-2, LL-37, sIgA levels associated with a decrease in faecal calprotectin level were observed in the BB-12 group.

Conclusions: Supplementation with BB-12 is effective in managing infant colic. The effect could derive from immune and non-immune mechanisms associated with a modulation of gut microbiota structure and function.

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1 | INTRODUCTION

Infant colic (IC) is a functional gastrointestinal disorder, affecting up to 25% of infants in the first 3 months of life, with a typical peak of prevalence at about 6 weeks of age.[1] Although IC is a benign and usually self-limiting condition, it is a source of major distress for the infant, parents, family and health-care givers.[2] It is associated with maternal postpartum depression, early breastfeeding cessation, parental guilt and frustration, shaken baby syndrome, multiple physician visits, drugs use, formula changing, long-term adverse outcomes such as allergy, behaviour and sleep problems.[3,4] The incidence of IC seems to be the same between sexes, and no definitive correlation with type of feeding, gestational age, socioeconomic status and season of the year have been demonstrated.[3,4] Despite decades of research, at present, the pathogenesis of IC remains poorly understood and is thought to be multifactorial; however, a growing body of evidence suggests that alterations of gut microbiota can contribute to the development of this condition.[4,5] Distinct microbial patterns have been found in IC. A lower diversity and stability of the gut microbiota was reported in subjects with IC during the first 2 weeks of life.[4,5] These alterations suggest that a state of gut dysbiosis might play a role in the expression of IC symptoms, modulating various neural, endocrine, immune and humoral signalling pathways.[4-6]

Considering that dysbiosis could play a role in the pathogenesis of IC, there is an interest in gut microbiota modulation, including the use of probiotics, for the management of IC. *Bifidobacterium animalis* subsp. *lactis*, (BB-12®) is a well-known probiotic, which positively modulates the composition of the intestinal microbiota and the function of the immune system.[7] These features likely make this probiotic potentially useful for the treatment of IC. In a previous trial, BB-12 added to a low lactose partially hydrolysate formula containing prebiotics resulted effective in reducing the duration of crying in subjects with IC.[8] To further explore the potential efficacy of BB-12 in the treatment of IC we designed this double blind, placebo-controlled randomised trial.

2 | METHODS

This randomised, double blind, placebo-controlled clinical trial was conducted from 11 November 2016 to 6 November 2017 at the Department of Translational Medical Science—Pediatric Section of University of Naples "Federico II", Naples, Italy. The trial was conducted in collaboration with a group of family paediatricians, operating in the city area of Naples, who care for children up to 14 years of age in the Italian Public Health System. The family paediatricians were asked to refer to the Department potentially eligible infants and to provide support to the parents of those eventually enrolled by the hospital. Before the start of the study, all investigators involved in the trial attended an investigator meeting during which the study protocol was illustrated and discussed, and all definitions and procedures, including the key factors in the management of IC (such as parental education, reassurance and empathy)[9] were shared.

The study protocol, the subject information sheet and the informed consent form were reviewed and approved by the ethics committee of our institution.

The study was conducted in accordance with the Good Clinical Practice Standards and study monitoring was performed by the contract research organisation (CRO), blinded to the treatment assignment.

The study was registered in the Clinical Trials Protocol Registration System at ClinicalTrials.gov with the identifier NCT02988791.

2.1 | Study subjects

Otherwise healthy exclusively breastfed infants aged ≤7 weeks, with signs and symptoms possibly related to IC according to Rome III Criteria,[10] regularly followed by the family paediatricians involved in the trial were considered eligible for the study. Infant colic (IC) criteria include all of the following: paroxysms of irritability, fussing or crying that start and stop without obvious cause; episodes lasting 3 or more hours per day and occurring at least 3 days per week for at least 1 week; and no failure to thrive.

The exclusion criteria were the following: age ≥7 weeks, birth weight <2500 g, gestational age <37 weeks, Apgar score at 5 minutes <7, partially or total formula feeding, stunted growth/weight loss (<100 g/week from birth to last reported weight), neurological diseases, suspected or confirmed food allergy, gastroesophageal reflux disease, use of probiotics, prebiotics, antibiotics or gastric acidity inhibitors at any time before enrolment, fever and/or infectious diseases at any time before enrolment, current systemic infections, history of congenital infections, chronic intestinal diseases, cystic fibrosis or other forms of primary pancreatic insufficiency, gastrointestinal malformations, metabolic diseases, genetic diseases and chromosomal abnormalities, primary or secondary immunodeficiency, insufficient reliability or presence of conditions that made the patient's compliance with the protocol unlikely and participation in other studies.

2.2 | Data collection and intervention

At the baseline, after obtaining informed consent from the parents/tutors of each infant, the health status of all the study subjects was carefully assessed by physicians involved in the trial. Previous pharmacological treatment and presence of infectious diseases or other diseases were ruled out by means of complete anamnestic evaluation and clinical examination, including vital signs, neurological status, growth status, nutritional status, hydration, skin evaluation, otoscopy, evaluation of oral cavity, respiratory/abdomen/lymphnode examination and genital examination. Anamnestic, demographic, anthropometric and clinical data were collected and reported in a specific clinical chart.

