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Lactobacillus acidophilus for infant colic and microbiota modulation: a randomized clinical trial

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ABSTRACT

Background: Infantile colic affects up to 25% of infants during the early weeks of life. Dysbiosis plays a prominent role in its pathogenesis, supporting the potential therapeutic role of probiotics. We aimed to evaluate the efficacy of *Lactobacillus acidophilus* NCFM versus placebo in infant colic and microbiota modulation. **Methods:** A double-blind, placebo-controlled randomized trial was conducted with 60 infants with colic, randomly assigned to receive five drops of L. acidophilus NCFM (1x109 CFU) or placebo for 28 days. Data on responders (subjects with a

colicky full-force crying/fussing time reduction of ≥50% from baseline), daily fussing and crying time, adverse effects, and maternal and child characteristics were collected. Changes in gut microbiota and fecal calprotectin were also evaluated. The study was registered at ensaiosclinicos.gov.br (RBR-5pp4nks). **Results:** The primary endpoint (proportion of responders at Day 28) was not significantly different between groups (p=0.240). A higher responder rate was observed in the intervention group at Day 14 in exploratory analyses (p=0.045); however, this result was attenuated after correction for multiple comparisons. No statistical difference was observed in calprotectin concentration (p = 0.687), infant mood (p = 0.720), fussing and crying time (p=0.524). A higher diversity was observed in the breastfed and formula-fed groups utilizing probiotics, but not in the formula-fed group receiving placebo. A high relative abundance of Akkermansia was identified in the probiotic group. Ruminococcus was identified in both groups before and after the intervention. **Conclusions:** Intervention did not significantly improve the proportion of responders at Day 28, the primary endpoint. An apparent benefit at Day 14 emerged in secondary. The use of L. acidophilus NCFM was able to improve some aspects of infants' intestinal microbiota. Also, there was no statistical difference in calprotectin and infant mood. The use of L. acidophilus NCFM was safe for this population.

Clinical Trial Registration: The study was registered at ensaiosclinicos.gov.br (RBR-5pp4nks), retrospectively registered at 09/09/2023.

Keywords: Probiotics, *Lactobacillus acidophilus*, colic, infant, microbiota.

1 BACKGROUND

Infantile colic can be considered a behavioral event, defined by Rome IV criteria for clinical research purposes as a healthy infant with crying or fussiness for ≥3 hours per day on ≥3 days within 7 days during a telephone or face-to-face screening interview with a researcher or clinician; in the selected infants, a total 24-hour crying plus fussing time of ≥3 hours is then confirmed using at least one prospectively kept 24-hour behavior diary. [1] Infantile colic affects

approximately 4–25% of infants in the first weeks of life and is a significant source of stress for parents. [2, 3] This stress has been associated with maternal depression and anxiety, [4] early discontinuation of breastfeeding, [5] and, in extreme cases, shaken baby syndrome. [6]

The pathophysiology of infantile colic is multifactorial and remains only partially understood. Epidemiological studies have reported an association between maternal migraine and infant colic, suggesting that genetic, neurobiological, and environmental factors may contribute to its development. [7] These observations support the concept of a shared vulnerability within the gut–brain axis, in which early disturbances in gastrointestinal function and nociceptive pathways may be linked to migraine and other pain syndromes.

Environmental factors implicated in infantile colic have focused largely on the intestinal microbiota and its role in the microbiota–gut–brain axis. Lower gut microbiota diversity and a greater prevalence of fecal coliforms, together with a reduced prevalence of *Lactobacillus*, have frequently been associated with infantile colic. [8, 9] More recently, observational data have suggested that an increase in Ruminococcus in the gut microbiota of infants may be associated with the development of colic. [10] These findings indicate that dysbiosis—characterized by qualitative and quantitative alterations in microbial composition—may contribute to abnormal gas production, mucosal immune activation, and altered visceral pain perception in early life.

In parallel, fecal calprotectin has emerged as a useful, non-invasive biomarker of intestinal inflammation in both adults and children. It is a calcium-binding protein derived predominantly from neutrophils and excreted in the stool, with higher levels reflecting increased neutrophil migration to the intestinal lumen. Elevated fecal calprotectin concentrations have been reported in various inflammatory and some functional gastrointestinal conditions, and may reflect subclinical mucosal inflammation or increased intestinal permeability in infants. Within the framework of the microbiota–gut–brain axis, intestinal inflammation and barrier dysfunction are considered potential mediators linking dysbiosis to visceral hypersensitivity and pain. [11,12]

To address infantile colic and its consequences, several clinical trials have evaluated probiotics as a therapeutic strategy. Supplementation with different probiotic strains has been reported to reduce crying and fussing time in some studies. [13-19] The specific use of *Lactobacillus acidophilus* SD-5221 (L. acidophilus) has shown promise as a treatment option, primarily because of its capacity to modulate visceral pain. [20-22] This strain has demonstrated the ability to ferment human milk oligosaccharides (HMOs), [23] producing metabolites such as acetate and lactate, similar to those generated by Bifidobacterium. [24] The production of these compounds has been associated with suppression of opportunistic microorganisms, [25, 26] modulation of intestinal motility and perfusion,[25] promotion of an anti-inflammatory milieu, [27] immunomodulation, [28] and modulation of microbiota–gut–brain communication, potentially leading to attenuation of abdominal pain. [29]

In addition, probiotics may influence the expression of opioid and cannabinoid receptors in intestinal cells, [21] in a manner analogous to some actions of morphine, [22] thereby affecting neuronal activity and brain function. Such modulation suggests that L. acidophilus NCFM may indirectly impact the central nervous system and help regulate an infant's response to pain and discomfort. [20]

Therefore, the aim of this study was to evaluate the efficacy and safety of L. acidophilus NCFM supplementation in treating infantile colic, with specific attention to clinical response (responders), infant mood, intestinal inflammation as assessed by fecal calprotectin, and modulation of the intestinal microbiota.

2 METHODS

2.1 Study design and population

This randomized, double-blind, placebo-controlled clinical trial was registered at ensaiosclinicos.gov.br (RBR-5pp4nks) on September 9, 2023, i.e., after the completion of

participant recruitment. The protocol, including the primary outcome (proportion of responders at day 28) and main secondary outcomes, had been specified in writing before the start of recruitment and was not modified in response to accumulating trial data. The protocol and procedures presented in the project are in full accordance with the Brazilian legislation (Resolution CNS 466/2012) and the declaration of Helsinki regarding ethical standards in conducting research involving human beings. Ethical approval was granted by the Research Ethics Committee of the Universidade Federal de Goiás (UFG), Brazil (protocol CAAE 40846520.5.00005078). It was conducted in Goiânia, Brazil. The study protocol is presented in Figure 1 in accordance with the CONSORT statement. Infants were recruited at Nascer Cidadão and Dona Iris Maternity Center.

