Beneficial Microbes, 2018; 9 (6): 917-925

Wageningen Academic Publishers

Regulatory T cells and Toll-like receptor 2 and 4 mRNA expression in infants with colic treated with *Lactobacillus reuteri* DSM17938

F. Savino[1*], I. Galliano[2], M. Garro[1], A. Savino[1], V. Daprà[2], P. Montanari[2] and M. Bergallo[2]

[1]Department of Paediatrics, Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino, Piazza Polonia, 94, 10126 Turin, Italy; [2]Dipartimento delle Scienze di Sanità Pubblica e Pediatriche, Università degli Studi di Torino, Scuola di Medicina, Piazza Polonia, 94, 10126 Turin, Italy; francesco.savino@unito.it; fsavino@cittadellasalute.to.it

Received: 15 December 2017 / Accepted: 12 July 2018

RESEARCH ARTICLE

Abstract

Regulatory T cells induce immune homeostasis and the expression of Toll like receptors (TLRs); subsequent inflammatory cytokine release may be involved. Recent studies have shown a microbial imbalance in the gut of colicky infants (with a prevalence of gram-negative bacteria, such as *Escherichia coli*), and accumulating evidence has shown the efficacy of a probiotic (*Lactobacillus reuteri*) in breastfed subjects, but the underlying mechanism remains undefined. The study enrolled 59 infants younger than 60 days, of whom 34 subjects had colic and 25 were healthy controls. With a double-blind, placebo-controlled randomised study performed in our unit from October 2016 to July 2017, infants with colic were randomly assigned to receive oral daily *L. reuteri* DSM17938 (1×10^8 cfu) or placebo for 28 days. Peripheral blood was collected to assess the expression of FoxP3, TLR2 and TLR4 mRNA using real-time TaqMan RT-PCR at baseline and after the study period. Our findings showed increased mRNA expression of the transcription factor forkhead box P3 (FoxP3) in infants treated with *L. reuteri* DSM 17938 for 28 days ($P<0.009$) and increased TLR2 and TLR4 mRNA expression in both treated and placebo subjects. After *L. reuteri* administration for 28 days in infants with colic, we observed a significant decrease in daily crying time (302.3±19.86 min/day on day 0 vs 76.75±22.15 min/day on day 28, $P=0.001$). This study provides evidence that the observed increase in FoxP3 expression and reduction in crying time might be responses to probiotic treatment, while the increase in TLR2 and TLR4 mRNA expression might be related to age. Exploiting these new findings may lead to an unprecedented level of therapeutic control over immune tolerance using probiotics.

Keywords: infantile colic, *Lactobacillus reuteri* DSM 17938, mRNA Treg cells, Toll-like receptors, TLR2, TLR4

1. Introduction

Infantile colic is considered a common self-limiting and benign disorder in early infancy and represents one of the most common problems during the first four months of life. Wessel defined colic as crying and/or fussing for more than three hours per day and for more than three days per week (Wessel *et al.*, 1954). Recently, colic has been defined as recurrent and prolonged periods of crying, fussing or irritability that occur without obvious cause and cannot be prevented or resolved by caregivers in the absence of infant failure, fever or illness. The observation period for the diagnosis has been reduced to one week (Benninga *et al.*, 2016). Nevertheless, the aetiology of colic is still not understood but likely involves the dysregulation of gut motility, gut microflora and tolerance (Camilleri *et al.*, 2017).

Many researchers have demonstrated increased levels of *Escherichia coli* in the gut microbiota of infants with colic (Savino *et al.*, 2009, 2011), and some trials have been performed to treat colic with a probiotic; *Lactobacillus reuteri* DSM17938 has been shown to be effective in breastfed infants (Savino *et al.*, 2010; Sung *et al.*, 2018; Xu *et al.*, 2015). Understanding these potential mechanisms may lead to the introduction of diagnostic measures that can improve the choices regarding tailored therapy for infantile colic (Lu and Ni, 2015).

How the microbiota influences the immune system and the development of immune-mediated diseases late in early infancy is not yet completely known (Lu and Ni, 2015), but studies based on animal models have shown that factors released from the microbiota can activate dendritic cells, which favour T lymphocyte responses (Cervantes-Barragan *et al.*, 2017; Gensollen *et al.*, 2016). The relationship between the gut microbiota and the immune system has been investigated, particularly in relation to inflammatory bowel disease, but few data are available for infant colic (Van der Sloot *et al.*, 2017).

Interestingly, regulatory T cells (Tregs) are a functionally specialised subset of CD4(+) T cells and were found to play a key role in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against the commensal flora (Nakanishi *et al.*, 2018). Tregs maintain immune tolerance and homeostasis *via* cell-cell interactions and the secretion of interleukin (IL)-10 or other anti-inflammatory cytokines that inhibit the activation of effector T cells. Remarkably, Treg cells may play a crucial role in inhibiting intestinal inflammation, maintaining immune tolerance, and providing protection from colitis (Ma *et al.*, 2016).