Then, infants were required to follow a 1-week pre-enrolment period. If after this period the diagnosis of IC was confirmed, the subject was randomised to one of the following study groups: Group 1, parental reassurance and education plus BB-12 (*Bifidobacterium*

animalis subsp. *lactis* BB-12®, DSM 15954, 1 × 10^9 CFU/daily dose in oil maltodextrin suspension; Bifidolactis Infant, Sofar SpA); Group 2, parental reassurance and education plus placebo (oil maltodextrin suspension). Parents were requested to administer to their infants six drops of the assigned study product, once a day, for 28 days directly in the mouth, preferably in the morning before feeding. Instructions for keeping and maintaining the product were also provided according to the manufacturer's indications. Study products were provided by Sofar SpA. The patient's parents, investigator staff, persons performing the assessments and data analysts were blinded to the identity of the treatment at all times, ie allocation, intervention, laboratory analysis and statistical analysis. The packaging, colour, weight, smell and taste of the investigational product and of the placebo were identical and thus ensured blind conditions. The bottles containing the probiotic or the placebo were labelled with consecutive numbers without any reference to the group assignment, which was known only to the CRO and statistician who generated the list and to the technician who prepared the packages. The patient's parents were provided with a diary and they were instructed on how to complete it daily with data concerning administration of daily dose of the study product, number and duration of crying episodes, number of bowel movements and consistency of baby's stool (according to the Bristol stool scale),[11] sleep duration, possible adverse events. Each study subject was evaluated by the hospital paediatricians involved in the trial for a total of six visits over a 5-week period; unscheduled visits were performed if necessary. At each visit, the hospital paediatricians performed a full clinical examination of the infant and assessed and collected data from the diary. Compliance was assessed by evaluating the diary provided by the parent. In addition, parents were also asked to return used bottles to further assess the compliance to assigned treatment.

The possible influences of maternal dietary factors or changes in dietary habits were assessed by analysing data from 7-days food diary collected during the week before treatment (V0-V1). In addition, possible changes in maternal diet were also assessed during the last week of the study (V4-V5). All diaries were assessed by experienced dietitians unaware of the study aims and blinded to group assignment. Stool samples (3 g) were collected at enrolment before the start of therapy, and after 4 weeks of treatment. All samples were collected from diapers in sterile plastic tubes and stored at −80°C until analysis.

At each visit, the parents of the patients were asked to report any side effects, unexpected symptoms or unexpected events related or not to the treatment occurred after the last visit. All Adverse events (AEs) that occurred from the start of the study until the final visit or after 30 days from the last administration of study treatment were registered. For each AE, the nature, date and time of onset, duration, severity and correlation with treatment was established, and any changes in the dosage or other treatments have been noted in detail on the case report form (CRF).

Parents were instructed to avoid the use pre/pro/synbiotics or any anti-colic medications during the study, and eventual use of other medications by the study subjects was reported in the diary. All study procedures and assessments were performed as shown in Figure 1, panel a.

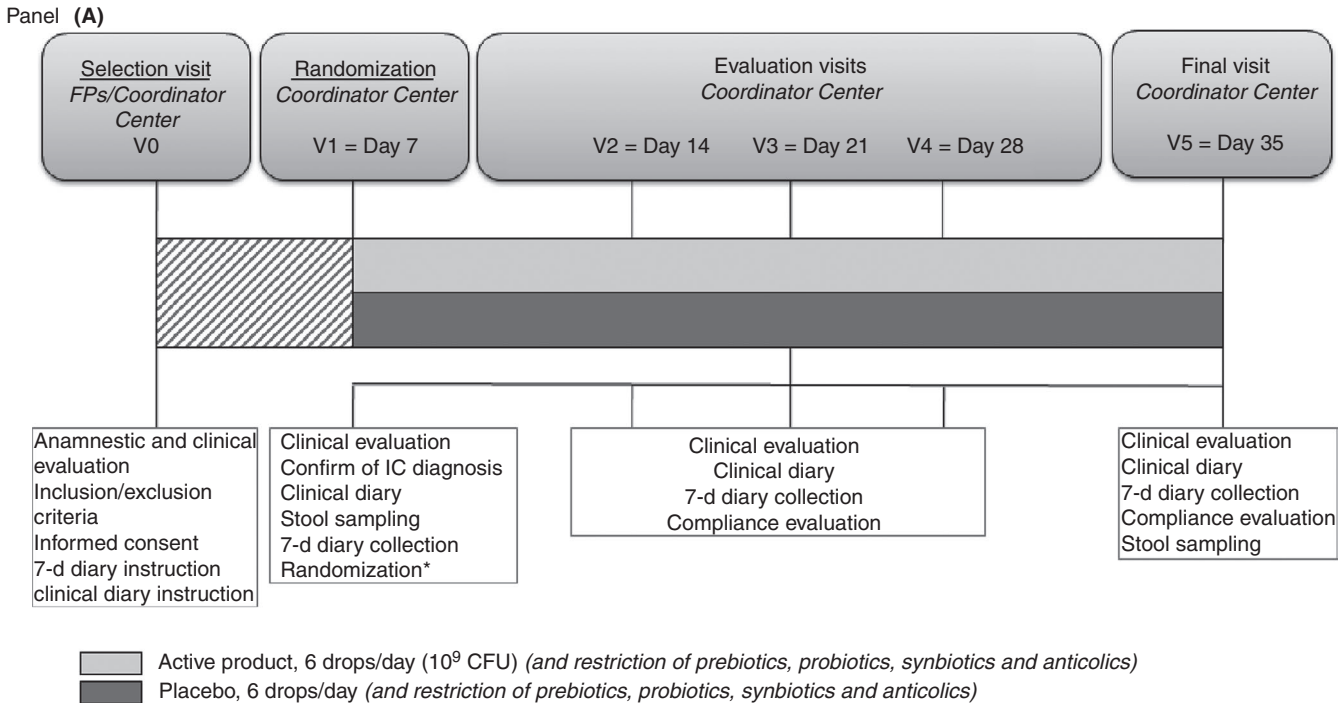

FIGURE 1 Panel (A). The design of the study. Panel (B). The flow of subjects during the phases of the study

