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RESEARCH ARTICLE

White blood cells, TNF-α, and interleukin-6 in subjects with infantile colic treated with *Lacticaseibacillus rhamnosus* GG (ATCC 53103): a randomised prospective study

F. Savino[1], *M. Passerini*[2], *S. Gambarino*[3], *A. Clemente*[3], *M. Dini*[3], *P. Montanari*[3], *I. Galliano*[3] *and M. Bergallo*[3]*

[1]Early Infancy Special Care Unit, Regina Margherita Children Hospital, A.O.U. Città della Salute e della Scienza di Torino 10126 Torino, Italy; [2]University of Turin, Piazza Polonia 94 Turin, Italy; [3]Department of Public Health and Pediatric Sciences, Paediatric Laboratory, Medical School, University of Turin, 10126 Turin, Italy; *massimiliano.bergallo@unito.it

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Abstract

Recent metanalysis reported that certain probiotic strains, such as *Limosilactobacillus reuteri* and *Lacticaseibacillus rhamnosus* (LGG), seem effective for treatment of infantile colic of exclusively breastfed infants; some reports have also linked probiotics to have an immunological effect, however further investigation are needed to fully understand the exact mechanism. The objective of this study was to assay white blood cells, tumour necrosis factor (TNF)-α and interleukin (IL)-6 values in peripheral blood in subjects treated in a randomised, double-blind, placebo-controlled trial for infantile colic with LGG. Fifty-eight infants were enrolled and followed for a study period of 28 days. Parent were asked to record daily crying time using a structured cry diary. Peripheral white blood cells was assessed and RNA (mRNA) expression of TNF-α and IL-6 was measured using TaqMan real-time PCR-maternal amplification. Infants with colic treated with LGG showed a reduction in daily crying duration after 28 days of treatment and a reduction in values of IL-6 ($P < 0.005$) and TNF-α ($P < 0.05$); we observe also a significantly decreasing of IL-6 in the placebo group while decrease of TNF-α was not significant in this group. A significant decreased values of monocytes ($P < 0.05$) was observed in infants treated with LGG. Our data therefore showed, in addition to crying time reduction, a significant decrease of TNF-α and a significant reduction of monocytes cells in colicky infants treated with LGG, compared to placebo group. This observation supports the hypothesis that probiotics may have anti-inflammatory properties. Further studies are needed to better understand the influence of probiotic on immunity cells.

Keywords

chemokines – probiotics – infantile colic – cytokines – monocytes

1 Introduction

Infant colic is one of the most common problems occurring in the first months of life, with a prevalence of up to 15% (Robin *et al.*, 2018; Wolke *et al.*, 2017). Colic is a condition characterised by excessive crying episodes in infants in the afternoon and evening and was initially defined by Wessel *et al.* (1954). More recently, it has been

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revised and included in functional gastrointestinal disorders by the Roma criteria IV, which state that an infant with colic has no evidence of failure to thrive (Benniga *et al.*, 2016). The etiopathogenesis of infant colic is not fully understood; immaturity of digestive functions and motility may be a causative factor (Camilleri *et al.*, 2017), and this could also be related to increased dysbiosis, gut inflammation or imbalance of the gut microbiota (Rhoads *et al.*, 2018). Severe infantile colic may be an early symptom of atopic condition, white blood cells and eosinophils could be taking a count for a possible role in pathogenesis. The immunological model of infantile colic focuses on possible allergens, such as cows' milk proteins in breast milk or infant formula, as a possible cause of symptoms (Gupta *et al.*, 2007; Hall *et al.*, 2012; Perry *et al.*, 2011). The current evidence does not support the use of drugs in the treatment of infantile colic for side effects and for scanty evidence of efficacy (Biagioli *et al.*, 2016), also for dietary modification is quite a bit of evidence (Gordon *et al.*, 2018). To date, several clinical trial data have been published supporting a potential effect of probiotics in infant colic treatment, *Limosilactobacillus reuteri* is the strain most researched for this condition, with positive results for reducing the symptoms (Liu *et al.*, 2022; Pereira *et al.*, 2022). Furthermore, a meta-analysis has been recently published reporting the beneficial effects of probiotics on crying time per day of subject with infantile colic and also on inflammatory markers (Sheldon *et al.*, 2022). Previously Pärtty *et al.* (2017) have reported an association between infant colic and low-grade inflammation: infants with colic had increased concentrations of interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β in serum as compared with healthy subjects. We choose to use *Lacticaseibacillus rhamnosus* GG (LGG), the genus formerly known as *Lactobacillus*, because it has been more studied and investigated with clinical trial documenting its clinical and immunological effects from many years (Capurso *et al.*, 2019). In fact, it has been seen that *Lacticaseibacillus* GG used in adult inflammatory disease have been shown to be able to reduce inflammatory markers, such as IL-6 and tumour necrosis factor (TNF)-α (Plaza-Díaz *et al.*, 2017), and it has also been shown to reduce elevated faecal concentrations of tumour necrosis factor in patients with atopic dermatitis and cow milk allergy (Isolauri *et al.*, 2001). Both IL-6 and TNF-α can be considered as two important signalling factors for inflammation in infant colic (Sheldon *et al.*, 2022). In a recent meta-analysis of randomised clinical trials on the treatment of infantile colic Skonieczna-Zydecka *et al.* (2020) reported the efficacy of probiotics on symptom and on faecal inflammatory markers, such as calprotectin. The present study aims to investigate *in vivo* in infants suffering from infantile colic and treated with LGG, the reduction of colic symptoms (crying duration per day) and the change in inflammatory markers TNF-α, IL-6 and white blood cells after a study period of 28 days.