Microbial exposure is a potent source of early immune stimulation and appears to be essential for the development of normal immune function. Toll-like receptors (TLRs) are molecular regulators by which the immune system may protect the host from pathogens. Understanding the functions of specific TLRs is important for finding compounds useful for re-establishing gut homeostasis (Hug *et al.*, 2018). TLRs are the major pattern recognition receptors that mediate the sensing of a wide range of microorganisms and are pivotal in eliciting an immune response to microbial gut presence; thus, TLRs can be considered an interface among the intestinal epithelial barrier, microbiota, and immune system (Round and Mazmanian, 2010). TLR4 is an innate immune receptor that is responsible for the recognition of the gram-negative cell wall component lipopolysaccharide, while bacterial lipopeptides are recognised by TLR2, a receptor of gram-positive bacteria, which together with TLR4, initiates proinflammatory signalling cascades, resulting in the induction of nuclear factor κB and, consequently, various cytokines and chemokines (Mogensen, 2009, Round and Mazmanian, 2010). Several studies have shown that TLR2, TLR4 and the gut microbiota play important roles in ulcerative colitis and other inflammatory bowel diseases (Fan and Liu, 2015, Toiyama *et al.*, 2006). Recently, Hoang *et al.* 2018) reported a positive effect of *L. reuteri* DSM 17938 against experimental necrotizing enterocolitis through TLR2.

L. reuteri, a gram-positive bacterium, is a probiotic species with antimicrobial activity (Wu *et al.*, 2013). The strain DSM 17938 reduces pain perception via the transient receptor potential vanilloid 1 channel (TRPV1) (Perez-Burgos *et al.*, 2015), thereby influencing the activity of the potassium-dependent calcium channel IK (Ca) in enteric neurons. This probiotic is able to inhibit the contractility of the colon (Haileselassie *et al.*, 2016; Kunze *et al.*, 2009), and as observed recently *in vitro* and in animal models, *L. reuteri* elicits an anti-inflammatory effect (Liu *et al.*, 2012). Cervantes-Barragan *et al.* (2017) reported that *L. reuteri* is able to expand Treg cells in the lamina propria and epithelium of the gut.

In this study, we investigated the effects of supplementation with the probiotic *L. reuteri* DSM 17938 on Treg and TLR expression (TLR 2 and TLR4) in a group of colicky infants treated in a double-blind, placebo-controlled clinical trial.

Table 1. Characteristics of the study population.

	Infant with colic (n=30)	Infant without colic (n=25)	*P*-value[1]
Mean age (day ± SD)[2]	27.4±13.85	21.63±11.63	0.69*
Gender			
Female, n (%)	18 (60.00)	11 (44.00)	0.285#
Male n (%)	12 (40.00)	14 (56.00)	0.285#
Birth weight (g ± SD)	3,242±342.74	3,180±304.74	0.54*
Gestational age (weeks ± SD)	38.5±1.4	38.9±1.4	0.46*
Nationality			
Italian, n (%)	25 (83.33)	21 (84.00)	1#
Foreign, n (%)	5 (16.66)	4 (16.00)	1#

[1] # = Fisher's test; * = Mann-Whitney test.

[2] SD = standard deviation.

2. Materials and methods

Trial design

This study was conducted as a double-blind, randomised placebo-controlled trial. From October 2016 to July 2017, 59 infants younger than 60 days seen as an outpatient at the Newborn Unit at our Department of Paediatrics (Regina Margherita Children's Hospital, Turin, Italy) were enrolled. The infants underwent blood tests during routine outpatient examinations.

According to the Wessel criteria (Wessel *et al.*, 1954), the 59 term infants were assigned to two groups: 34 in the colic group, and 25 in the control group (without colic). The characteristics of the enrolled subjects are reported in Table 1. We excluded infants with a birth weight of less than 2,500 g who had major medical problems or if the mother had received antibiotic therapy, had an allergy to cow's milk protein, had gastroenteritis, or was taking antibiotics or *L. reuteri* at the start of the trial.

The parents of the enrolled infants were informed about the purpose, benefits, and possible risks of the study and provided written informed consent. The study was conducted in accordance with the protocols of the International Council for the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guidelines, applicable regulations, guidelines governing clinical studies, and the ethical principles of the Declaration of Helsinki. The study protocol was approved by the local ethics committee (Comitato Interaziendale AA.SS.OO. O.I.R.M./S. Anna-Ordine Mauriziano di Torino, n. 4698).

Intervention

The treatment included five drops of *L. reuteri* DSM 17938 (0.2×10^8 cfu/drop) in sunflower oil suspension (as indicated by manufacturer Noos s.r.l., Rome, Italy) for one month. The placebo comprised maltodextrin in the same oil suspension and had the same appearance, colour, and taste as the treatment. The dosage of *L. reuteri* was the same as that used in our previous trials, which showed its effectiveness in the management of colic (Savino, 2010).

Randomisation, allocation concealment, and blinding

Colicky infants were randomly assigned to receive *L. reuteri* DSM 17938 or placebo using a computer-generated randomisation list created by an independent statistician (Mr. R. Calabrese). Randomisation was stratified using the random-digit method based on computer-generated numbers. A statistician prepared the computer-generated randomisation schedule using a 2-treatment randomisation scheme with random blocks of varying size (Stata 9 (Stata Corp, College Station, TX, USA), the Ralloc procedure). The trial was double-blinded, and the treatment allocation was concealed from all study investigators and participants at every phase, including the measurement of outcomes. A hospital pharmacist assigned a study identification number to each enrolled infant and dispensed the study product according to the randomisation schedule.

Objectives

The primary outcome were the levels of Treg cells and TLR2 and TLR4 mRNA expression in the groups of infants with and without colic. The secondary outcome was the impact of supplementation for 28 days with *L. reuteri* DSM17938 or placebo on Treg cells and TLR mRNA expression.

Study design

At recruitment (day 0), each child was visited, and the parents were interviewed to complete a medical history card with personal data. After the visit, we collected a tube of peripheral blood *via* a routine manner. Based on the clinical examination and the Wessel criteria, the children were divided into two groups: infants with colic (n=34) and healthy control infants (n=25) (Table 1). The parents were asked to complete a cry diary recording the frequency of colic episodes and daily full-force crying (in minutes).