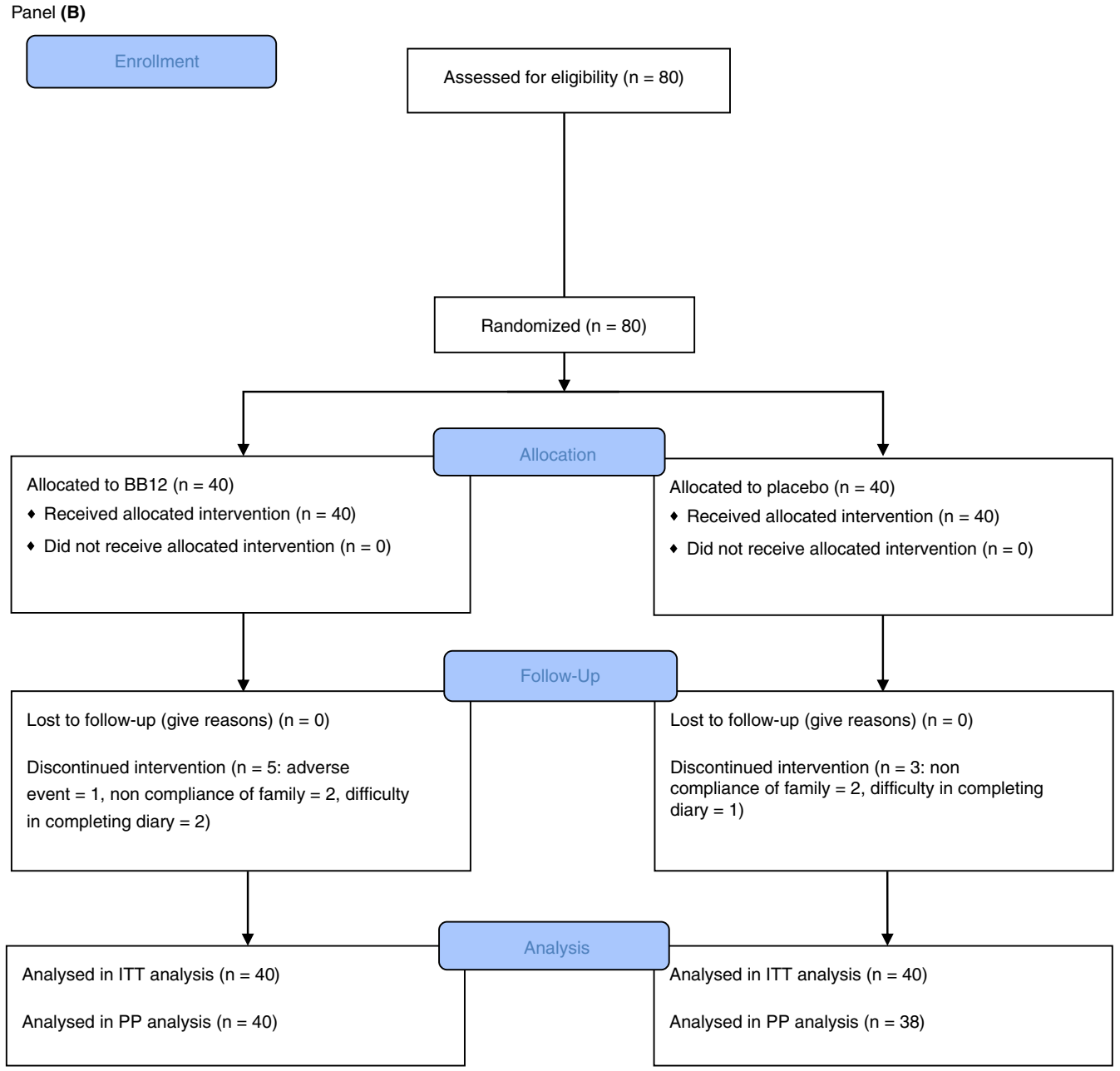

FIGURE 1 (Continued)

2.3 | Study outcomes

The primary outcome of the study was the proportion of infants with a treatment success rate, defined as a reduction of ≥50% of mean daily crying duration after 28 days of intervention.

The secondary outcomes were: the mean number of crying episodes; sleep duration; number of bowel movements and stool consistency. Study groups were also compared for gut microbiota structure, faecal levels of human beta-defensin 2 (HBD-2), cathelicidin (LL-37), secretory IgA (sIgA), calprotectin and butyrate at enrolment and after 28 days of treatment. Safety and the possible occurrence of adverse events were also assessed.

2.4 | Faecal analytical methods'

Human beta-defensin 2, LL-37 and sIgA were measured from the supernatants of faecal homogenates, using commercial kits as previously described.[12] HBD-2 was measured using a HBD-2 (Human) ELISA kit (Phoenix Pharmaceuticals, Inc), LL-37 using an ELISA human kit (Hycult biotechnology) and sIgA using indirect enzyme immunoassay (Salimetrics LLC). The results were expressed as ng/g for HBD-2, LL-37 and as μg/g of supernatant for sIgA.

Faecal calprotectin level was determined using a commercial ELISA kit (Calprest, Eurospital) as previously described,[13] and the result was expressed as mg/Kg of faeces.

TABLE 1 Main features of the study population at enrolment

	Group 1 BB-12	Group 2 Placebo
N.	40	40
Male, n (%)	22 (55)	21 (52.5)
Spontaneous delivery, n (%)	15 (37.5)	21 (52.5)
Gestational age, mean week (SD)	38.5 (1.1)	38.5 (1.2)
Birth weight, mean kg (SD)	3280.7 (367.5)	3412 (442.7)
APGAR score at 5 min, mean (SD)	8.95 (0.4)	8.83 (0.4)
Age, days, mean (SD)	32.9 (5.3)	33.0 (5.0)
Familial risk for allergy, n (%)	19 (47.5)	18 (45)
Functional gastrointestinal disorders in first-degree relatives, n (%)	3 (7.5)	7 (17.5)
Exposure to passive smoking, n (%)	11 (27.5)	11 (27.5)

Faecal butyrate level was assessed using gas chromatography as previously described[14] and expressed as mM.

