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BENEFICIAL MICROBES 16 (2025) 157–169

Beneficial Microbes
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RESEARCH ARTICLE

Effects of *Limosilactobacillus reuteri* DSM 17938 in neonates exposed to antibiotics: a randomised controlled trial

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Received 9 March 2024 | Accepted 10 September 2024 | Published online 31 October 2024 | Published in issue 15 February 2025

Abstract

Perinatal antibiotic exposure potentially leads to gut microbiota dysbiosis, which is associated with functional gastrointestinal disorders (FGIDs). We aimed to investigate the effects of *Limosilactobacillus reuteri* DSM 17938 supplementation on the development of FGIDs, crying and sleep duration, and the gut microbial composition in infants exposed to antibiotics during the neonatal period. In this randomised, double-blind, placebo-controlled study, we included 89 term neonates treated with antibiotics. Neonates received the study product for six weeks. FGIDs, assessed by the Infant Gastrointestinal Symptom Questionnaire, crying and sleep duration were assessed at four and eight weeks, and six months after enrolment. Faecal samples were collected six weeks and twelve months after enrolment. The gut microbial community composition was analysed using 16S amplicon sequencing and qPCR. The proportion of infants with FGIDs was greater in the control group, although the difference between the groups was significant only six months after enrolment. At all time points, the probiotic group presented a longer sleep duration and shorter crying time than the control group, but the difference was not statistically significant. Probiotic consumption had no significant effect on the gut microbiota composition except for increased *L. reuteri* DSM 17938 abundance in the probiotic group at six weeks after enrolment. At specific time points after supplementation with *L. reuteri* DSM 17938, a reduction in the prevalence of FGIDs was observed in the probiotic group. However, no observable effect on the gut microbiota was detected during the intervention. We believe that probiotic supplementation in neonates during and after antibiotic treatment to minimise the negative effects of antibiotics on gut function during this vulnerable period of human development warrants further investigation. The trial is registered at ClinicalTrials.gov (NCT02865564)

Keywords

probiotic – infantile colic – functional gastrointestinal disorders – gut microbiota

1 Introduction

Microbial colonisation during the neonatal period offers a unique opportunity to shape the microbial community that is crucial for host homeostasis. Alterations in the microbiota are associated with various diseases in early life, especially necrotizing enterocolitis, neonatal sepsis, infantile colic and other functional gastrointestinal disorders (FGIDs) (Hofman *et al.*, 2022; Pantazi *et al.*, 2023; Senn *et al.*, 2020). Infantile colic symptoms, such as excessive crying, fussing and sleep distress, are commonly reported in infants. While the pathogenesis of infantile colic remains multifactorial and unclear, recent evidence suggests that the gut microbiota may play a role in its development (Zeevenhooven *et al.*, 2018).

Infants exposed to antibiotics during the perinatal period have an increased abundance of the phylum *Proteobacteria* and a decreased abundance of the phylum *Actinobacteria*, especially the family *Bifidobacteriaceae* (Zimmermann and Curtis, 2020). A reduction in the overall diversity of the gut microbiota and the presence of selected drug-resistant microbes have been reported (Morreale *et al.*, 2023; Pantazi *et al.*, 2023; Zimmermann and Curtis, 2020). Moreover, antibiotic exposure during pregnancy and delivery has been demonstrated to be associated with infantile colic, possibly due to the effect of antibiotics on early gut colonisation (Leppälehto *et al.*, 2018).

The effects of probiotics on the prevention of necrotizing enterocolitis, neonatal sepsis and infantile colic have been studied (Aceti *et al.*, 2017; Pärtty *et al.*, 2018; Sharif *et al.*, 2023). However, its ability to prevent of FGIDs in infants exposed to antibiotics during the neonatal period has not been adequately addressed in randomised controlled trials (RCTs). The use of probiotics in antibiotic-exposed neonates is based on reports that probiotics may help to restore the normal microbiota in patients receiving antibiotics (Korpela *et al.*, 2018), potentially preventing the disruption of postnatal gut colonisation leading to dysbiosis and the development of FGIDs in antibiotic-exposed neonates. In addition, probiotics have been reported to help prevent colic symptoms in infants (Ong *et al.*, 2019). Many RCTs have demonstrated the beneficial effects of *Limosilactobacillus reuteri* (before reclassification known as *Lactobacillus reuteri*) DSM 17938 on FGIDs and colic symptoms (Sung *et al.*, 2017); consequently, we sought to investigate the extent to which these effects were also present in antibiotic-treated infants.