Of the group of infants with infantile colic, 18 received supplementation with *L. reuteri* DSM 17938, and 16 were given the placebo. At the follow-up visit on day 28, the cry diaries and a blood sample (for routine analysis) were gathered for each subject. In the placebo group, 4 infants were lost to follow up for fever (Figure 1). This discrepancy between the number of enrolled patients and the results presented was due to the amount of blood samples available because for some subjects, there was not enough material to perform the test.

Sample collection

A tube of haemochrome was collected at recruitment for each child; for infants with colic, a tube of haemochrome was also collected at the control visit after 28 days. Each sample was transferred to sterile Eppendorf tubes and stored in a freezer at -80 °C until use.

RNA extraction

Total RNA was extracted from 200 µl of blood using a Maxwell automated extractor (Promega, Madison, WI, USA) and the Simply RNA Blood Kit protocol without modification. One µg of total RNA was reverse transcribed with 8 µl of 10× buffer, 4.8 µl of 25 mM $MgCl_2$, 2 µl of ImpromII (Promega), 1 µl of 40 U/l RNase inhibitor, 0.4 µl of 250 µM random hexamers (Promega), 2 µl of dNTP

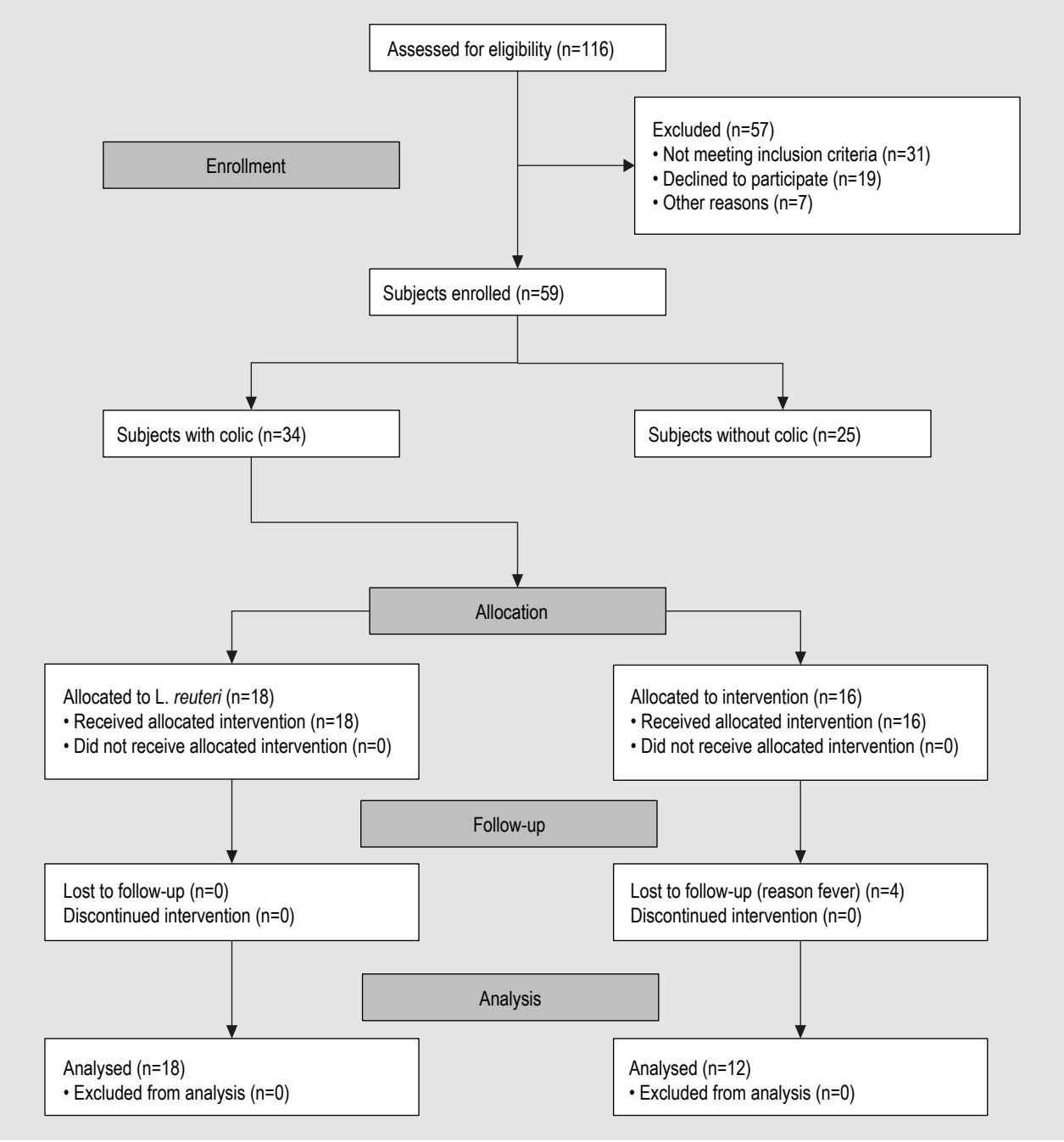

Figure 1. Flow diagram of the subjects' progression during the study.

mix (100 mM each, Promega) and double-distilled water in a final volume of 20 µl. The reaction was carried out in a GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C for enzyme inactivation; the cDNAs were stored at -80° C until use.