Gut microbiota structure was characterised using high-throughput sequencing of 16S rRNA gene amplicons. DNA extraction, library preparation, sequencing and data analysis were carried out as recently described.[15] Briefly, raw reads were joined using FLASH[16] and quality-filtered by Prinseq,[17] trimming out bases with Phred score <20 and shorted than 300 bp. Operational taxonomic units (OTU) picking and taxonomic assignment was carried out in QIIME v. 1.9.[18] Taxonomic identification of OTUs was achieved using Greengenes 13_5 database and OTUs were collapsed at the different taxonomic levels. Alpha-diversity indices (number of OTUs, Shannon and Chao1 indices) were computed in QIIME. OTU table produced in QIIME was imported in R environment for further analyses. The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive of the National Center for Biotechnology Information, under accession number SRP11.

2.5 | Sample size, randomisation and statistical analysis

Sample size was calculated taking into account the effect size estimated from a previous trial on IC.[19] We calculated that 33 infants per group were needed to detect an absolute difference of 35% of the treatment success rate (from 15% in the placebo group to 50% in the active group), with a power of 0.80 at an alpha level of .05 (Pearson's Chi-square, two-tailed test). Assuming a dropout rate up to 20%, we calculated that 40 infants per group had to be enrolled into the study. Patients were randomised in a 1:1 ratio to one of the two treatment groups according to a randomisation list generated by a specific software (SAS for Windows release 9.4-64-bit).

Descriptive statistics are reported as means and standard deviations for continuous variables and as numbers and proportions for dichotomous variables. Percentages were computed considering subjects with nonmissing information, if not differently specified.

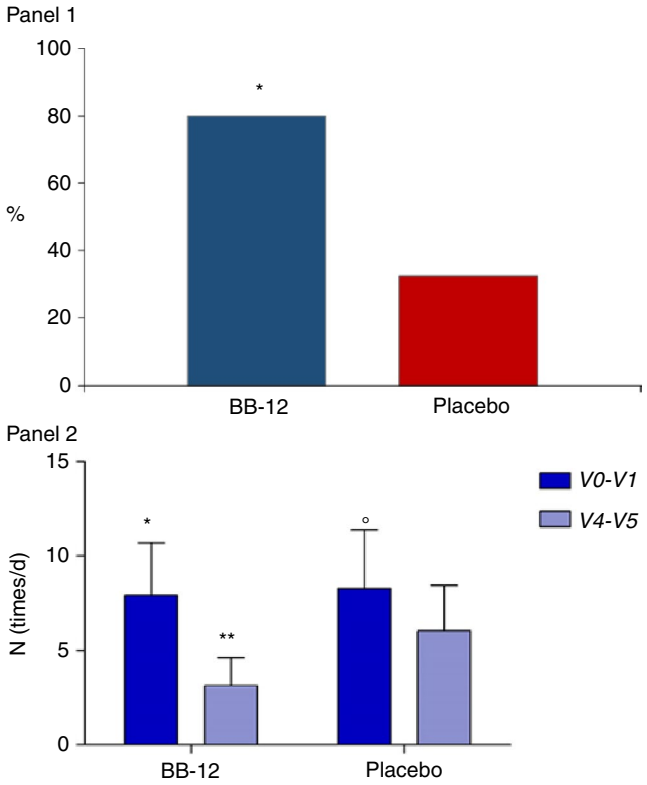

FIGURE 2 Panel 1. The results of the main study outcome (ITT analysis): the rate of infants with reduction of ≥50% of duration of crying after 28 days of treatment. Eighty percent of the BB-12 group and 32.5% of the placebo group showed a ≥50% reduction in crying duration after 28 days of treatment. The between-group difference was significantly in favour of BB-12 and the asterisk indicates a significant difference (* = BB-12 vs placebo, $P < 0.0001$). Panel 2. The mean number of crying episodes during the week before treatment (V0-V1, blue bars) and during the last week of treatment (V4-V5, light blue bars) in infants enrolled in the two study groups. Values are expressed as mean and SD and symbols indicate a significant difference (* = BB-12 V0-V1 vs BB-12 V4-V5, $P < 0.05$; ** = BB-12 V4-V5 vs Placebo V4-V5, $P < 0.05$; ° = Placebo V0-V1 vs Placebo V4-V5, $P < 0.05$)

The following analysis sets were defined: per protocol set (PP), all randomised infants who completed the study without any significant protocol violation; intention-to-treat set (ITT), all randomised infants who received at least one dose of study treatment; safety set, all randomised infants who received at least one dose of study treatment. A subject who came back for the first visit after the start of treatment (V2) was considered as having received at least one dose of study treatment. The analysis of primary outcome was performed on the ITT population, and on the PP population as supportive. The analyses of the secondary outcomes were performed on the ITT population only. The safety analysis was performed on the safety set population.

Mean daily crying duration, the mean number of daily crying episodes, the mean daily duration of sleep (in minutes) and stool frequency were described for each week by means of descriptive statistics for continuous data and were calculated on nonmissing

values; observations with values equal to zero were included in the computation. *T* test was performed comparing the change between the two study groups at each week.

Stool consistency was evaluated as the number and the proportion of patients with at least one stool sample of each type per week according to the Bristol scale.

Safety data were summarised by treatment on the Safety set population. The incidence of Adverse Events during the study was reported. Anthropometric data were summarised by treatment group by means of descriptive statistics for continuous variables at V1 and V5. Weight (g) and length (cm) were calculated at each visit. Changes from V0 at V1 and V5 were provided.