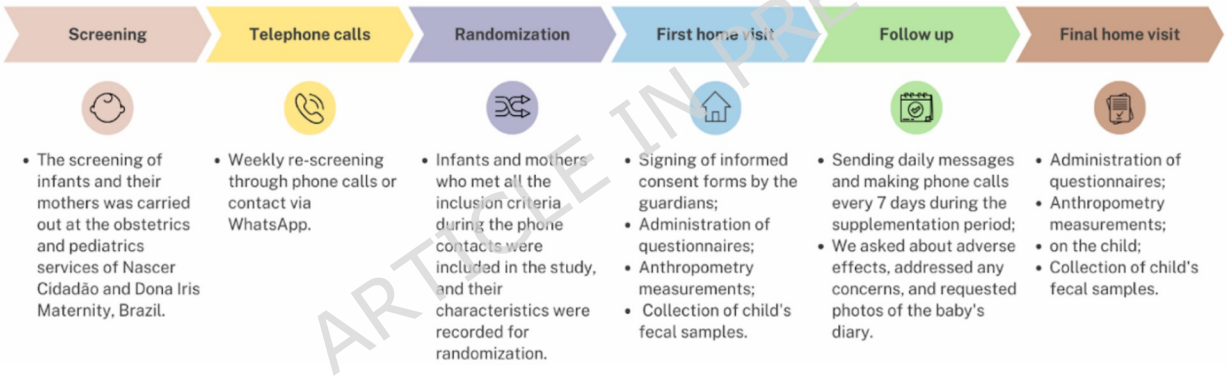

Figure 1 – Study Protocol

The eligibility criteria were born at term (gestational age ≥37 weeks), birth weight above 2500 g, and a diagnosis of colic according to the Rome IV criteria[1], aged 2–12 weeks at enrollment. The exclusion criteria were acute or chronic severe illnesses; gastrointestinal diseases/malformations and gastroesophageal reflux; medical diagnosis of cow's milk protein allergy; maternal use of probiotics or antibiotics during delivery and breastfeeding; infant use of probiotics and antibiotics since birth; underage mothers; and use of antibiotics during the study by infants or breastfeeding mothers. Parents or guardians signed written informed consent. The infants were randomly assigned to two groups: (a) the *L. acidophilus* NCFM® group, which

received daily oral administration of five drops of *L. acidophilus* NCFM® (DuPont Danisco® – Flora B® – Cifarma Científica Farmacêutica LTDA, ATCC Strain deposit number: SD-5221) in sunflower oil and a medium-chain triglyceride suspension ($1x10^9$ colony-forming units (CFUs) per five drops); and (b) the placebo group, which included infants who received daily oral administration of five drops of sunflower oil and a medium-chain triglyceride suspension. Caregivers were instructed to offer the supplement once daily for 28 days, preferably in the morning, and not to offer the infants any other probiotic products. There were no dietary restrictions for mothers during the intervention period. Adherence was monitored by phone messages and diary entries.

2.2 Randomization

Eligible infants were randomized in a 1:1 ratio to receive either *Lactobacillus acidophilus* NCFM® or placebo. An independent researcher generated the random sequence using Research Randomizer (Version 4.0) [31], with stratification by feeding type (exclusive breastfeeding vs. exclusive formula feeding or mixed breastfeeding) and block sizes of 8. Allocation cover-up was ensured by keeping the sequence with a third party not involved in recruitment, and investigators enrolling participants had no access to these data. Another person not involved in the trial, packaged the probiotic and placebo in indistinguishable containers labeled only with participant identification codes. Study products were dispensed according to these codes. The placebo was identical to the probiotic in appearance, color, odor, taste and volume. Participants, caregivers, investigators, and statisticians remained blinded throughout follow-up, and the allocation code was released only after data collection and primary analyses were completed.

2.3 Study outcomes

The primary outcome was the proportion of responders at day 28, defined as infants with a ≥50% reduction in mean daily crying time compared with baseline, and the corresponding response

rate. All other time points (e.g., Day 7 and Day 14) were pre-specified as secondary outcomes. Between-group differences in the proportion of responders at each time point were compared using the chi-square test (or Fisher's exact test when appropriate). Because responder status was evaluated at multiple time points, we applied a Holm–Bonferroni correction to control the familywise error rate across these comparisons. Unadjusted and adjusted p-values are reported for secondary time points, and these analyses are interpreted as exploratory. All tests were two-sided, with a significance level of $\alpha=0.05$ for the primary endpoint. Other secondary endpoints were not powered for definitive inference. Secondary outcomes were: (a) microbiota modulation, (b) fecal calprotectin level measured at baseline and day 28, (c) child's mood, and (d) fussing and crying time (minutes/day) recorded by caregivers using a diary registry at baseline and throughout follow-up. All randomized infants were included in the intention-to-treat (ITT) population, defined as all 96 participants analyzed according to their original group assignment, irrespective of adherence, protocol deviations, withdrawal, or post-randomization exclusion. The per-protocol (PP) population comprised infants who received the allocated intervention, had outcome data at Day 28, and did not present major protocol violations. Adverse events, including gastrointestinal symptoms (vomiting, bloating, diarrhea) were monitored during the 28-day supplementation period.

2.4 Analysis of colic symptoms

Daily crying and fussing time, bowel movement number and characteristics (modified Bristol scale) [32], and the child's abdominal symptoms and mood, as reported in the baby diary, were recorded.

To support adherence, caregivers received daily reminders via WhatsApp to administer the probiotic/placebo and complete the diary. Also, blinded weekly phone calls were conducted to resolve questions and to review entries. Growth parameters (weight gain during the period), stool

characteristics (frequency and consistency), and symptoms of digestive intolerance (constipation or vomiting) were evaluated to assess tolerance of the probiotic and possible adverse effects.

2.5 Gut microbiota and calprotectin analysis

Intestinal microbiota and fecal calprotectin data were collected at the beginning of the experiment and after 28 days of supplementation. The samples were stored at -80°C for microbiota analysis and -20°C for calprotectin analysis. Fecal calprotectin was measured using the RIDASCREEN® Calprotectin Kit (R-BIOPHARM) according to the manufacturer's instructions.

DNA sequencing was performed by Neoprospecta® - Brazil. The 16S rRNA hypervariable region V3/V4 was amplified through polymerase chain reaction using gene-specific primers with the following Illumina adapters: 341F GTGCCAGCMGCCGCGGTAA and 806R GGACTACHVGGGTWTCTAA.