Relative quantification by real-time PCR

Relative quantification of the mRNA expression levels of the selected genes was achieved by TaqMan amplification and normalisation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as the reference gene) using an ABI PRISM 7500 real-time system (Life Technologies, Austin, TX, USA). The expression levels of TLR2 and TLR4 were quantified using real-time PCR. Approximately 100 ng of cDNA was amplified in a total volume of 20 µl containing 2× GoTaq® qPCR Master Mix (Promega), 500 nmol specific

primers and 200 nmol probe, as previously reported by Flacher *et al.* (2006).

Primer TLR2 Forward: GGCCAGCAAATTACCTGTGTG; TLR2 Reverse: AGGCGGACATCCTGAACCT; TLR2 Probe: 6FAM-CCATCCCATGTGCGTGG-TAMRA. Primer TLR4 Forward: GCAGTGAGGATGATGCCAGGAT; TLR4 Reverse: GCCATGGCTGGGATCAGAG; TLR4 Probe: 6FAM-TGTCTGCCTCGCGCC-TAMRA.

The amplifications were performed in a 96-well plate at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each sample was run in triplicate. Relative expression of the target gene in patients was compared with that in normal subjects using the 2-ΔΔCt method, and relative expression was expressed in arbitrary units (AU). The mRNA expression levels of FoxP3 and GAPDH were quantified by real-time PCR as previously described by Mareschi *et al.* (2016).

Statistical analysis

The sample size was calculated to allow the determination of a clinically relevant reduction in the average daily crying time of 75 min between the groups. With α=0.05 and β=0.80, 12 patients were needed per group. The data were analysed using GraphPad Prism 5® (La Jolla, CA, USA). Quantitative variables are reported as the mean and standard deviation or as the median (Me) and variation range/interquartile range. Responders were defined as subjects with a 50% reduction in crying time from baseline. Qualitative variables are reported as absolute frequencies and percentages. The data samples were analysed using the Mann-Whitney test. All tests were two-tailed, and differences were considered significant at $P<0.05$.

3. Results

Primary outcome

The primary outcomes were the mRNA levels of Treg, TLR2 and TLR4 in a group of infants with and without colic. Our findings did not show differences in the mRNA levels of Treg, TLR2 and TLR4 between the two groups of infants (Figure 2). Treg levels were 1.23±1.29 in infants with colic and 1.10±0.61 in infants without colic (P=0.286). TLR2 expression levels were 1.48±1.05 AU and 1.40±1.18 AU in infants with colic and without colic, respectively (P=0.583). TLR4 expression levels were 1.23±0.74 AU and 1.26±0.87 AU in infants with colic and without colic, respectively (P=0.915).

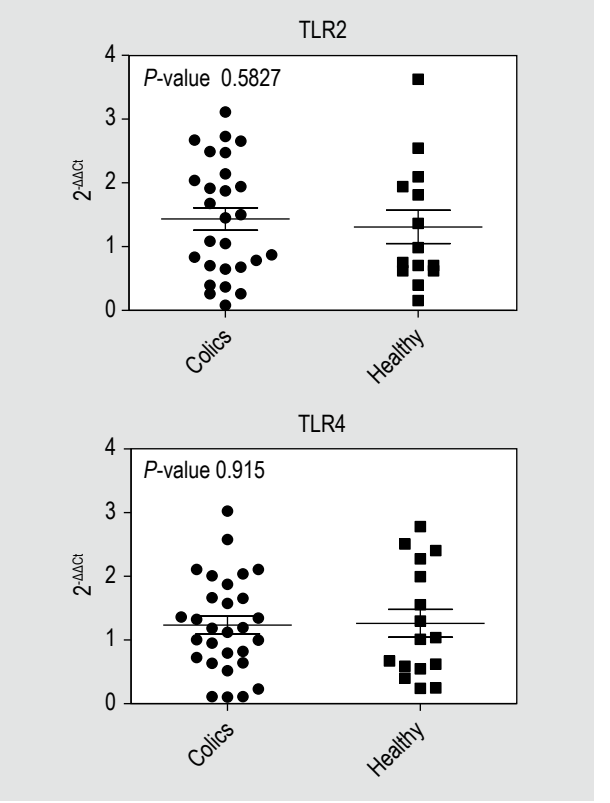

Figure 2. Mann-Whitney T-Test. Comparison of the expression values of Toll-like receptor (TLR)2 and TLR4. (A) TLR2 mRNA in colic infants vs healthy infants, *P*=0.583; (B) TLR4 mRNA in colic infants vs healthy infants, *P*=0.915.

Secondary outcome

The characteristics of subjects treated with *L. reuteri* or placebo are reported in Table 2. After *L. reuteri* administration for 28 days in infants with colic, we observed a significant decrease in daily crying time (302.3±19.86 min/day on day 0 vs 76.75±22.15 min/day on day 28, P=0.001) and an increase in Treg mRNA (1.23±1.29 AU on day 0 and 1.86±1.13 AU on day 28, P=0.009) (Figure 3). We also observed an increase in TLR2 and TLR4 mRNA levels: TLR2 expression was 1.66±1.09 AU on day 0 and 4.86±5.94 AU on day 28 (P=0.0044); and TLR4 expression was 1.33±0.74 AU on day 0 and 4.23±4.49 AU on day 28 (P=0.006).

The proportion of responders (50% reduction in crying time from baseline) was significantly higher in the *L reuteri* group than in the placebo group on day 28 (14 vs 4; P=0.0243) (Table 3).

In the placebo group, we did not observe a significant decrease in daily crying time (312.69±15.69 min/day on day 0 vs 194.32±33.85 min/day on day 28, $P>0.05$); the FoxP3 mRNA expression level did not show any changes (0.92±1.12 AU on day 0 and 0.72±0.25 AU on day 28,

Table 2. Characteristics of subjects treated with *Lactobacillus reuteri* or placebo.