2 Material and methods

Trial design

The trial was an interventional randomised double-blind placebo-controlled trial. Protocol of the study has been approved 22 July 2021 by Comitato Etico interaziendale AUO Città della salute AUO ospedale Mauriziano di Torino (Corso Bramante, 88/90 – 10126 Torino, Italy), ref: P.I./Cod. Fisc. 10771180014, Prot. n° 0078279 Titolario A/2.4.8.

Inclusion/exclusion criteria

The study was designed to include a specific subset of infants. The inclusion criteria were as follows: the infants had to be between 2 and 10 weeks of age, with a gestational age ranging from 37 to 40 weeks. Their birth weight had to fall within the range of 2,700 to 4,200 g. It was also required that the infants were exclusively breastfed and demonstrated normal growth patterns.

On the other hand, certain conditions led to the exclusion of infants from the study. Infants who were artificially fed or predominantly breastfed were not considered for the study. Any clinical evidence of chronic illness or gastrointestinal disorders also led to exclusion. Furthermore, infants who had received antibiotics, drugs, or probiotics in the period preceding recruitment were also excluded from the study. This rigorous selection process ensured that the study was conducted on a homogeneous and carefully selected group of infants. All subjects were recruited at the outpatients department of paediatrics of Regina Margherita children Hospital città della salute e della scienza di Torino, Italy.

Colic was diagnosed when infant were at least 3 episode of unexplained full force crying lasting more than 3 h a day on at least 3 day, according to the ROMA IV criteria (Benniga *et al.*, 2016). At enrolment each infants underwent a medical examination by a paediatrician and parents completed a questionary to obtain data concerning type of delivery, birth weight, and gestational age. Daily crying time of the 3 days before (registered by parents using a parental diary) and the use

of any medication to threat colic. Paediatricians give to the parents a participant information sheet and parents gave a written consent to the inclusion of their infant in the study. The study was approved in 22/07/2021 prot. N. CS2/109 by the local ethical committee (Comitato Eticointeraziendale – Azienda Universitaria Ospedaliera Città della salute – Azienda Universitaria Ospedaliera ospedale Mauriziano di Torino, Torino, Italy).

Sample collection

Venous blood sample were collected from each infant during the morning after a 3 h fasting period in occasion of a routine clinical blood sampling programmed to lessen the disruption to infants. On the first day of the study, a tube of haemachrome was collected from each infant at recruitment and again at the follow-up visit after 28 days. The samples were placed in sterile Eppendorf tubes (Merck RGaA, Darmstadt, Germany) and kept frozen at –80 °C until analysis.

Intervention

The infants were randomised to receive the probiotic product (*Lacticaseibacillus rhamnosus GG* (ATCC 53103)) or a placebo for 28 days study period. The probiotics dosage was 5 drops of *L. rhamnosus* GG (ATCC 53103) corresponding to 5×10^9 cfu per day. Randomisation was performed by the pharmacist using a random-digit method based on a computer-generated numbers. He used a two-treatment randomisation scheme with a random block of varying size (Stata statistical software: release 9; STATA Corp LP, College Station, TX, USA; Ralloc procedure). A paediatrician allocated infants to the following available product on entry in to the trial and impatient received the study product directly from our children hospital. Probiotic study product was a suspension of *L. rhamnosus* ATCC53103 in a mixture of maize, oil and mono and diglyceric oil provided in a 5-ml dark bottle fitted with a dropper cap. The placebo product was an identical mixture of maize, oil and mono and diglyceric oil with a similar smell and taste of the probiotic. Parents were asked to administer both formulations during the morning before the first feeding, once a day for a period of 28 days.

Blood count

Assay of blood count was performed using methodology of the ADVIA Hematology System 2120i (Siemens Healthineers, Erlangen, Germany).

Genetic marker selection and samples preparation

Total RNA extraction

Total RNA was extracted from whole blood using the automated extractor Maxwell following the RNA Blood Kit protocol without modification (Promega, Madison, WI). This kit provides treatment with DNase during the RNA extraction process. To further exclude any contamination of genomic DNA, RNA extracts were directly amplified without reverse transcription to validate the RNA extraction protocol. RNA concentration and purity were assessed by traditional UV spectroscopy with absorbance at 260 and 280 nm (ND-1000 spectrophotometer, Biochrom Enterprise Waterbeach, Cambridge, United Kingdom). The total RNA was stored at –80 °C until use.

Reverse transcription

400 ng of total RNA were reverse-transcribed with 2 µl of buffer 10X, 4.8 µl of $MgCl_2$ 25 mM, 2 µl ImpromII (Promega, Madison, WI, USA), 1 µl of RNase inhibitor 20U/l, 0.4 µl random hexamers 250 µM (Promega), 2 µl mix dNTPs 100 mM (Promega), and dd-water in a final volume of 20 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C for the inactivation of enzyme; the cDNAs were stored at –20 °C until use.