The aim of this study was to evaluate the effects of *L. reuteri* DSM 17938 supplementation on the development of FGIDs, crying and sleep duration, and the gut microbiota in infants exposed to antibiotics during the early neonatal period. We expected that neonates receiving *L. reuteri* DSM17938 during and after antibiotic therapy for a total of six weeks would have fewer FGIDs symptoms, shorter daily crying, longer daily sleep duration and different gut microbial compositions than those in the placebo group.

2 Materials and methods

Study design

We conducted a randomised, double-blind, placebo-controlled trial registered at ClinicalTrials.gov (identifier: NCT02865564) and approved by the National Medical Ethics Committee of the Republic of Slovenia (No. 89/03/15). The recruitment of neonates took place in three Slovenian neonatal units from November 2016 to March 2019. The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and the legal requirements of the study country. Informed consent was obtained from the parent or legal guardian of each neonate after written and verbal study information was provided.

Study subjects

Neonates who needed antibiotic treatment were considered eligible for the study. The aim was to recruit 100 participants who were allocated to the study or placebo group at a 1:1 ratio. The inclusion criteria were antibiotic administration for suspected early or late neonatal sepsis for ≥ five days (ampicillin/flucloxacillin and gentamicin), age < 21 days, and gestational age ≥ 37 weeks. The exclusion criteria were antibiotic treatment for < five days, prematurity, congenital malformations or syndromes, perinatal hypoxia (Apgar at 5 min < 5) and probiotic administration prior to randomisation.

Randomisation, intervention, and study procedure details

At the beginning of antibiotic treatment, neonates were randomly assigned to receive an active study product (containing *L. reuteri* DSM 17938 at a dose of 1×10^8 cfu per day) or a placebo study product (consisting of an identical formulation in all respects except that the bacteria were excluded) according to a computer-generated randomisation scheme. The study products were oil suspensions without vitamin D manufactured and supplied free of charge by BioGaia (Lund, Sweden). The manufacturer was not involved in the study conception, design or conduct and had no influence on the analysis or interpretation of the data. Allocation to the placebo or intervention group was revealed to the study investigators after the last follow-up visit.

The participants in both groups received five drops (1×10^8 cfu) of the study product daily for six weeks, starting on the first day of antibiotic treatment, during hospitalisation and in the home environment. The study product was administered by nurses in the hospital and by parents at home before feeding. Before discharge from the hospital, the nurses instructed the parents on how to administer the study product. The study products were stored in a refrigerator. If antibiotic treatment was discontinued before five days, the participant was excluded from the study. Moreover, the participants were excluded from the study if their parents introduced additional probiotics during the intervention or observation period.

Outcome measures, data, faecal sample collection, and microbial DNA analyses

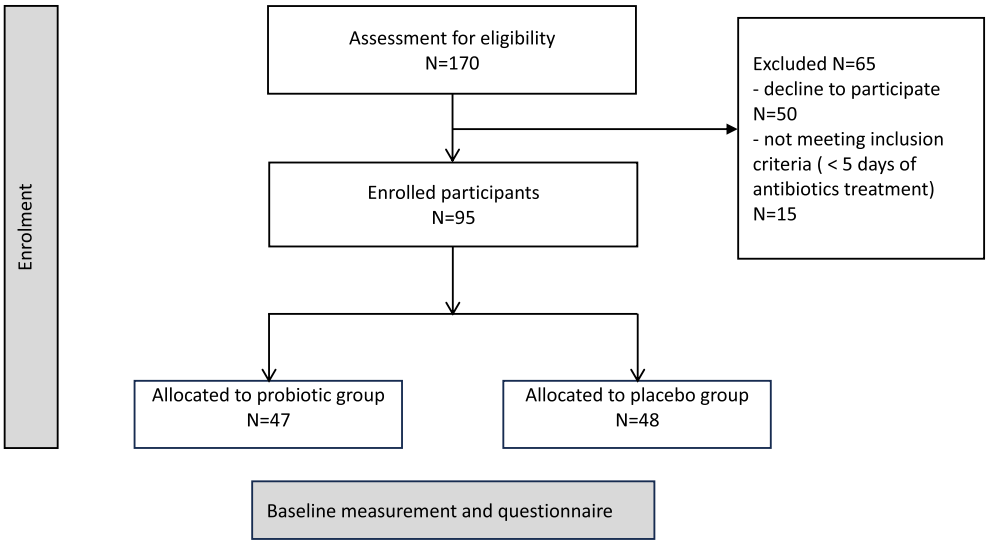
The primary outcomes were the incidence of FGIDs, evaluated by the Infant Gastrointestinal Symptom Questionnaire (IGSQ) (Riley *et al.*, 2015), and daily crying and sleep duration, whereas the secondary outcome was the composition of the faecal microbiota.