	L. reuteri (n=18)	Placebo (n=12)	*P*-value[1]
Mean age (day ± SD)[2]	25.4±12.78	27.9±11.55	0.63*
Gender			
Female, n (%)	10 (55.6)	8 (66.7)	0.709#
Male n (%)	8 (44.4)	4 (33.3)	0.709#
Birth weight (g ± SD)	3,090±362.63	3,389±384.47	0.58*
Gestational age (weeks ± SD)	38.3±1.5	38.7±1.6	0.48*
Nationality			
Italian, n (%)	16 (88.89)	9 (75.00)	0.364#
Foreign,n (%)	2 (11.11)	3 (25.00)	0.364#

[1] # = Fisher's test; * = Mann-Whitney test.
[2] SD = standard deviation.

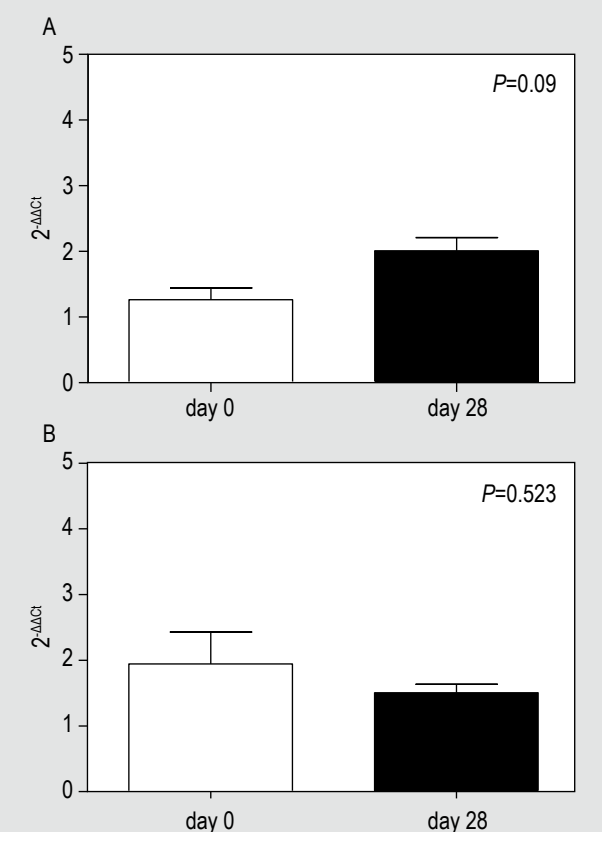

Figure 3. Expression of mRNA Treg on day 0 and day 28 in infants treated with *Lactobacillus reuteri* or placebo. $2^{-\Delta\Delta ct}$ represented by vertical bars. The data were analysed using the Mann-Whitney U test. *P*≤0.05 was considered statistically significant.

P=0.523). In contrast, we observed increased levels of TLR2 and TLR4 mRNA expression (Figure 4). TLR2 expression was 1.22±0.97 AU on day 0 and 4.24±2.11 AU on day 28 (*P*=0.007), and TLR4 expression was 1.13±0.93 AU on day 0 and 3.94±2.88 AU on day 28 (*P*=0.0379).

Table 3. Effectiveness (number of responders) of *Lactobacillus reuteri* vs placebo.

	Responders	Non responders	n	χ^2test[1]
L. reuteri	14 (77%)	4 (23%)	18	0.0243
Placebo	4 (33%)	8 (67%)	12	0.0243
Total	18	12	30	

[1] Fisher's test (significant at *P*<0.05).

4. Discussion

Recent studies of interactions between different components of the immune system and gut microbiota have expanded our knowledge on gut inflammation. Imbalanced relationships among these components may promote aberrant TLR signalling, particularly in relationships contributing to intestinal inflammatory processes such as inflammatory bowel disease and colitis (Inoue *et al.*, 2017; Török *et al.*, 2017). However, data on intestinal inflammatory conditions in infantile colic have not been well defined (Pärtty *et al.*, 2017).

Our findings suggest that *L. reuteri* supplementation induces anti-inflammatory actions by increasing the mRNA levels of Treg, a component of the adaptive immune system, although it did not appear to affect TLR expression. Some studies conducted in animal models have revealed an important effect of this probiotic on the expression of Treg: this strain was able to reprogram intraepithelial T cells into immunoregulatory T cells (Cervantes-Barragan *et al.*, 2017; Ma *et al.*, 2016). Furthermore, an increase in Treg was observed after probiotic administration in a necrotizing enterocolitis mouse model (Liu *et al.*, 2012). The effects on lymphocytes might result from increased secretion of IL-10,