Transcription values of IL-6 and TNF-α by real-time PCR assay

The relative expression of transcript levels of IL-6 and TNF-α was determined using the primers and probes indicated in Table 1. In brief, 40 ng of cDNA was amplified in a reaction with a total volume of 20 µl containing 2.5 U goTaQ MaterMix (Promega), 1.25 mmol/l $MgCl_2$, 500 nmol of specific primers, and 200 nmol of specific probes. All amplifications were performed in a 96-well plate at 95 °C for 2 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min. Each sample was run in triplicate. The relative expression of target gene transcripts was determined by the 2-ΔΔCt method (Livak *et al.*, 2001). GAPDH was selected as the reference gene because it has been shown to have good efficiency and excellent reproducibility with consistent expression in human leukocyte samples (Spinsanti *et al.*, 2008) and was previously used in our studies (Bergallo *et al.*, 2019). Briefly, after normalising the PCR result of each target gene with the housekeeping gene, the method comprises an additional calibration of this value with the median of the expression of the same gene deter-

TABLE 1 Primers and probes used to assess the transcription levels IL-6, TNF-α, and of GADPH

Name	Primer/probe	Sequence
IL-6	Forward	5′-GGTACATCCTCGACGGCATCT-3′
	Reverse	5′-GTGCCTCTTTGCTGCTTTCAC-3′
	Probe	JOE-5′-TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT-3′-TAMRA
TNF-α	Forward	5′-GCTGCACTTTGGAGTGATCG-3′
	Reverse	5′-GTTTGCTACAACATGGGCTACAG-3′
	Probe	CY5-5′-CCCAGGCAGTCAGATCATCTTCTCGA-3′-BHQ1
GAPDH	Forward	5′-CGAGATCCCTCCAAAATCAA-3′
	Reverse	5′-TTCACACCCATGACGAACAT-3′
	Probe	6FAM-5′-TCCAACGCAAAGCAATACATGAAC-3′-TAMRA

mined in a pool of controls after normalisation with the housekeeping gene. The results, expressed in arbitrary units (called relative quantification (RQ)), show the variations of the target gene transcripts compared to the standard set of controls. Since we measured Ct for every target in all samples, we argue that our methods were suitable for IL-6 and TNF-α detection and quantifications. All analyses were performed in a laboratory of biosafety level 2 (BSL-2) according to the NHI (NIH guidelines, 2019) and WHO (WHO, 2020) guidelines (Table 1).

Statistical analysis

The continuous variables are reported with mean ± standard deviation (SD), median and interquartile range (IQR); the categorical variables are reported with frequencies and percentages. To examines if continuous variables are normally distributed, we use the Kolmogorov-Smirnov normality test (K-S).

Sample size was calculated based on the finding of previous studies (Savino *et al.*, 2010, 2018, 2020) using a difference between groups of a 50-min reduction in daily average crying time, which was considered a clinically relevant difference. With $\alpha = 0.05$, $\beta = 0.20$ and an estimated SD within groups of 55 min, 20 patients were needed in each group.

We used a paired t-test for paired sample to compare means from the same group at different times in parametrical variables, and we used the Wilcoxon signed-rank test to compare median ranks from the same group at different times in non-parametrical variables.

We used the Student's t-test for an independent sample to evaluate differences between the means of the two groups in parametrical variables, and we used a Mann-Whitney U test to evaluate differences between the medians of the two groups in non-parametrical variables. All tests were 2-tailed and considered significant at values of $P < 0.05$.

A Mann-Whitney test was used to compare the relative transcription levels of IL-6 and TNF-α pre and post-treatment with *L. rhamnosus* GG (ATCC 53103) and placebo. Statistical analyses were done using the Prism software (GraphPad Software, 5.0, La Jolla, CA, USA). In all analyses, $P < 0.05$ was taken to be statistically significant.

3 Results

Subjects

A total of 108 infants were assessed for eligibility from 22 July 2021 to 28 February 2023. We excluded 50 infants (Figure 1) of which 33 subjects were not meeting inclusion criteria, 15 declined to participate at the study and 2 for other reasons – family transferred to another city. We used a blocked randomisation to form the allocation list for the two comparison group. Pharmacist have used a computer random number generator to select random permuted blocks with a block size of four and an equal allocation ratio. A total of 58 infants were enrolled and randomised: 33 infants in the LGG group and 25 infants in the placebo. In the LGG group one infant was lost in follow up, resulting in 32 infants analysed at the end of the study period, in placebo group four infants were lost in follow up, 21 subjects were analysed in placebo group at the end of the study period.

The baseline characteristics of the two study groups of infants involved in the study are reported in Table 2 and data of the type of delivery, gender, age at enrolment, birth weight, weight average at enrolment, daily crying time at enrolment are reported in Table 2. This data did not differ between the two groups of infants.