The raw reads were analyzed using QIIME2 (Release 2020.8) [33] using default parameters for trimming and joining paired ends. The DADA2 (Divisive Amplicon Denoising Algorithm 2) plugin was used to denoise the reads, remove chimeric sequences, and generate amplicon sequence variants (ASVs), which were clustered into operational taxonomic units (OTUs) at 99% similarity using the VSEARCH (Vectorized Search) plugin. Taxonomic assignment to each OTU was performed using the SILVA [34] database (Release 138), with filtering to exclude mitochondria, chloroplasts, and eukaryotic taxa.

2.6 Sample size calculation and statistical analysis

A sample size calculation, aiming for 80% power and α=0.05, utilized responder rates from the study of Szajewska et al. (2013) at day 28 (100% for probiotic, 62.5% for placebo). This yielded a requirement of 16 participants per group, reflecting the 37.5% absolute difference in responder

rates. Adjusting for a 20% attrition rate, approximately 20 participants per group would be needed, totaling 40 participants, to ensure sufficient power.

Associations between categorical variables were evaluated by using the chi-square test or Fisher's exact test. All reported P values are 2-sided; differences were considered significant when $P \leq 0.05$. The data were analyzed using Statistical Package for the Social Sciences, SPSS 20 (SPSS Inc., Chicago, IL).

Data analysis and visualizations of the microbiota were carried out in R studio (version R 3.6.1) via the phyloseq, vegan, ggplot2, and microbiome packages. The α diversity and Chao1 and Shannon indices are presented, and statistical significance was determined via the Mann–Whitney test with Dunn's adjustment. β diversity was calculated using unweighted and weighted UniFrac (Unique Fraction metric) distances and represented in a principal coordinate analysis (PCoA) plot for each analysis. Statistical analysis of β diversity was based on permutational multivariate analysis of variance (PERMANOVA) via the functions adonis and pairwise adonis.

3 RESULTS

Between August 2021 and June 2023, 3,369 infants were born during the recruitment period. Of these, 3,273 were excluded after screening for one or more of the following reasons: maternal or infant antibiotic exposure, absence of infantile colic during follow-up, low birth weight, prematurity, probiotic use by the mother or infant, gastrointestinal disease, residence outside the study area, missed contact, or decline to participate. A total of 96 infants were randomized (Figure 2). Of these, 16 infants withdrew from the study: 7 from the *L. acidophilus* group and 9 from the control group. Additionally, we excluded 15 infants from the analysis, 8 in the *L. acidophilus* group and 7 in the control group, for the following reasons: careless or absent entries in the baby diary (n= 3), use of antibiotics by the breastfeeding mother or infant (n=5), use of

another probiotic by the infant (n=5), and a medical diagnosis of reflux (n=2) For the ITT analysis, all 96 randomized infants were included and analyzed according to their original group assignment. The PP population consisted of 32 infants in the intervention group and 28 infants in the control group who completed follow-up without major protocol deviations.

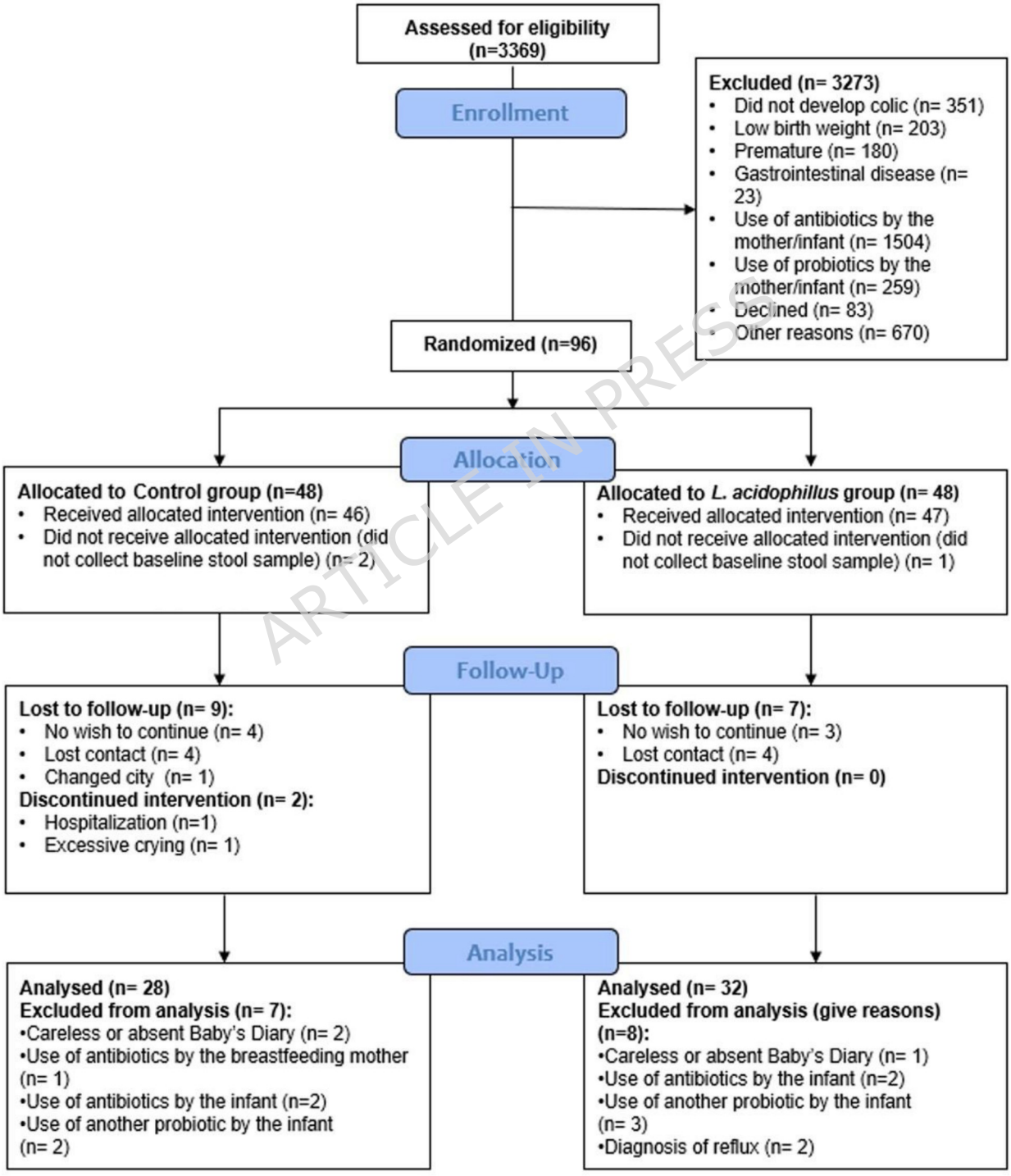

Figure 2 - Consolidated Standards of Reporting Trials (CONSORT) flow diagram.

The baseline demographic and clinical characteristics of the two groups were similar, as shown in Table 1.