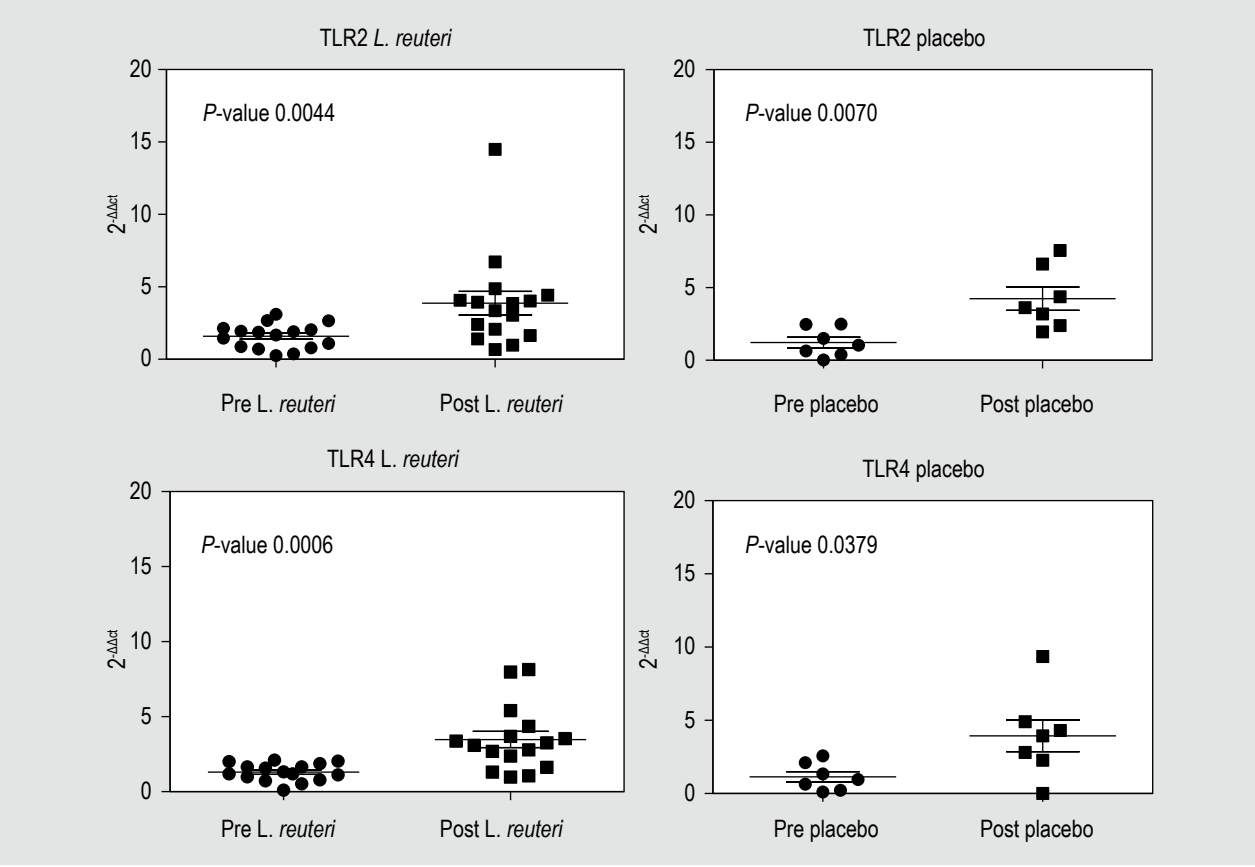

Figure 4. Mann-Whitney T-Test. Comparison of the expression values of Toll-like receptor (TLR)2 and TLR4. Effect of *Lactobacillus reuteri* or placebo treatment on TLR2 and TLR4 expression levels.

a cytokine that is reduced in several models of colitis and in infants with colic but is also essential for the differentiation of lymphocytes into Treg cells (Pärtty *et al.*, 2017).

The observed increase in TLR2 and TLR4 mRNA after the study period in both groups of infants might be due to their age rather than the probiotic treatment (Savino *et al.*, 2018). In fact, the changes imposed by the colonisation and replication of intestinal microbiota may be the main factor that governs the dynamic expression of TLR2 and TLR4 during development. It has been shown that physiological expression of TLR2 and TLR4 is determined by the gut microbiota (Kawai and Akira, 2010). We had to consider that all enrolled patients were exclusively breastfed and that maternal breast milk contains a large number of immune regulatory factors, including soluble CD14, which plays a critical role in the recognition of TLR2 and TLR4 in cells lacking membrane-CD14, such as intestinal epithelial cells (He *et al.*, 2016).

Recently, the effects of probiotics on TLR expression in an animal model of ulcerative colitis have been reported by Yao *et al.* (2017). These authors suggested that probiotics regulate the balance of intestinal flora and inhibit the TLR-mediated immune response, thus alleviating the pathogenesis of intestinal inflammatory reactions (Yao *et al.*, 2017). However, our results did not support the idea that the inflammatory state of infantile colic is related to the expression of TLRs, as there was no difference in TLR2 and TLR4 expression between infants with colic and infants without colic after treatment with *L. reuteri*.

Our interest in innate immune pathways was based on evidence that (microbial) TLR activation in early life can modify innate immune function. Only preliminary evidence exists to support the notion that the anti-inflammatory effects of probiotics are mediated through TLR-receptor signalling in experimental models of colitis (Rachmilewitz *et al.*, 2004). Probiotics are viable non-pathogenic microorganisms that confer health benefits to the host by balancing the microflora. The benefits of probiotic bacteria are mediated by various mechanisms, such as by decreasing colonisation, intestinal pH, and invasion by pathogenic organisms as well as by modifying the host immune response. For example, *Lactobacillus plantarum* and *L. reuteri* attenuate virulence by competitively excluding the binding of pathogenic organisms to the host epithelium and by acidifying the luminal environment. In humans, there are reports that changes in colonisation patterns are associated with increased TLR expression (particularly

TLR2) (Taylor *et al.*, 2006). Nevertheless, we did not observe any evidence that probiotic supplementation to treat infantile colic for 28 days had any significant effect on the expression of either TLR2 or TLR4 mRNA.