Clinical data: daily crying time

Daily crying duration reported by parents using a diary was downloaded and analysed (Table 3). Our data con-

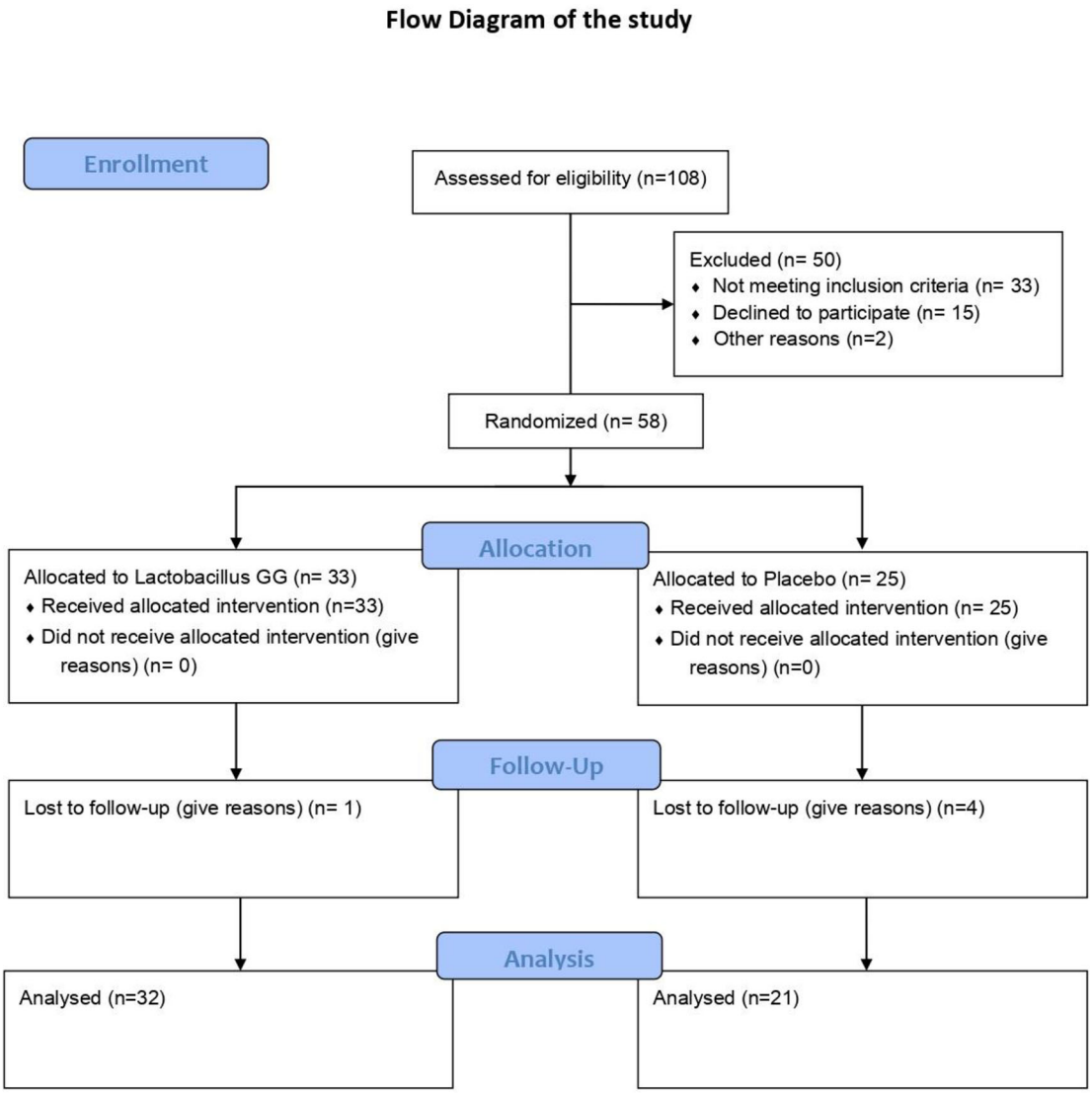

FIGURE 1 Flow diagram of the study.

TABLE 2 Characteristics of the infants involved in the study[1]

Variable	Colic treated with LGG (n = 32)	Colic with placebo (n = 21)	*P*-value
Type of delivery (vaginal/ caesarean)	20/12	13/8	0.663[#]
Gender (male, female)	21/11	12/9	0.185[#]
Age at enrolment (days)	37 (18-45)	33 (16-55)	0.306[¶]
Birth weight (g.)	3,300 (2,750-3,970)	3,250 (2,830-4,030)	0.14[¶]
Weight average at enrolment (g)	4,270 (3,370-5,130)	4,120 (3,170-5,250)	0.154[¶]
Daily crying time (minutes) at enrolment	263 (185-335)	253 (175-356)	0.16[¶]

[1] Data are reported as median (range) or numbered. Statistical analysis: [#] Fisher's exact test, [¶] Mann-Whitney test.

TABLE 3 Crying and fussing time Paired Sample T test analysis between day 0 and day 28, and between LGG and Placebo on day 28

Group	Day 0 mean	Day 28 mean	Difference between day 0 and 28	*P*-value
LGG (min)	263.0	102.0	–161.0	$P < 0.05$
Placebo (min)	253.0	221.0	–32	$P < 0.05$
Difference between LGG and Placebo		119		
P-value		$P < 0.05$		

firmed that LGG crying time at day 0 and LGG crying time at day 28 were normally distributed (K-S test $P < 0.05$) and placebo crying time at day 0 and placebo crying time at day 28 were also normally distributed (K-S test $P < 0.05$).

We compared the differences between the mean values of LGG whine time at day 0 (263.0 min) and the mean values of LGG whine time at day 28 (102.0), and the difference is statistically significant (161.0, $P = 0.001$).

We compared the differences between the means of Placebo crying time at Day 0 (253.0) and the means of Placebo crying time at Day 28 (221.0) and the difference is not statistically significant (32, $P = 0.106$) (Table 3).