Table 1. Baseline characteristics of the participants in the two study groups.

Variables	*L. acidophilus* *n=28*	*Control* *n=32*	P
Male, n (%)*	15 (46.9)	15 (53.6)	0.60[a]
Gestational age (weeks)	39 (38.5-40)	39 (38-40)	0.30[b]
Birth weight (g)	3224.6 ± 414.8	3189.3 ± 336.3	0.72[c]
Age on day 0 (days)	28.9 ± 11.9	31.7 ± 9.2	0.10[b]
Weight on day 0 (g)	4095.5 ± 621.4	4306.7 ± 577.1	0.18[c]
Length on day 0 (cm.)	54.2 ± 2.9	54.3 ± 2.1	0.92[c]
Exclusive breastfeeding on day 0, n (%)	25 (78.1)	20 (71.4)	0.57[a]

Continuous variables are presented as the mean ± standard deviation or *median (interquartile range (IQR)). P value was obtained by [a] the chi-square test, [b] the Mann–Whitney test, or [c] Student's t test, all with a significance level of 5%.

All infants resided in urban areas and had no prior clinical or surgical events. In both the *L. acidophilus* and control groups, infants were delivered vaginally at term and had appropriate weights for their gestational age. None of the infants or breastfeeding mothers had received antibiotics or probiotics from infant birth until their enrollment in the study, and this remained consistent throughout the trial period.

3.1 Outcomes

Between-group differences in the number responders throughout the treatment were evaluated (Table 2). Primary outcome: at Day 28, the proportion of responders did not differ significantly between the intervention and control groups (17.9% vs. 6.4%; unadjusted p=0.240). Secondary time points (exploratory analyses): at Day 14, a higher proportion of children in the intervention group were responders compared with the control group (22.2% vs. 3,3%; unadjusted p=0.045). After adjustment for multiple comparisons across the evaluated time points, the p value for the Day 14 comparison was p=0.180.

Table 2. Effectiveness (number of responders) of *L. acidophilus* versus placebo throughout the study.

	L. acidophillus n=28 (%)	*Control n=32 (%)*	p (ITT*)	p (PP**)	P (PP adjusted §)
Day 7	6(22.2)	7(22.6)	0.764[a]	0.974[a]	0.974
Day 14	6(22.2)	1(3.3)	0.050[b]	0.045[b]	0.180
Day 21	1(3.6)	5(16.7)	0.092[b]	0.195[b]	0.585
Day 28	5(17.9)	2(6.4)	0.239[b]	0.240[b]	0.585

[a] – Chi-square test; [b] – Fisher's exact test; *ITT – number needed to treat; **PP – per protocol; §PP adjusted by Holm–Bonferroni correction.

The proportion of responders changed throughout the treatment (Table 2). On day 7, responder rates were similar between groups: 6 infants (22.2%) in the probiotic arm and 7 (22.6%) in the placebo arm (p=0.974). By day 14, the probiotic group maintained 6 responders (22.2%), whereas the placebo group dropped to only 1 responder (3.3%), yielding a statistically significant difference favoring *L. acidophillus* (p=0.045). At day 21, responder rates were low and not significantly different—1 responder (3.6%) with *L. acidophillus* versus 5 (16.7%) with placebo (p=0.195). By day 28, responders increased again in the probiotic arm to 5 infants (17.9%) compared with 2 (6.4%) in placebo, but this difference did not reach significance (p=0.240). In

the intention-to-treat (ITT) analysis, all 96 randomized infants (48 in each group) were included and those who discontinued the study were considered non-responders. Using this conservative approach, the proportions of responders in the L. acidophilus and placebo groups were, respectively, 12.5% vs 14.6% at day 7 (p = 0.764), 12.5% vs 2.1% at day 14 (p = 0.050), 2.1% vs 10.4% at day 21 (p = 0.092), and 10.4% vs 4.2% at day 28 (p = 0.239). Comparison of the median (95% confidence interval (CI)) fussing and crying times showed that these times were similar between the control and *L. acidophilus* groups at each study time point (Table 3).

Table 3. The median fussing and crying times (minutes/day) in the control and *L. acidophilus* groups

Weeks of study (median (95% CI)	*L. acidophillus*	*Control*	P between group [a]
Number evaluated	*n=28*	*n=32*	
1st week	126.6 (120.26-183.74)	139.64 (115.17-191.85)	0.847
2nd week	118.93 (89.35-142.16)	120.00 (68.70-122.99)	0.620
3rd week	73.64 (68.70-122.99)	80.00(64.44-133.67)	0.722
4th week	70.00 (44.63-106.22)	76.90 (46.36-101.82)	0.524
p intragroup [b]	<0.001	<0.001	

[a] Mann–Whitney

The calprotectin levels were similar at baseline and after 28 days in both groups (*L. acidophilus*: 410.8 ± 497.5 mg/dL vs. control: 425.3±565.2 mg/dL, P=0.91 at baseline; *L. acidophilus*: 535.5 ± 653.1 mg/dL vs. control: 549.3 ± 541.2 mg/dL, P=0.69 after 28 days). A reduction in crying time and an increase in time the child a good mood was observed in both groups over 28 days (Figure 3).