Recently, Zhang *et al.* (2017) reported that *L. reuteri* did not shape the double-positive intraepithelial lymphocytes T cell receptor (DP-IEL-TCR) repertoire but generated indole derivatives of tryptophan that activated the aryl-hydrocarbon receptor in CD4(+) T cells, thus allowing Thpok down-regulation and differentiation into DP IELs. Thus, *L. reuteri*, together with a tryptophan-rich diet, can reprogram intraepithelial CD4(+) T cells into immunoregulatory T cells. This experimental observation allowed us to clarify our findings of increased mRNA levels in infants treated with *L. reuteri* (Zhang *et al.*, 2017).

Strengths and limitations of the study

To the best of our knowledge, this was the first double-blind, randomised controlled trial of probiotic intervention in breastfed infants with colic that aimed to evaluate the mRNA expression levels of Treg and the transcription factors TLR2 and TLR4.

The amount of blood sampled was often low due to the age of the enrolled infants. To confirm the observed data in peripheral blood, further studies of other members of the TLR family should be investigated. Furthermore, the study was designed to investigate the transcription profiles of several mRNAs in peripheral blood without detecting the corresponding translated proteins.

5. Conclusions

In conclusion, at baseline, infants with and without colic showed the same mRNA levels of FoxP3 and TLR2/TLR4. The data obtained after treatment with *L. reuteri* showed an increase in mRNA FoxP3 expression (marker of regulatory T cells) in infants who received the probiotic and exhibited decreased crying time per day, but no differences in TLR expression were observed following *L. reuteri* treatment compared to placebo treatment. Further investigations are needed to better understand the role of immunity and TLRs in subjects with infantile colic.

Acknowledgements

We are grateful to Dr R. Calabrese for his time and collaboration on statistical analyses.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Benninga, M.A., Faure, C., Hyman, P.E., St James Roberts, I., Schechter, N.L. and Nurko, S., 2016. Childhood functional gastrointestinal disorders: neonate/toddler. Gastroenterology 150: 1443-1455.

Camilleri, M., Park, S.Y., Scarpato, E. and Staiano, A., 2017. Exploring hypotheses and rationale for causes of infantile colic. Neurogastroenterology and Motility 29: e12943.

Cervantes-Barragan, L., Chai, J.N., Tianero, M.D., Di Luccia, B., Ahern, P.P., Merriman, J., Cortez, V.S., Caparon, M.G., Donia, M.S., Gilfillan, S., Cella, M., Gordon, J.I., Hsieh, C.S. and Colonna, M., 2017. *Lactobacillus reuteri* induces gut intraepithelial CD4(+) CD8αα(+) T cells. Science 357: 806-810.

Fan, Y. and Liu, B., 2015. Expression of Toll-like receptors in the mucosa of patients with ulcerative colitis. Experimental and Therapeutic Medicine 9: 1455-1459.

Flacher, V., Bouschbacher, M., Verronèse, E., Massacrier, C., Sisirak, V., Berthier-Vergnes, O., De Saint-Vis, B., Caux, C., Dezutter-Dambuyant, C., Lebecque, S., Valladeau, J., Bouschbacher, M. and Verronèse, E., 2006. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and gram-positive bacteria. Journal of Immunology 177: 7959-7967.

Gensollen, T., Iyer, S.S., Kasper, D.L. and Blumberg, R.S., 2016. How colonization by microbiota in early life shapes the immune system. Science 352: 539-544.

Haileselassie, Y., Navis, M., Vu, N., Qazi, K.R., Rethi, B. and Sverremark-Ekström, E., 2016. *Lactobacillus reuteri* and *Staphylococcus aureus* differentially influence the generation of monocyte-derived dendritic cells and subsequent autologous T cell responses. Immunity, Inflammation and Disease 4: 315-326.

He, Y., Lawlor, N.T. and Newburg, D.S., 2016. Human milk components modulate Toll-like receptor-mediated inflammation. Advances in Nutrition 7: 102-111.

Hoang, T.K., He, B., Wang, T., Tran, D.Q., Rhoads, J.M. and Liu, Y., 2018. Protective effect of *Lactobacillus reuteri* DSM 17938 against experimental necrotizing enterocolitis is mediated by Toll-like receptor 2. American Journal of Physiology – Gastrointestinal and Liver Physiology 315: G231-G240.

Hug, H., Mohajeri, M.H. and La Fata, G., 2018. Toll-like receptors: regulators of the immune response in the human gut. Nutrients 10: E203.

Inoue, R., Yajima, T. and Tsukahara, T., 2017. Expression of TLR2 and TLR4 in murine small intestine during postnatal development. Bioscience, Biotechnology and Biochemistry 81: 350-358.

Kawai, T. and Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature Immunology 11: 373-384.

Kunze, W.A., Mao, Y.K., Wang, B., Huizinga, J.D., Ma, X., Forsythe, P. and Bienenstock, J., 2009. *Lactobacillus reuteri* enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. Journal of Cellular and Molecular Medicine 13: 2261-2270.

Liu, Y., Fatheree, N.Y., Mangalat, N. and Rhoads, J.M., 2012. *Lactobacillus reuteri* strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-κB signaling in the intestine. American Journal of Physiology – Gastrointestinal and Liver Physiology 302: G608-G617.

Lu, C.Y. and Ni, Y.H., 2015. Gut microbiota and the development of pediatric diseases. Journal of Gastroenterology 50: 720-726.

Ma, Y.H., Zhang, J., Chen, X., Xie, Y.F., Pang, Y.H. and Liu, X.J., 2016. Increased CD4(+)CD45RA(-)FoxP3(low) cells alter the balance between Treg and Th17 cells in colitis mice. World Journal of Gastroenterology 22: 9356-9367.