Tumour necrosis factor-α

The TNF-α mRNA expression values in the peripheral blood were compared in the LGG treated group and placebo group based on relative quantification. The expression of TNF-α was significantly reduced in patients treated with LGG ($P = 0.0298$) at day 28, as shown in Figure 2A. Mean values and SD of TNF-α for pre-treatment was 1.17 ± 0.74 and for post-treatment 0.84 ± 0.83. The expression of TNF-α values does not differ significantly in subjects receiving placebo from day 0 to day 28 ($P = 0.0663$), as shown in Figure 2B. Mean values and SD of TNF-α for pre-placebo was 1.30 ± 0.69 and for post-placebo 1.50 ± 0.50.

Interleukin-6

The expression of IL-6 was significantly reduced in patients treated with LGG (p = 0.0063) at day 28, as shown in Figure 2C. Mean values and SD of IL-6 for pre-treatment was 1.57 ± 1.55 and for post-treatment 0.61 ± 0.71. The expression of IL-6 was significantly reduced in patients receiving placebo ($P < 0.0001$) at day 28, as shown in Figure 2D. Mean values and SD of IL-6 for pre-placebo was 1.40 ± 1.02; and for post-placebo 0.25 ± 0.10.

Leucocytes

Numbers of monocytes significantly decreased in patients treated with LGG ($P = 0.145$) at day 28, as shown in Figure 3. Numbers of lymphocytes significantly increased in patients treated with LGG ($P = 0.0305$) at day 28, as shown in Figure 3. Mean values and SD of leucocytes for pre-treatment was 10,089 ± 3,251 and for post-treatment 9,508 ± 2,223. Mean values and SD of % neutrophils was for pre-treatment 21.44 ± 8.35 and for post-treatment 17.95 ± 5.84. Mean values and SD of % lymphocytes for pre-treatment was 61.15 ± 11.45 and for post-treatment 67.65 ± 6.84. Mean values and SD of % eosinophils for pre-treatment was 4.07 ± 1.44 and for post-treatment 4.16 ± 3.19. Mean values and SD of % monocytes for pre-treatment was 9.27 ± 4.82 and for post-treatment 6.16 ± 1.17. Leucocytes, neutrophils and eosinophils did not show any significant variation during the treatment.

Numbers of lymphocytes significantly increased in patients receiving placebo ($P < 0.0310$) at day 28, as shown in Figure 4. Mean values and SD of leucocytes for pre-placebo was 9,402 ± 3,622 and for post-placebo 9,111 ± 2,669. Mean values and SD of % neutrophils for pre-placebo was 31.17 ± 12.39 and for post-placebo 22.65 ± 10.06. Mean values and SD of % lymphocytes for pre-placebo was 51.81 ± 12.87 and for post-placebo 62.67 ± 12.65. Mean values and SD of % eosinophils for pre-placebo was 3.40 ± 2.06 and for post-placebo 2.90 ± 2.13. Mean values and SD of % monocytes for pre-placebo was 10.40 ± 4.33 and for post-placebo 8.62 ± 3.14. Leucocytes, monocytes, neutrophils and eosinophils did not shown any significant variation in this group.

4 Discussion and conclusions

The crosstalk between the mucosa, immune system, and microbiota is crucial during the first months of life, this topic has brought great interest not only form clinicians, but also from researchers. In 2007, a possible influence between probiotic bacteria and host immune system has been hypothesised (Blaise *et al.*, 2007). More recently, Rhoads *et al.* demonstrated that infants with colic exhibit dysbiosis and gut inflammation (Rhoads *et al.*, 2018). Infants' immune system is dominated by T helper 2 cells. Walker *et al.* demonstrated how breast