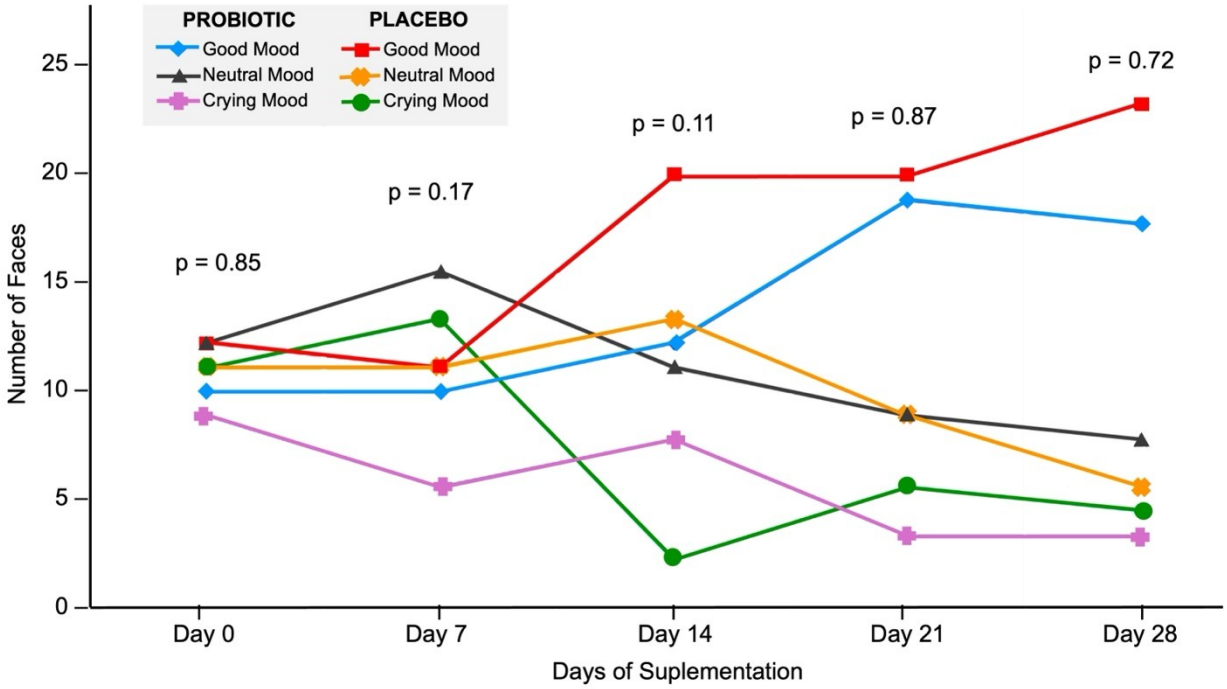

Figure 3 – Reduction in crying time and an increase in time the child a good mood along time study.

3.2 Compositional variability of the microbiota

Six main phyla were identified in gut: Firmicutes, Bacteroidota, Actinomycetota, Proteobacteria, Fusobacteriota, and Verrucomicrobiota. No significant differences were observed in the α diversity measurements. Weighted and unweighted UniFrac distances were used to evaluate β diversity among the stool samples (Figure 4).

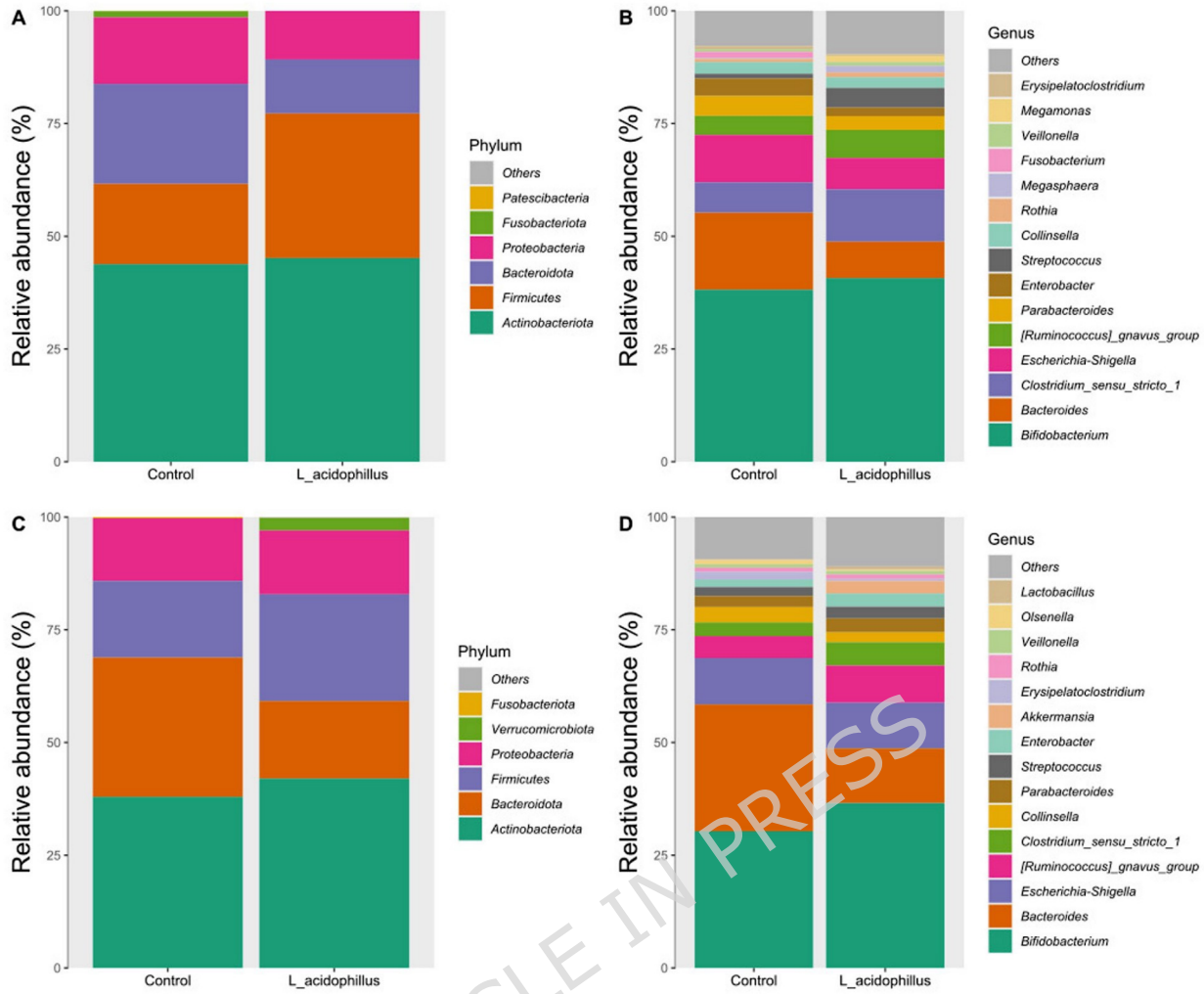

Figure 4 - Relative abundance of various phyla and genera in the gut in the control and *L. acidophilus* groups: A and B, most abundant phyla and genera before the intervention; C and D, most abundant phyla and genera after the intervention.

Comparisons between the subgroups revealed differences in β diversity, highlighting that after 28 days of placebo administration in the control group, analyses revealed statistically significant differences in the β diversity of the intestinal microbiota between exclusively breastfed infants and infants receiving both breastmilk and formula or exclusively formula. Significant differences in the β diversity were observed in the exclusively breastfed and formula-fed groups that utilized *L. acidophilus* but not in the formula-fed group that received placebo (Figure 5).