Other secondary outcomes were differences in the faecal levels of HBD-2, LL-37, sIgA, calprotectin and butyrate between the two groups. These values were compared between the groups at enrolment and after 28-days of treatment with an independent t test.

Furthermore, subjects were classified in responders or nonresponders to treatment by using the *k*-means clustering ($k = 2$). The best number of clusters was defined using the NbClust function (*NbClust* R package) on a matrix containing variation (V5-V1) of the following variables: duration of crying, LL-37, sIgA, faecal butyrate, calprotectin and HBD-2. Nonparametric Kruskal-Wallis and pairwise Wilcoxon tests were carried out in order to find differences in microbial taxa, butyrate or immunity peptides between placebo and BB-12 or between responders and nonresponders. All p-values were corrected for multiple-comparison testing when appropriate.[20]

The level of significance for all statistical tests was two-sided, $P < 0.05$. All data were collected in a dedicated database and analysed by a statistician, using SAS® for Windows release 9.4 (64-bit) or later (SAS Institute Inc) or SPSS for Windows (SPSS Inc, version 23.0) and GraphPad Prism 7.0.

3 | RESULTS

The flow of the subjects during the study is reported in Figure 1, panel b. Eighty infants were enrolled and randomised, 40 per group; eight subjects did not complete the study due to noncompliance of the family (n = 3), difficulty in completing the diary (n = 3), lost to follow-up (n = 1) or adverse event (n = 1). Seventy-two subjects completed the study: 35 in BB-12 group and 37 in placebo group. All infants were included in the ITT and safety populations since they were randomised and received at least one dose of study treatment. Seventy-eight infants were included in the PP population (40 in the BB-12 group and 38 in the placebo group) since they were randomised and took part in the trial without any significant protocol violation. Two patients in the Placebo group (patient 01_12 and patient 01_42) violated the protocol criteria and were excluded from the PP population. Only one adverse event was reported during the study and occurred in a patient treated with BB-12: the patient had a respiratory distress induced by an upper respiratory tract infection, classified as a serious adverse event, mild in severity and led to permanent study treatment discontinuation. The event resolved spontaneously and it was not related to treatment.

Baseline demographic and anamnestic features were similar comparing the two study groups (Table 1). All infants were from families of middle socioeconomic status and lived in urban areas. No prior clinical or surgical events were reported. All infants in the probiotic and control were breast fed during the entire study period. None of the infants received any treatments for colic before study entry and during the trial period. Seven-days food diaries were available from all mothers of the babies included in the study, and no dietary changes were observed during the week before treatment (V0-V1) and the last week (V4-V5) of the study.

In Figure 2, panel 1 shows that the BB-12 group presented a significantly higher reduction in crying duration after 28 days of treatment compared to the placebo group ($P < 0.0001$). Also in the PP population, treatment was successful in 80.0% of the BB-12 group and in 31.5% of the placebo group. Similarly, the between-group difference was significantly lower in favour of BB-12 ($P < 0.0001$). The rate of responders (decrease in the mean daily crying duration (in minutes) of ≥50% from the baseline measurement) was significantly higher in the BB-12 group starting from the 3rd week of treatment (Figure S1).

The mean daily duration of crying episodes was consistently shorter in the BB-12 group at each week and decreased from week to week in both the ITT and PP population. Mean change from baseline (ITT population) was significantly greater in the BB-12 group than in the placebo group: −129.9 ± 43.7 (range: −210.0 to −31.4) and −84.3 ± 51.4 (range: −192.8 to 22.1) respectively ($P = 0.0001$). The mean number of daily crying episodes was also lower in the BB-12 group than in the placebo group at each week and decreased from V1 to V5. Mean change from baseline at V5 was significantly greater in the BB-12 group: −4.7 ± 3.4 (range: −16.1 to 0.4) vs −2.3 ± 2.2 (range: −7.0 to 1.1) in placebo group ($P = 0.001$) (Figure 2, panel 2).

The sleeping time increased from baseline, with a mean change at V5 of 36.5 ± 98.8 minutes per day in the BB-12 group (range: −225.7 to 345.0 minutes) and 47.9 ± 108.6 minutes per day (range: −265.0 to 225.0 minutes) in the placebo group.

No significant differences were observed comparing the mean change of daily stool frequency from baseline to V5 in the two study groups: −1.0 ± 0.9 (range −4.0 to 0.2) in BB-12 group vs −1.1 ± 0.8 (range: −2.5 to 1.1) in the placebo group.

During the first week, most patients had at least one type F stool defined as "fluffy pieces with ragged edges, a mushy stool" (67.5% in the BB-12 group and 85.0% in the placebo group), while during the last week most had at least one type E stool defined as soft blobs with clear-cut edges, passed easily (65.0% in the BB-12 group and 72.5% in the placebo group).

In the Figures S2-S5 and Table S1 are reported the evolution week by week of the following variables: mean daily crying duration, mean number of daily crying episodes, mean daily bowel movements, stool consistency and sleep duration. A statistical difference in favour of BB-12 group was observed in mean daily crying duration starting from V2 and in mean number of daily crying episodes from V3.

The anthropometric parameters increased within the normal range from visit to visit and they were very similar in the two groups. No use of antibiotics was reported.