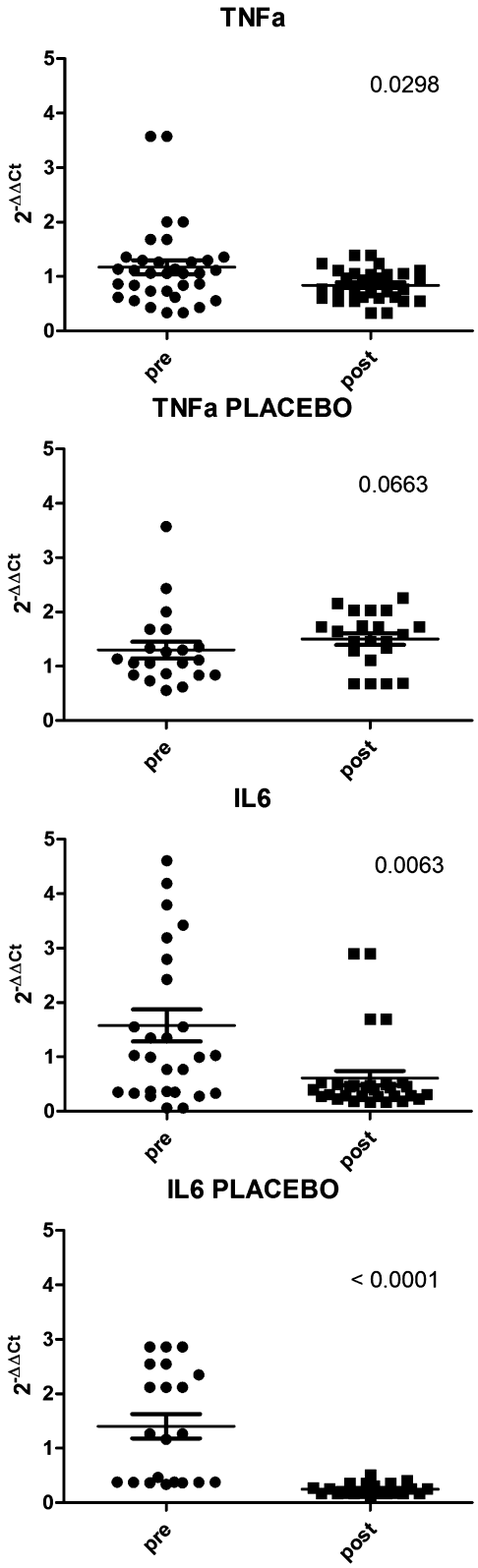

FIGURE 2 Tumour necrosis factor (TNF)-α mRNA expression values in (A) LGG group and (B) Placebo group pre- and post-treatment (28 days). Interleukin (IL)-6 mRNA expression values in (C) LGG group and (D) Placebo group pre- and post-treatment (28 days).

milk facilitates a harmonious growth of intestinal bacteria in newborns and stimulates a balanced Th1/Th2 immune response during the breastfeeding period. This transition results in a shift from the predominant Th2 phenotype, which is characteristic of intrauterine life and the early postnatal period, to a more balanced one.

Coliform bacteria, particularly *Escherichia coli*, were found to be more abundant in colicky infants than the healthy ones (Savino *et al.*, 2009), and it has been documented that the presence of pathogenic microbes, such as *E. coli*, can stimulate innate immune cells, including dendritic cells, macrophages, and natural killer (NK) cells. These immune cells secrete abundant cytokines, such as TNF-α (Sonnenberg *et al.*, 2019). To investigate this hypothesis, we have designed this study aimed at evaluating the potential impact of probiotics on inflammatory cytokines and white blood cells. Interestingly *LGG* and *Bifidobacterium lactis* BB-12 have been reported to modulate immune response in humans (Alanzi *et al.*, 2018), in animals (Trevisi *et al.*, 2011) and *in vitro* (Bhoi *et al.*, 2008). It has recently been reported that LGG and *Bifidobacterium longum* alleviate colitis in mice by regulating IFN-γ and TNF-α and IL-10 expression ratio (Ma *et al.*, 2021). Consistent with these reports, in the current study, although in a small number of subjects, patients treated with LGG had a significant decrease in TNF-α.

Previous clinical studies and meta-analyses have reported the beneficial effects of certain probiotics, mainly of the genus Lactobacillus (*Limosilactobacillus reuteri* and *Lacticaseibacillus rhamnosus* GG), in reducing clinical symptoms in colic infants (Liu *et al.*, 2022, Sheldon *et al.*, 2022). Further, we found that infants with colic treated with *L. reuteri* for 30 days had significantly decreased crying time and increased FOXP3 blood levels, resulting in a decreased RORγ/FOXP3 ratio along with a decrease in fecal calprotectin (Savino *et al.*, 2018). FOXP3 can be considered as a Treg marker, since an increase in FOXP3 levels and a decrease in RORγ/FOXP3 ratio result in an increase of IL-10 secretion, which is a critical cytokine for differentiating lymphocytes into Treg cells. It is noteworthy to consider that Pärtty *et al.* have shown that subjects with infantile colic have a low level of IL-10, a cytokine known for its anti-inflammatory properties.

Cervantes-Barragan *et al.* in 2018, reported that species of lactobacilli, including *L. bulgaricus* and *L. reuteri*, can modulate responses of intestinal and adaptive lymphocytes, and ameliorate inflammation (Cervantes-Barragan *et al.*, 2018). Our findings for the present paper support the results of these studies: the inflammatory