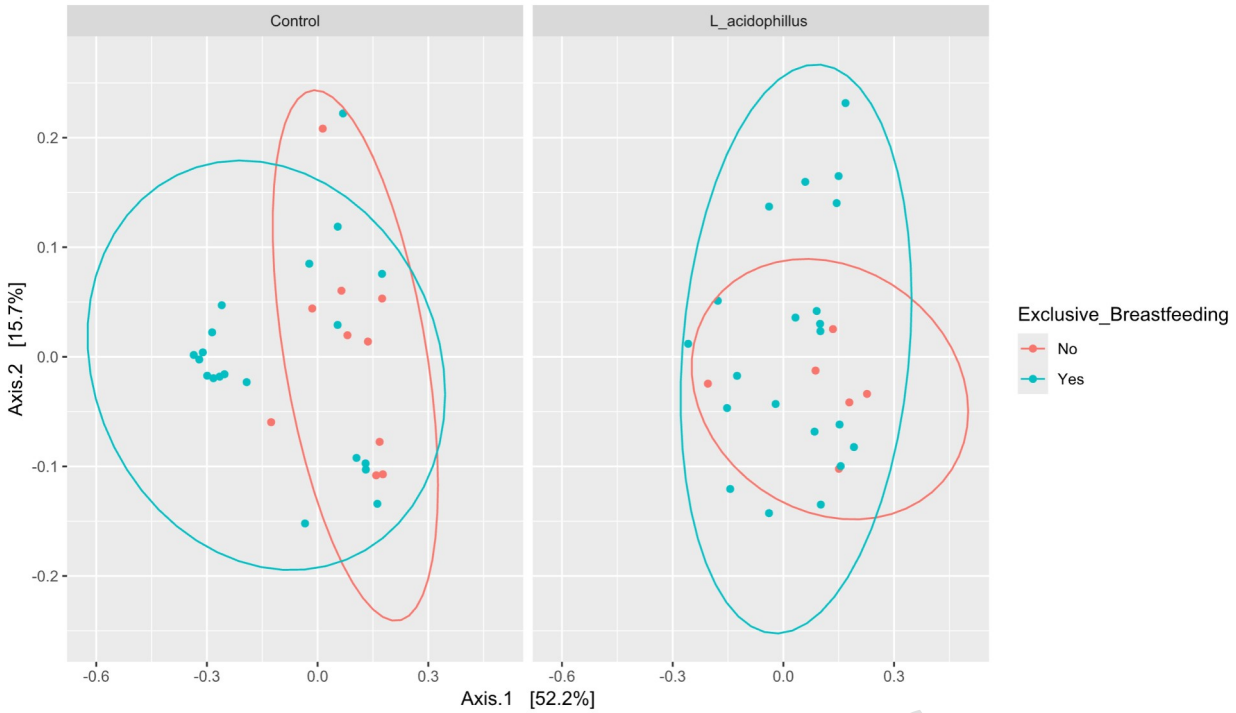

Figure 5 – β diversity (weighted unifrac) in the exclusively breastfed and formula-fed groups before and after the intervention. Control > p= 0,011; *L. acidophilus* > p= 0,705.

After intervention, linear discriminant analysis (LDA) revealed a significant difference in the relative abundances of the bacterial genera *Akkermansia* (control group – 0.0%, *L. acidophilus* group – 2.77%), *Lactobacillus* (control group – 0.23%, *L. acidophilus* group – 0.72%) and *Atopobium* (control group – 0.23%, *L. acidophilus* group – 0.41%). However, the relative abundance of *Akkermansia* remained very low (2.77%). Given this low proportion and the limited power of our study to detect microbiota–clinical correlations, this finding should be considered exploratory and does not, by itself, support a clinically effect on gut microbiota composition. (Figure 6).

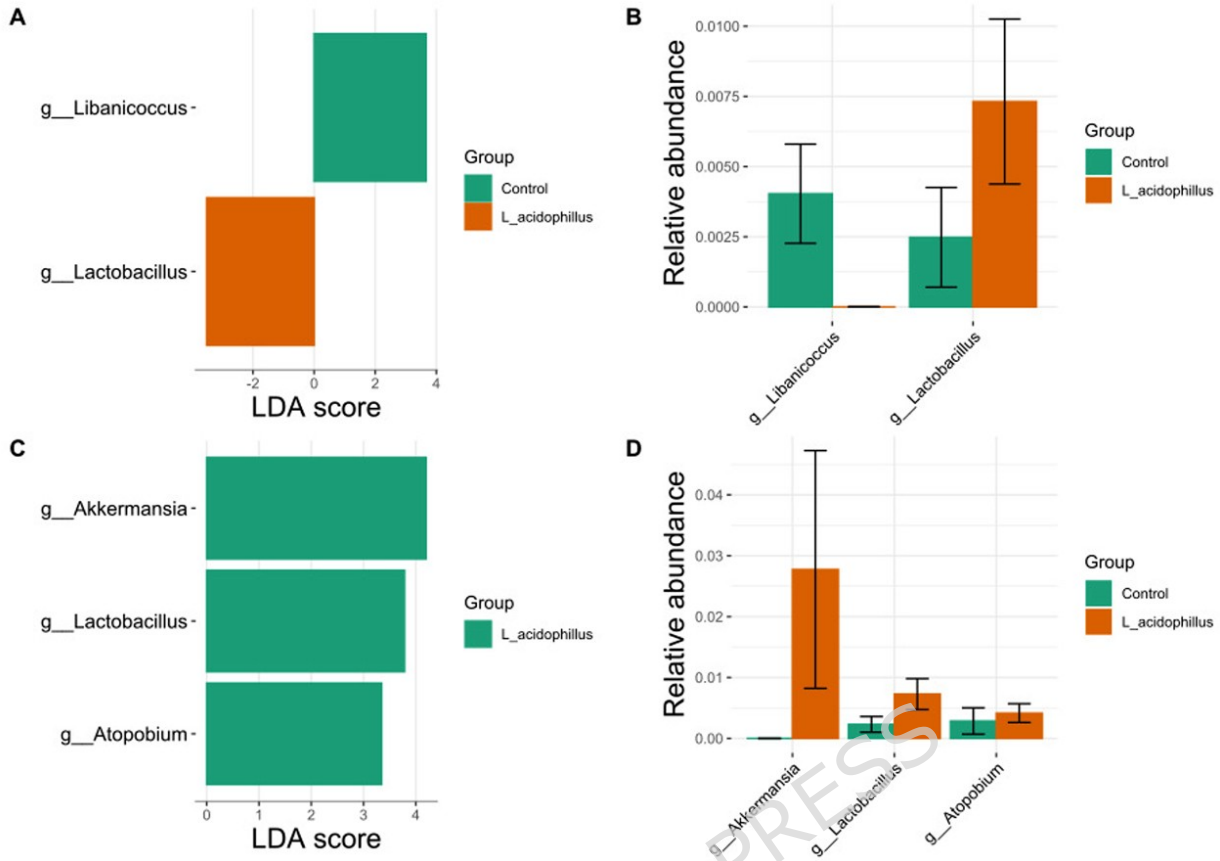

Figure 6 - Linear discriminant analysis (LDA) score and relative abundance of bacterial genera that were significantly different between the control and *L. acidophilus* groups, according to the LDA effect size (LEfSe) model.