Demographic, clinical, anthropometric and feeding data were collected for each patient at enrolment and at follow-up visits. Follow-up visits with faecal sample collection were performed six weeks after enrolment and at 12 months of age (Figure 1). Four and eight weeks after the start of antibiotic therapy and at six months of age, parents completed an online questionnaire on the following clinical data: infant diet, possible allergies and dietary supplements, antibiotic use and infectious diseases, diaper dermatitis, compliance with the study product, crying and sleep duration in the last 24 h and the IGSQ. An SMS message with a link to the web questionnaire and a personal code were sent to the parents. A member of the study team reminded nonrespondents by telephone. Additional clinical interviews were conducted by the clinical investigator at the 12-month follow-up visit.

Faecal samples were collected in sterile sealed containers and kept refrigerated for less than 24 h before being stored at −80 °C for further analyses. First, suspensions of faecal samples (0.1-0.5 g) were prepared in Ringer's solution (1:100 dilution). After centrifugation (3,600×*g*/10 min/10 °C), the pellet was lysed and sonicated as described by Matijašić *et al.* (2014). DNA extraction was performed according to the automated Maxwell 16 System protocol (Promega, Madison, WI, USA).

Similarly, the standard DNA for selected bacterial groups and for the *L. reuteri* DSM 17938 strain applied in real-time PCR assays (Supplementary Table S1) was prepared as described previously (Obermajer *et al.*, 2015, 2017). Real-time PCRs were carried out in a total volume of 20 µl and consisted of 1 µl of 10-fold diluted sample DNA, 0.2 µM forwards/reverse primers (Supplementary Table S1) and KAPA SYBR Fast Master Mix (2×) Universal (KapaBiosystems, Boston, MA, USA). A single 96-well plate assay included reactions of standard DNA dilutions (1:2) as well as diluted samples (1:10) and nontemplate controls (NTCs) run in duplicate. The analysis of a specific microbial group (all bacteria, *Bifidobacterium*, *Enterobacteriaceae*, *Lactobacillus*, *L. reuteri* DSM 17938) included 6 assays carried out on a CFX96 Touch real-time PCR instrument (Bio-Rad, Hercules, CA, USA). CFX Maestro 2.2 software (Bio-Rad) was used to analyse the acquired fluorescence data using the baseline subtracted setting and single threshold mode. The sample concentrations (target copies per g of faeces) and corresponding limits of detection (LODs) of the assay and reaction parameters were calculated according to Obermajer *et al.* (2017) (Supplementary Table S1). In different target reactions, NTCs crossed the threshold of at least 2 logs after the end cycle (30) or after the last sample detected for all bacteria target reactions. The standard dilutions ensured a linear range of at least 2.5 logs for the quantification of the selected bacterial groups.

Metagenomes were obtained via target amplicon sequencing of the variable region V3-V4 of the 16S rRNA gene using the primer pair Bakt_341F (5'-CCTACGGG NGGCWGCAG-3')-Bakt_805R (5'-GACTACHVGGGTAT CTAATCC-3') (Klindworth *et al.*, 2013). Sequencing was performed on a MiSeq instrument (2 × 300 bp). The raw sequence reads were quality filtered with USEARCH v.11.0.667 (Edgar, 2010). UCHIME denoising with default settings was used to obtain zero-radius operational tax-



Allocation

	Probiotic group	Placebo group
Discontinued intervention		
- parents wish	N=3	N=1
- introduced additional probiotic	/	N=2
Finished intervention period	N=44	N=45

Follow up

Period	Assessment	Probiotic group	Placebo group
Four weeks	Online questionnaire	N=43	N=44
Six weeks	Clinical check up - Faecal sample - Infants body measurements	 N=42 N=40	 N=43 N=44
Eight weeks	Online questionnaire	N=42	N=45
Six months	Online questionnaire	N=42	N=45
Twelve months	Clinical check-up: - Faecal sample - Infants body measurements - Clinical interview	 N=38 N=41	 N=38 N=41

FIGURE 1 Study flow diagram and sample attrition.

onomic units (ZOTUs) (Edgar, 2010). The taxonomy was inferred using the RDP trainset (version 18). The sequencing data are available from the NCBI under accession number PRJNA954698.