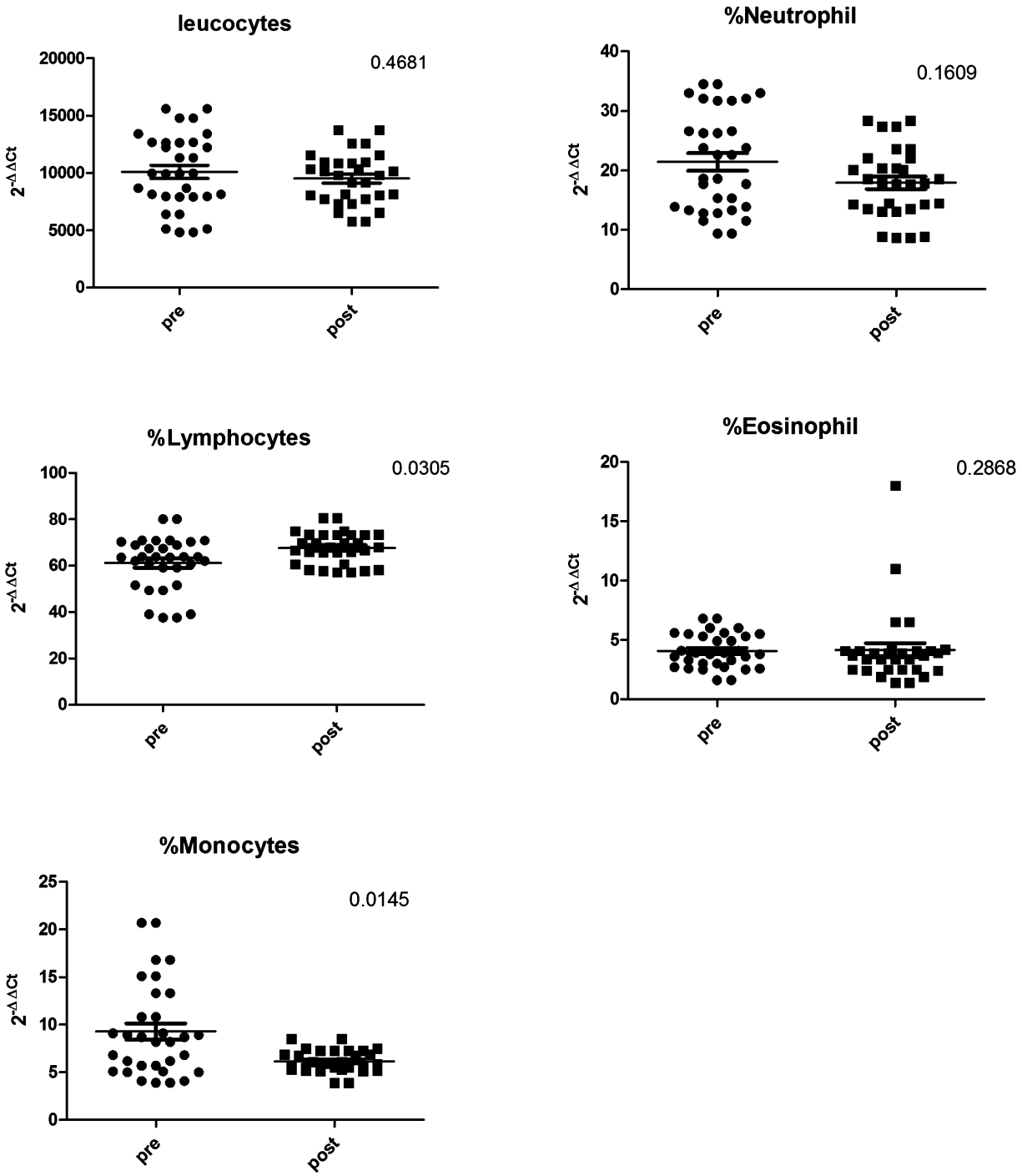

FIGURE 3 Leucocytes values in LGG group pre- and post-treatment (28 days).

marker TNF-α and monocyte cells were lowered using *Lacticaseibacillus* GG. On other hand previous studies have shown a possible effect of LGG and TNF-α reduction and prevention of disruption of intercellular tight junctions in epithelial cell cultures (Donato *et al.*, 2010). Interestingly, some reports have also noted a possible association between LGG and anti-inflammatory effects in human colic mucosa and *ex vivo* organ culture model: this study showed a dose-dependent effect of *L. rhamnosus* on cytokine RNA expression (Pagnini *et al.*, 2018). These effects demonstrated in *ex vivo* organ culture model seem to be found also in our group of infants with colic treated with LGG.