3.3 Adverse effects

There were no unexpected or severe adverse events in either group. One mother from the *L. acidophilus* group reported an increase in the crying time and withdrew from the study. No significant differences in median weight gain per day (*L. acidophilus* = + 42.0 ± 11.11 g vs. control: + 40.0 ± 17.8 g, P = 0.665), frequency of crying/fussing episodes (*L. acidophilus* = + 1.9 ± 1.5 episodes/day vs. control: 2.2 ± 2.1, p = 0.822) or symptoms of digestive intolerance (*L. acidophilus* = 2 (7.7%) – constipation vs. control: 1 (5.0%) P = 0.785) were observed between the groups. There were no reports of increases in regurgitation or vomiting during the study.

4 DISCUSSION

This trial revealed that the *L. acidophilus* NCFM® did not produce a statistically significant increase in the proportion of responders at Day 28, the predefined primary endpoint. A possible benefit at Day 14 was observed only in secondary, exploratory analyses. There was no statistically significant difference in the level of calprotectin or infant mood after 28 days of study. Additionally, *Akkermansia* was predominant in the *L. acidophilus* NCFM® group, and β diversity was significantly greater in the exclusively breastfed placebo group, the breastfed *L. acidophilus* group, and the formula-fed *L. acidophilus* group than in the placebo formula-fed group. The use of *L. acidophilus* NCFM® was safe for this population.

Infant colic has a natural history in which symptoms improve as the baby grows. However, providing relief from symptoms can help families by reducing stress, repeated visits to healthcare services and others' problems [3]. The gradual increase in the number of children with a 50% reduction in crying or more compared with baseline can be positive, another notable factor is the difference at day 14, where the *L. acidophilus* NCFM® group had significantly more responders. An additional finding of our study was the considerable volatility in responder rates across the follow up visits, particularly in the probiotic group. This pattern is unlikely to reflect a simple, monotonic biological effect of the intervention and is more consistent with a combination of sampling variability, the inherently fluctuating clinical course of infant colic, and the use of a dichotomous responder definition. Because our sample size at each time point was modest, even a small number of infants crossing the responder threshold between visits can produce large relative changes in the observed proportions. Taken together, the observed instability in responder rates, the non-significant primary endpoint at day 28, and the sensitivity of our binary responder definition all argue for a cautious interpretation of the time course data. We therefore regard the intermediate time point findings, including the apparent early benefit at day 14, as exploratory and hypothesis generating rather than confirmatory evidence of a consistent treatment effect. A study with a larger sample size could resolve this uncertainty. A meta-analysis including 32 studies published from 1960 to 2015 involving 2332 children

revealed that probiotic use (*Limosilactobacillus reuteri* DSM 17938) had superior outcomes compared with other interventions, such as massage, dietary modifications in mothers, acupuncture, and pharmacological changes. [35] It is important to note that, although the results appear promising, the use of probiotics for infant colic remains controversial, particularly with regard to strains other than *L. reuteri* DSM 17938.[36] For a formal recommendation of a probiotic strain, more than one randomized placebo-controlled clinical trial in different populations is needed to prove the expected benefit.

Calprotectin is a marker of intestinal inflammation, although nonspecific, and calprotectin levels are frequently associated with infantile colic.[11, 12] Findings suggest that in young infants, high fecal calprotectin levels are normal,[37] and high calprotectin levels are associated with favorable development of the gut microbiota and immune system in infants.[38] However, other studies have demonstrated a relationship between increased levels of calprotectin and the diagnosis of infantile colic [11, 12] as well as a reduction in calprotectin levels concurrent with the resolution of colic after probiotic supplementation. [18] In our study, both groups presented high and variable calprotectin values, with no significant reduction after supplementation; our data reinforce that calprotectin alone is not a good marker of clinical response in colic. The complex interplay among the intestinal microbiota, immune system, and calprotectin levels in infants is still not fully understood, and individual variations in these factors, such as feeding type,[39] may influence the response to probiotic supplementation. Further research is needed to elucidate the mechanisms underlying the association between calprotectin levels and infantile colic.

In recent years, alterations in the infant microbiota have been elucidated as a possible pathogenic mechanism of infantile colic.

Studies have shown that there is an increased in microbial abundance and biodiversity, reflected by higher alpha diversity, in infants with colic compared to those without colic.

The bacterial α diversity in the infants in the current study was likely to be more closely associated with other specific factors, such as exclusive breastfeeding, rather than the resolution

of colic or the use of probiotics. This outcome aligns with the findings of Rhoads et al., who reported similar α diversity in children with and without colic, along with lower α diversity in breastfed children (Shannon, P = 0.0001; Simpson, P = 0.0004).[40]

Various studies have attempted to identify a microbial signature for infants with colic. It has been reported that these patients have a lower relative abundance of *Actinobacteriota* and *Bifidobacterium*[10, 41]. However, this study observed that their relative abundance was increased in patients with colic before intervention. This demonstrates that other factors may interfere with the determination of this signature, such as geographical aspects, maternal diet, and lifestyle habits.

This study demonstrated lower β diversity in the formula-fed placebo group than in the formula-fed *L. acidophilus* NCFM® group. Several studies have shown conflicting results regarding the role of probiotics in infant formula use to mimic the beneficial effects of exclusive breastfeeding on the microbiota. Establishing this causality is difficult because microbiota imprinting is highly complex and influenced by multiple factors, including type of delivery, dietary practices, environmental variables, and microbial exposures.[42,43]

Not all infants who received probiotics responded in the same way at 28 days of supplementation: individual variations in probiotic responses can further contribute to the temporal variability in β diversity outcomes. Notably, in the present study, probiotics played a pivotal role in shaping the infant colonic microbiota, especially in formula-fed infants.

Importantly, the *L. acidophilus* NCFM® group presented an increased LDA score for *Akkermansia*.

Akkermansia can modulate the immune system by strengthening anti-inflammatory responses through increased interleukin (IL)-10 levels and elevated expression of tight junction proteins, making the gut less permeable to pathogen translocation[43,44]. Additionally, the abundance of the *Akkermansia* genus is linked to various benefits, such as improved therapeutic responses to oncological immunotherapy and reduced incidences of depression, osteoporosis, chronic kidney

diseases, and tuberculosis[43]. With respect to gut–brain axis disorders, evidence indicates that individuals with irritable bowel syndrome who experienced an increase in *Akkermansia* abundance after fecal microbiota transplantation reported reduced pain intensity, highlighting the positive role of this genus in these conditions[44]. Furthermore, in children, higher abundance of *Akkermansia* has been associated with milder asthma symptoms, increases in linear growth, and a less severe course of atopic dermatitis, among other advantages.[43,44] Nevertheless, the observed increase in *Akkermansia* in the probiotic group, although statistically detectable, occurred at a very low relative abundance (2.77%) and should therefore not be over-interpreted in terms of clinical relevance. Although this is the first study to provide evidence of *Akkermansia* relative abundance in children supplemented with *L. acidophilus* NCFM®, our trial was not designed to determine whether *L. acidophilus* directly promoted *Akkermansia* growth. In addition, unmeasured or incompletely controlled factors—such as feeding method (breastfeeding versus formula) and other environmental exposures—may have contributed to the observed shift. Consequently, the *Akkermansia* signal in our data should be regarded as hypothesis-generating, and further mechanistic studies are needed before any clinically meaningful interpretation can be made.