Mareschi, K., Castiglia, S., Sanavio, F., Rustichelli, D., Muraro, M., Defedele, D., Bergallo, M. and Fagioli, F., 2016. Immunoregulatory effects on T lymphocytes by human mesenchymal stromal cells isolated from bone marrow, amniotic fluid, and placenta. Experimental Hematology 44: 138-150.

Mogensen, T.H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical Microbiology Reviews 22: 240-273.

Nakanishi, Y., Ikebuchi, R., Chtanova, T., Kusumoto, Y., Okuyama, H., Moriya, T., Honda, T., Kabashima, K., Watanabe, T., Sakai, Y. and Tomura, M., 2018. Regulatory T cells with superior immunosuppressive capacity emigrate from the inflamed colon to draining lymph nodes. Mucosal Immunology 11: 437-448.

Pärtty, A., Kalliomäki, M., Salminen, S. and Isolauri, E., 2017. Infantile colic is associated with low-grade systemic inflammation. Journal of Pediatric Gastroenterology and Nutrition 64: 691-695.

Perez-Burgos, A., Wang, L., McVey Neufeld, K.A., Mao, Y.K., Ahmadzai, M., Janssen, L.J., Stanisz, A.M., Bienenstock, J. and Kunze, W.A., 2015. The TRPV1 channel in rodents is a major target for antinociceptive effect of the probiotic *Lactobacillus reuteri* DSM 17938. Journal of Physiology 593: 3943-3957.

Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., Akira, S., Takeda, K., Lee, J., Takabayashi, K. and Raz, E., 2004. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 126: 520-528.

Round, J.L. and Mazmanian, S.K., 2010. Inducible Foxp3^{+} regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences of the USA 107: 12204-12209.

Savino, F., Cordisco, L., Tarasco, V., Calabrese, R., Palumeri, E. and Matteuzzi, D., 2009. Molecular identification of coliform bacteria from colicky breastfed infants. Acta Paediatrica 98: 1582-1588.

Savino, F., Cordisco, L., Tarasco, V., Locatelli, E., Di Gioia, D., Oggero, R. and Matteuzzi, D., 2011. Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC Microbiology 11: 157.

Savino, F., Cordisco, L., Tarasco, V., Palumeri, E., Calabrese, R., Oggero, R., Roos, S. and Matteuzzi, D., 2010. *Lactobacillus reuteri* DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial. Pediatrics 126: e526-e533.

Savino, F., Galliano, I., Garro, M., Savino, A., Manzoni, P., Daprà, V., Montanari, P. and Bergallo, M., 2018. Changes in the messenger RNA expression of Toll-like receptors 2 and 4 in healthy infants according to age. American Journal of Perinatology 35: 583-588.

Sung, V., D'Amico, F., Cabana, M.D., Chau, K., Koren, G., Savino, F., Szajewska, H., Deshpande, G., Dupont, C., Indrio, F., Mentula, S., Partty, A. and Tancredi, D. 2018. *Lactobacillus reuteri* to treat infant colic: a meta-analysis. Pediatrics 141: e20171811.

Taylor, A., Hale, J., Wiltschut, J., Lehmann, H., Dunstan, J.A. and Prescott, S.L., 2006. Evaluation of the effects of probiotic supplementation from the neonatal period on innate immune development in infancy. Clinical and Experimental Allergy 36: 1218-1226.

Toiyama, Y., Araki, T., Yoshiyama, S., Hiro, J., Miki, C. and Kusunoki, M., 2006. The expression patterns of Toll-like receptors in the ileal pouch mucosa of postoperative ulcerative colitis patients. Surgery Today 36: 287-290.

Török, H.P., Bellon, V., Konrad, A., Lacher, M., Tonenchi, L., Siebeck, M., Brand, S. and De Toni, E.N., 2017. Functional Toll-Like Receptor (TLR)2 polymorphisms in the susceptibility to inflammatory bowel disease. PLoS One 12: e0175180.

Van der Sloot, K.W.J., Amini, M., Peters, V., Dijkstra, G. and Alizadeh, B.Z., 2017. Inflammatory bowel diseases: review of known environmental protective and risk factors involved. Inflammable Bowel Disease 23: 1499-1509.

Wessel, M.A., Cobb, J.C., Jackson, E.B., Harris Jr., G.S. and Detwiler, A.C., 1954. Paroxysmal fussing in infancy, sometimes called colic. Pediatrics 14: 421-435.

Wu, R.Y., Pasyk, M., Wang, B., Forsythe, P., Bienenstock, J., Mao, Y.K., Sharma, P., Stanisz, A.M. and Kunze, W.A., 2013. Spatiotemporal maps reveal regional differences in the effects on gut motility for *Lactobacillus reuteri* and *rhamnosus* strains. Neurogastroenterology and Motility 25: e205-e214.

Xu, M., Wang, J., Wang, N., Sun, F., Wang, L. and Liu, X.H., 2015. The efficacy and safety of the probiotic bacterium *Lactobacillus reuteri* DSM 17938 for infantile colic: a meta-analysis of randomized controlled trials. PLoS One 10: e0141445.

Yao, P., Tan, F., Gao, H., Wang, L., Yang, T. and Cheng, Y., 2017. Effect of probiotics on Toll-like receptor expression in ulcerative colitis rats induced by 2,4,6-trinitro benzene sulfonic acid. Molecular Medicine Reports 15: 1973-1980.

Zhang, M., Sun, K., Wu, Y., Yang, Y., Tso, P. and Wu, Z., 2017. Interactions between intestinal microbiota and host immune response in inflammatory bowel disease. Frontiers in Immunology 8: 942.