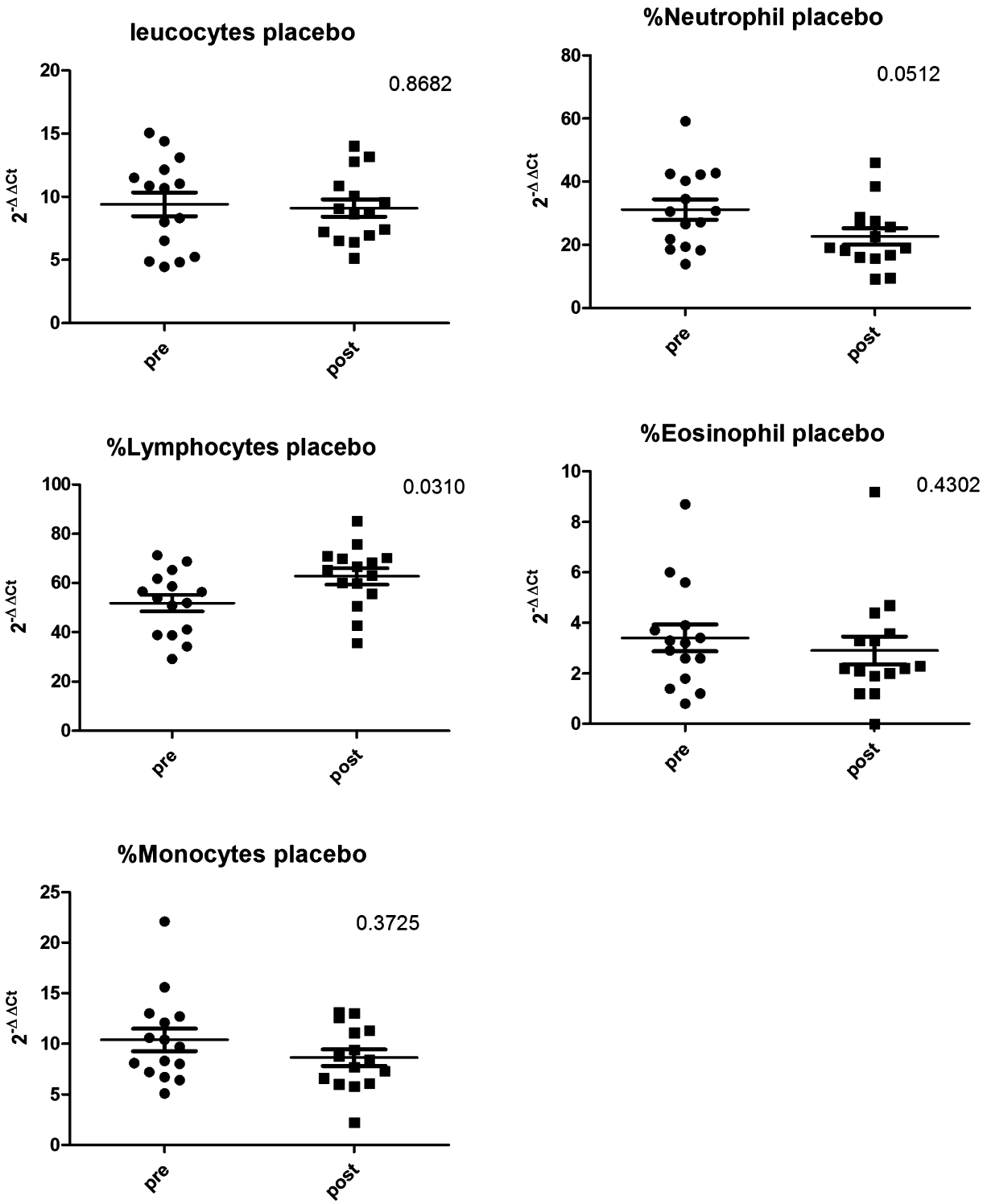

FIGURE 4 Leucocytes values in placebo group pre- and post-treatment (28 days).

As concerned IL-6, cytokine known to be involved in the inflammatory conditions (Sheldon *et al.*, 2022, Plaza-Díaz *et al.*, 2017), we observed a similar downward trend in values in both subjects treated with LGG and those receiving placebo. Therefore, we cannot support the hypothesis that probiotics (LGG) may play a role in lowering IL-6 in our patients. On the contrary, in the group of infants receiving LGG treatment, we observed a significant decrease of TNF-α after 28 days along with clinical improvement (significant decrease

in crying time per day); this decrease in TNF-α and clinical improvement were not observed in infants receiving placebo. It has been shown that the proinflammatory cytokine TNF-α plays an important role in regulating intestinal balance (Ruder *et al.*, 2019). In addition, our results show that subjects treated with probiotics after the study period had a significant decrease in monocyte cells. In this context, it may be of interest to consider that Al-Rashed *et al.* recently reported that TNF-α can stimulate a metabolic pathway called long-chain acyl-CoA synthetase 1 (ACSL1) that promotes a phenotypic shift to monocytes (Al-Rashed *et al.*, 2019). Our data on the concomitant decline of TNF-α and monocytes are consistent with this experimental observation.

Regarding lymphocytes, we noticed a substantial rise in both the probiotics-treated group and the placebo-treated group. Considering that the values in both groups fell within the normal range, we can hypothesise that the increase is a result of normal physiological growth corresponding to the infants' age. No significant modification has been observed for the values of leucocytes, neutrophils and eosinophils in both groups after the study period.

In reference to the crying time reported by parents using our parental diary, our results align with previous clinical trials involving probiotics. These studies demonstrated a significant reduction in crying time after the study period for subjects exclusively breastfed and treated with *Lactobacillus rhamnosus* GG (LGG), compared to those who received a placebo (Pärtty *et al.*, 2017; Savino *et al.*, 2020; Sung *et al.*, 2018). However, data on subjects treated with infantile formula are limited.

In summary, the results of the present study document a significant decrease in tumor necrosis factor alpha (TNF-α) and monocyte levels in subjects treated with the probiotic LGG for infantile colic. This modification was accompanied by clinical improvement compared to subjects treated with placebo. These data could also support a possible link between probiotic treatment and lower grade of inflammation.

Study limitations

To avoid confounding factor such as diet we decided to enroll only breastfed infants. We have verified that there is no difference in matching neither stratification for the two groups of infants.

Despite that, this study has some potential limitations. The effect of probiotics in our model is based on an interventional and prospective randomised clinical trial. One potential limitation is the sample size, which meets the required sample size for each group, but our result must be interpreted with caution and a study with a larger sample size is needed to confirm our results. In addition, we studied only TNF-α and IL-6, but the scenario of inflammatory cytokines included in intestinal inflammation is much broader (IL-1β, IL-10, IFN-γ, IL-8, MCP-1, MIP-1β, C-X-C motif).

Because colic is associated not only with inflammation but also with dysbiosis (Rhoads *et al.*, 2018), we did not examine gut microbiota and calprotectin levels in our study; these parameters may be of interest and another study may examine these data as well. These are the main limitations in this study that could be addressed in future research.

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Authors' contribution

F. Savino is responsible for conception of the study, assessment of patients, data analysis and writing and reviewing the manuscript. M. Passerini is responsible for enrolment and inclusion and exclusion of patients, data extraction, data analysis, writing and reviewing the manuscript. P. Montanari, Anna Clemente and Maddalena Dini are responsible for analysis, assessment of methodological quality and checking the results. S. Gambarino, and I. Galliano are responsible for performing analysis assessment of methodological quality and checking the data and figures. M. Bergallo is responsible for supervision, interpretation of results and figures checking the first and the final versions of the manuscript. All the authors contributed equally to the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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