3.1 | Faecal analytical methods'

Due to the limited amount of faecal sample collected by the parents we were able to measure HBD-2, LL-37, sIgA, calprotectin and butyrate levels only in 32 subjects treated with BB-12 and in 30 infants who received the placebo. In Figure 3 panel 1 shows faecal levels of HBD-2 (panel a), LL-37 (panel b), sIgA (panel c), butyrate (panel d) and calprotectin (panel e) in the two study groups at enrolment and after 28-days of treatment. A significant increase in HBD-2, LL-37, sIgA, calprotectin and butyrate was observed in the two study groups as a consequence of maturation of the immune system and gut microbiome function. However, based on the variation (V5-V1) of the main study outcome, HBD-2, LL-37, sIgA, butyrate and calprotectin faecal levels we identified two different clusters (Figure 3, panel 2). Cluster 1 included 10% of infants enrolled in the BB-12 group and 67% of infants enrolled in the placebo group. Whereas, Cluster 2 included 90% and 33% of BB-12 and placebo subjects respectively. We defined subjects in Cluster 2 as responders to the treatment, since they showed a significantly higher reduction of crying and calprotectin compared with subjects in Cluster 1, associated with a higher increase in HBD-2, LL-37, sIgA and butyrate faecal levels ($P < 0.05$; Figure 3, panel 3).

Due to the limited amount of faecal sample collected by the parents, gut microbiota structure was investigated only in a subset of the infants (23 subjects in BB-12 group and 10 in the placebo group). The overall gut microbiota structure remained unchanged in infants enrolled in the BB-12 or in the placebo group. No difference in alpha-diversity index was observed upon treatment ($P > 0.05$). However, we found a significant increase in *Bifidobacterium* only in the responder infants (Cluster 2) treated with BB-12 ($P < 0.05$), and a significant increase in *Proteobacteria* in subjects enrolled in the placebo group ($P < 0.05$). Interestingly, the variation of *Bifidobacterium* induced by BB-12 treatment correlated significantly with the reduction of crying time (Figure 4).

4 | DISCUSSION

The results of this trial suggest that the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 is effective in the treatment of IC. Administration of BB-12 at a daily dose of 1×10^9 CFU was associated with treatment success (defined as the percentage of infants who achieved a reduction in the daily average crying time ≥50%) and reduced crying time, with beneficial effects on sleep duration and on stool frequency and consistency. The clinical effect on daily average crying time was already evident on the first week of treatment in infants receiving BB-12. All these variables have been considered as clinically relevant in previous clinical trials and meta-analyses.[21-23]

The results are well in line with data of a previous open-labelled trial reporting that BB-12, added to a low lactose partially hydrolysed whey formula, decreased the duration of crying time in infants with colic.[8] Also the results on stool pattern are well in line with the data from previous trials showing that BB-12 has a beneficial action on transit time and stool consistency.[24-27]

The study has several strengths. Main strengths are the randomised, double blind, placebo-controlled design, the use of validated procedure for IC diagnosis and the use of a well-defined probiotic strain with a well characterised genome sequence.[7] The latter is relevant considering the surprisingly common problems on the quality of probiotic products used for a wide range of conditions recently reported by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Working Group for Probiotics and Prebiotics.[28] The concomitant evaluation of immunity and inflammation biomarkers, and of gut microbiota structure and butyrate production could be relevant, also in helping our knowledge on the probiotics action in IC. Infants treated with BB-12 showed a higher increase of all immunity biomarkers (HBD-2, LL-37 and of sIgA) compared to subjects in the placebo group, suggesting that this probiotic strain is able to exert an immunomodulatory action in the infant gut. These data are well in line with previous findings showing that BB-12 modulates proliferation of human peripheral blood mononuclear cells and cytokines expression,[29,30] with protective action against gastrointestinal infections in infants and children. In the context of IC, these effects could be responsible for a beneficial shaping of gut microbiota structure. It is well-known that a positive modulation of HBD-2, LL-37 and sIgA expressions into the intestinal lumen results in a positive influence on gut microbiota structure and butyrate production.[15] These effects seem particularly relevant in IC, where dysbiosis with increased presence of *Proteobacteria* and decreased presence of *Bifidobacteria* with reduced butyrate production have been demonstrated.[31,32] A pathogenetic mechanism proposed for IC, is the increased intestinal gas production which can be caused by fermentation of carbohydrates and proteins by *Protebacteria*.[31,32] Similarly, *Bifidobacteria* have been associated with decreased amounts of crying.[33] We found that BB-12 is able to counteract all these events, inhibiting the *Proteobacteria* increase and facilitating the *Bifidobacteria* increase and butyrate production in infant with colic. These effects were observed in the vast majority but not in all infants enrolled in the BB-12 group, supporting the hypothesis that other factors could influence these effects. The beneficial role of *Bifidobacteria* in IC was also demonstrated by the significant correlation with the reduction of crying time observed in this trial. *Bifidobacteria* are not able *to* produce butyrate, but through cross-feeding other commensal bacteria, they can increase butyrate levels potential influencing many aspects of gut physiology.[34] Butyrate is a major gut microbiota metabolite able to exert a wide range of beneficial actions at intestinal and extra-intestinal level.[34] Butyrate modulates intestinal transit time, visceral and central pain perception and gut-brain axis, and exerts a potent anti-inflammatory action.[35-46] The faecal