Another interesting point in this study was the presence of *Ruminococcus* in patients before and after the intervention. In a large-scale study involving over 1,000 Dutch families, they reported associations between infant gut microbiota and gastrointestinal symptoms, identifying high *Ruminococcus gnavus* relative abundance as a risk factor for colicky crying.[10]

At first glance, it could be said that the *L. acidophilus* NCFM® group intervention was not effective because it did not favorably modulate the microbiota, reversing this signaling. However, further studies are delving deeper into the role of *Ruminococcus* and its potential roles in the mechanism of health and disease in individuals.

Among the potential beneficial effects of *R. gnavus* is its ability to metabolize important oligosaccharides in human milk such as 2′-fucosyllactose (2′FL), 3-fucosyllactose (3FL), and N-

acetyl lactosamine (LacNAc), promoting the intestinal colonization of other beneficial microorganisms. They also aid in protein metabolism by preventing the oxidation of amino acids, which results in the promotion of protein synthesis and lean mass formation. In relation to the immune system, it has been observed that infants with atopic dermatitis have reduced intestinal colonization of *Akkermansia muciniphila, Ruminococcus gnavus,* and *Lachnospiraceae bacterium*[46,47].

In our cohort, *Ruminococcus* was present in both groups at baseline and persisted after the intervention, without a clear pattern indicating probiotic induced suppression or favorable modulation. These findings therefore do not support the notion that our intervention beneficially altered *Ruminococcus* in a way that could explain clinical improvement. It is possible that strain level differences or context dependent interactions modulate the functional impact of *Ruminococcus*, but such hypotheses remain speculative and were not addressed by our sequencing approach. Accordingly, we have refrained from characterizing the presence of *Ruminococcus* as beneficial and instead interpret our microbiota results as descriptive observations that should be confirmed and refined by larger, mechanistic studies.

A key methodological limitation of this study is the retrospective registration of the trial. Recruitment took place between August 2021 and June 2023, whereas protocol registration occurred on September 9, 2023. Although the primary and secondary outcomes, eligibility criteria, and main analytical approaches were defined a priori in the written protocol and were not altered on the basis of the observed results, retrospective registration does not fully prevent concerns regarding potential selective outcome reporting or analytical flexibility. This deviation from recommended practice may therefore limit the transparency and perceived robustness of the evidence and should be taken into account when interpreting our findings.

An important strength of this study is the use of an ITT approach that includes all randomized infants, supplemented by PP analyses as sensitivity checks. ITT analyses with handling of missing data, together with the similarity of ITT and PP results, suggest that dropout bias is

unlikely to fully explain the observed lack of effect at Day 28. Nonetheless, the proportion of missing data and protocol deviations may have reduced statistical power and could still have introduced some degree of bias. Future trials should aim to minimize loss to follow-up and pre-randomization ineligibility to further strengthen the validity of causal inferences, as well as allow for stratification by type of breastfeeding and the results found. To our knowledge, this is the first study to evaluate the isolated use of *L. acidophilus* NCFM® in the treatment of infant colic and to demonstrate the response rate and its effects at the intestinal microbiome.

5 CONCLUSION

In this randomized trial, the intervention did not significantly improve the proportion of responders at Day 28, the primary endpoint. An apparent benefit at Day 14 emerged in secondary, exploratory analyses, but this finding should be interpreted cautiously in light of the adjustment for multiple comparisons and the negative primary endpoint. Further studies are needed to clarify the potential short-term effects of the intervention and to confirm its clinical relevance in pediatric patients. Furthermore, the intervention positively modulated certain aspects of the infants' intestinal microbiota, specifically leading to an increased relative abundance of *Akkermansia*. However, no differential effects were observed regarding *Ruminococcus*, which remained consistent across both groups before and after the intervention. Moreover, the study found no statistically significant differences in fecal calprotectin levels or in the assessment of infant mood between the probiotic and placebo groups. Crucially, the use of *L. acidophilus* NCFM® was determined to be safe and well-tolerated within this infant population.

List of abbreviations:

16S rRNA: 16S ribosomal RNA

2′FL: 2′-fucosyllactose

3FL: 3-fucosyllactose

ASV / ASVs: amplicon sequence variants

CFU / CFUs: Colony Forming Units

CI: confidence interval

DNA: deoxyribonucleic acid

HMOs: human milk oligosaccharides

LDA: linear discriminant analysis

LacNAc: N-acetyl lactosamine

OTU / OTUs: operational taxonomic units

PCoA: principal coordinate analysis

PERMANOVA: permutational multivariate analysis of variance

DADA2: Divisive Amplicon Denoising Algorithm 2.

SPSS: Statistical Package for the Social Sciences

UFG: Universidade Federal de Goiás

UniFrac: Unique Fraction metric

VSEARCH: Vectorized Search

Declarations

Ethics approval: Research Ethics Committee of the Universidade Federal de Goiás (UFG), Brazil (protocol CAAE 40846520.5.00005078)

Consent for publication: Written informed consent was obtained from all parents or legal guardians prior to participation, including consent for publication where applicable, in accordance with the approval granted by the Research Ethics Committee.

Availability of data and materials: Deidentified individual participant data will not be made available.

Competing interests: The authors have no conflicts of interest relevant to this article to disclose.

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Authors contributions:

Dr. Sáskia Ribeiro Vaz conceptualized and designed the study, collected, analyzed and interpreted data, drafted the initial manuscript, and critically reviewed and revised the manuscript.

Dr. Marise Helena Cardoso Tofoli conceptualized and designed the study, collected, analyzed and interpreted data, wrote de manuscript and critically reviewed and revised the manuscript.

Prof. Melissa Ameloti Gomes Avelino conceptualized and designed the study, helped with data collection and interpretation, and critically reviewed and revised the manuscript.

Prof. João Felipe Mota supervised data collection and critically reviewed and revised the manuscript.

Dr. Humberto Bezerra de Araujo Filho helped with data (microbiota) analysis interpretation and critically reviewed and revised the manuscript.

Prof. Paulo Sucasas Costa conceptualized and designed the study, helped with data collection and interpretation, drafted the initial manuscript, and critically reviewed and revised the manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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