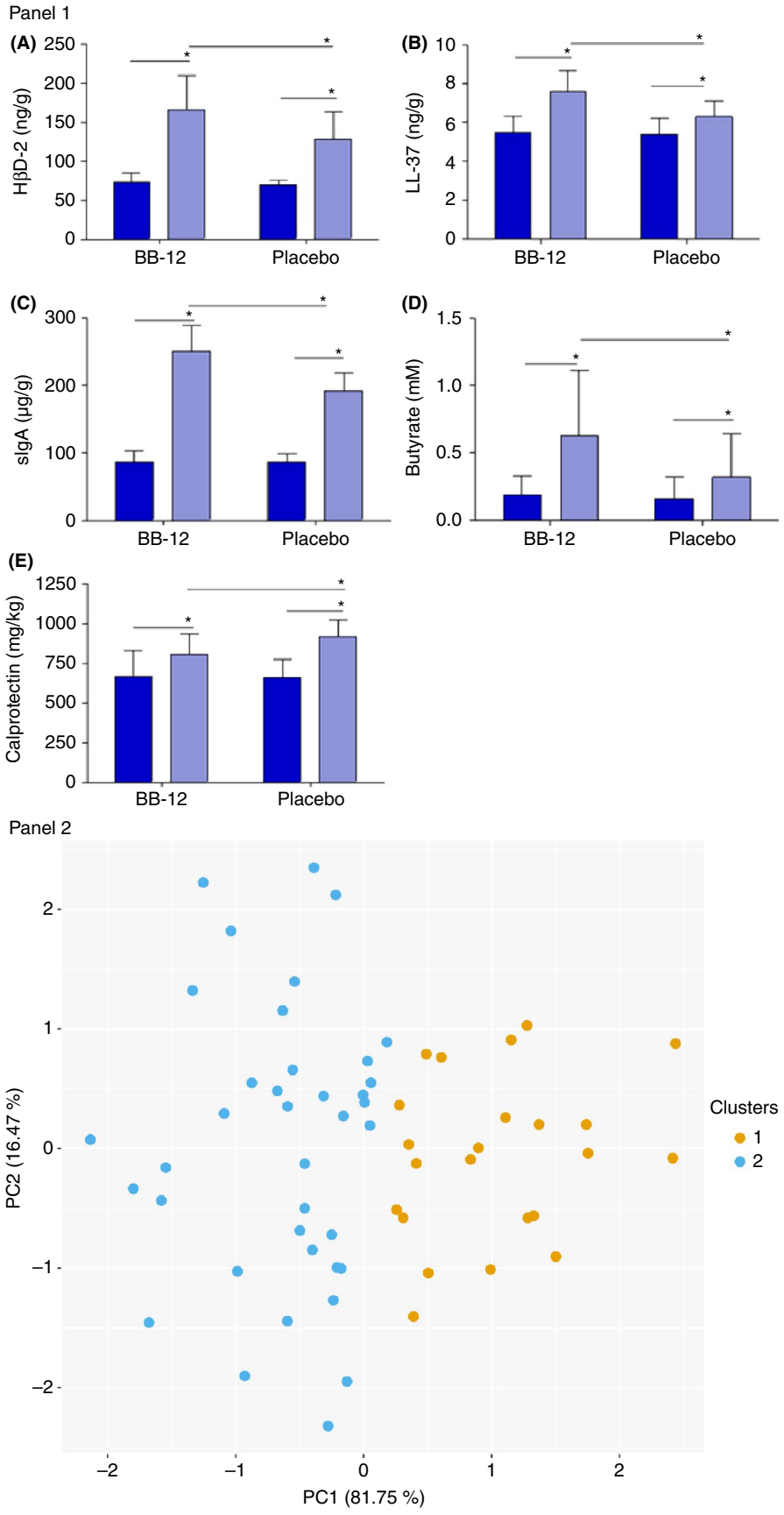

FIGURE 3 Panel 1. The values of innate and acquired immunity biomarkers, calprotectin and butyrate faecal levels at baseline during the week before treatment (V0-V1, blue bars) and during the last week of treatment (V4-V5, light blue bars) in the two study groups. Panel A: human β-defensin 2; panel B: cathelecidin (LL-37); panel C: secretory IgA; panel D: butyrate; panel E: calprotectin. Values are expressed as mean and SD and asterisks indicate a significant difference (* = $P < 0.05$). Panel 2. The *k*-means clustering ($k = 2$) of subjects based on the variation (V5-V1) of the crying time, beta-defensin 2, LL-37, sIgA, butyrate, faecal calprotectin levels after 28 days of treatment. Cluster 1 (yellow dot) included 10% of infants enrolled in the BB-12 group and 67% of infants enrolled in the placebo group. Whereas, Cluster 2 (blue dot) included 90% and 33% of BB-12 and placebo subjects respectively. Panel 3. The boxplots showing the variation (V5-V1) of crying time (in minutes), beta-defensin 2, LL-37, sIgA, butyrate and faecal calprotectin levels in subjects classified in Cluster 1 or 2. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 × IQR from the first and third quartiles respectively. Asterisks indicate a significant difference as obtained by pairwise Wilcoxon test ($P < 0.05$)

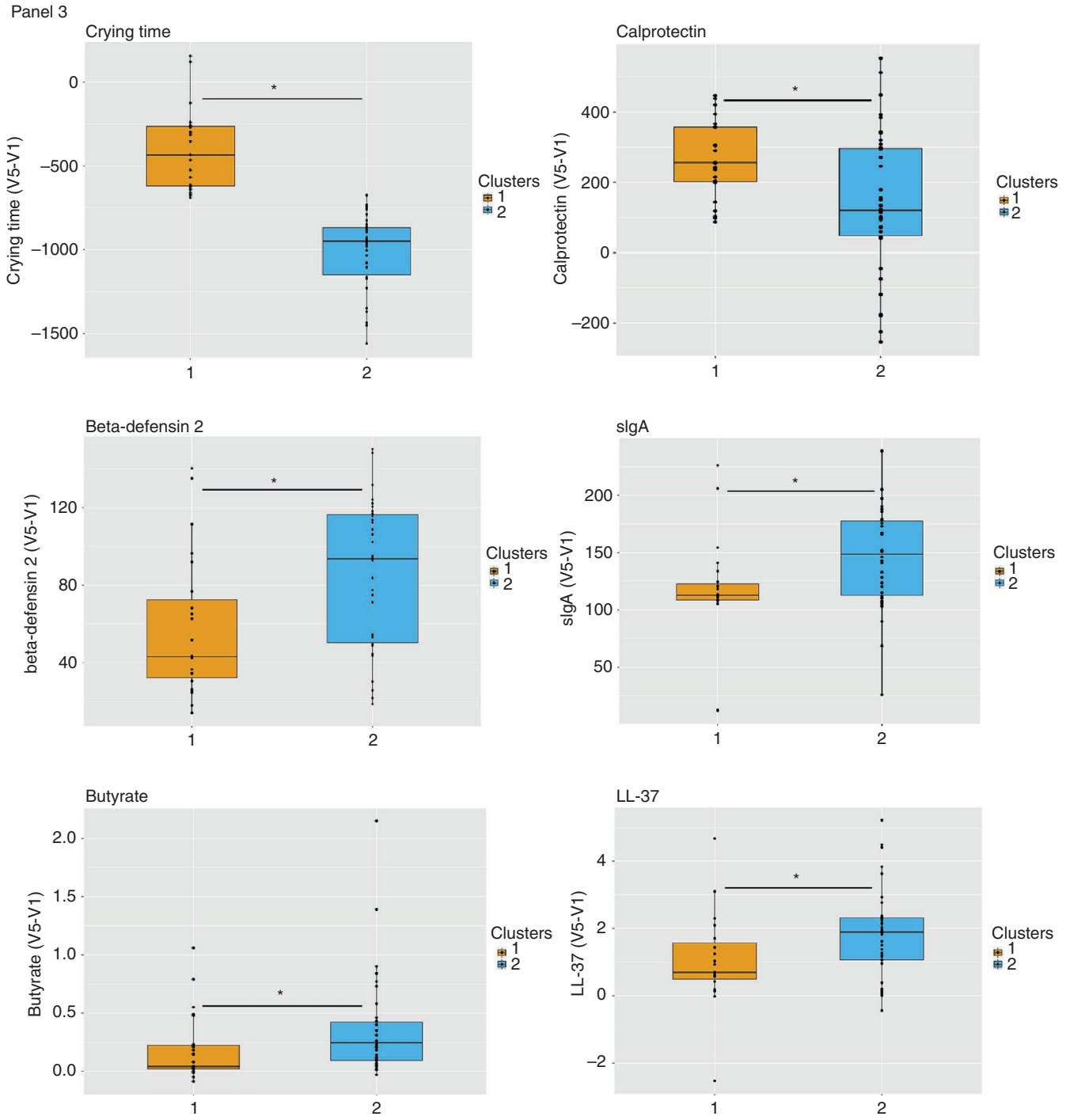

FIGURE 3 (Continued)

calprotectin features have been explored by only few authors with conflicting results.[47,48] We found a different modulation of calprotectin in responder infants to BB-12 intervention, suggesting that calprotectin could be involved in the modulation of the gut inflammatory state elicited by this probiotic.

Finally, it is well-known that butyrate modulates HBD-2, LL-37 and sIgA production,[15] supporting the hypothesis of multiple and connected actions elicited by BB-12 in IC.

The main limitations of this trial are related to the relatively small number of observations, the inclusion of subjects up to 7 weeks and the exclusion of formula feeding infants. All these points limit the generalisability of this study, and future trials may help in better elucidating the action of BB-12 in infant colic and gut homeostasis.

In conclusion, our study provides compelling evidences for the efficacy of *Bifidobacterium animalis* subsp. *lactis* BB-12 in the treatment of IC. These evidences further support the important role of gut microbiota as target of intervention against IC. It is relevant to underline that this trial studied a specific well-characterised probiotic strain, and that these findings cannot be extrapolated for other probiotic strains.

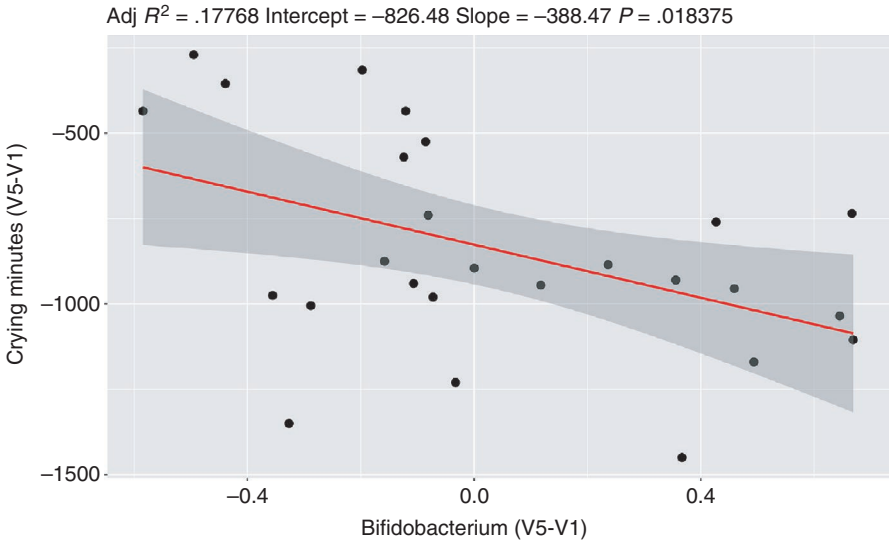

FIGURE 4 Linear regression of the duration of crying in minutes (V5-V1) as a function of the abundance of *Bifidobacterium* (V5-V1) ($P < 0.05$)

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AUTHORSHIP

Guarantor of the article: None.

Author contributions: RN and RBC conceptualised the study design, coordinated the research team and reviewed the manuscript. RN, RBC, FDF and DE drafted the manuscript and had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. RN, GC, AM, MM, CDS, AMI, SC, LC, LC, YM and GdG cared for the children. AC, CB, LP, CdC, FDF and DE performed laboratory analyses. RN and FDF performed the data analysis. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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SUPPORTING INFORMATION

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

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