Sample size

The sample size was calculated for the difference in the IGSQ score and crying duration between the study and placebo groups. On the basis of the data from the study of Indrio *et al.* (2014), 29 participants in each arm were required to detect a 53% difference in crying duration between the groups, with a power of 80% and a significance level of 5%. To detect a 15% difference in the mean IGSQ score between groups, assuming a mean IGSQ score of 20.9 (SD 5.3) in healthy infants (Riley *et al.*, 2015), a power of 80% and a significance level of 5%, the sample size was estimated at 46 participants per study arm. With a drop-off rate of 10%, the aim was to include a total of 100 infants.

Statistical analysis

Clinical and survey data analyses were performed using IBM SPSS Statistics, version 26 (IBM Corporation, Armonk, NY, USA). The chi-square test or Fisher's exact test (for small samples) was used to analyse the differences in categorical variables. The t test for independent samples was used to test the differences in normally distributed numerical variables. A nonparametric test was used to test the difference in asymmetrically distributed numerical variables.

The study and placebo groups were compared in terms of target intestinal bacteria and alpha and beta

microbial diversity. For the comparison of target intestinal bacteria, a logarithmic transformation was applied to each group-specific real-time PCR assay (number of group-specific DNA copies per gram of faeces) to normalise the distribution, and the difference in the median of target bacteria was assessed using a nonparametric test. $P < 0.05$ was considered significant.

Statistical analysis and visualisation of the microbiome data were performed in R (v4.2.2, R Core Team, 2013) with the 'vegan' and 'ggplot' packages.

3 Results

Sample attrition

Among the 95 neonates initially included in the study, 89 (probiotic: n = 44, placebo: n = 45) completed the intervention period, and 82 (probiotic: n = 41, placebo: n = 41) completed the observational period. The parents of 89 infants completed at least one online questionnaire. Faecal samples six weeks and one year after enrolment were collected from 85 and 76 participants, respectively (Figure 1).

Baseline characteristics

The demographic and clinical characteristics of the infants allocated to the probiotic and placebo groups are shown in Table 1. The groups were comparable in all clinical characteristics except for the birth measurements and breastfeeding at one year of age. The proportion of infants breastfeeding at one year of age was greater in the probiotic group (41.5% [95% confidence interval (CI); 39.5-43.5] vs 19.5% [95% CI; 17.5-21.5]) ($P = 0.031$) than in the placebo group.

Effects of probiotic supplementation on the development of FGIDs, crying and sleep duration

At all three evaluation time points, the probiotic group presented lower IGSQ scores and shorter durations of daily crying than did the placebo group, but the difference was not statistically significant. The proportion of infants with an IGSQ score > 30 (the score indicating the presence of clinically important digestive symptoms) (Riley *et al.*, 2015) was greater in the placebo group, and the difference between the groups was significant only six months after enrolment ($P = 0.026$) (Table 2). Although the duration of sleep was longer and the duration of daily crying was shorter in the probiotic group than in the placebo group at all three time points, the difference between the groups was not statistically significant (Table 3). Decreases in the IGSQ score and duration of crying during the six-month observation period were observed in both groups (Table 2). At 12 months of age, there were no differences between the groups in terms of sleep duration or bowel movement characteristics.

Real-time PCR quantification of bacteria in the probiotic and placebo groups

No significant differences were detected between the probiotic and placebo groups in the absolute abundances (number of target gene copies per g of faeces) of all bacteria, lactobacilli, *Bifidobacterium* and *Enterobacteriaceae* groups, as determined by real-time PCR, or in the ratio (group-specific DNA/total bacterial DNA (%)) during the observation period, except for one outcome. The strain-specific detection of *L. reuteri* DSM 17938 at six weeks revealed a greater prevalence (27.9% vs 2.4%, $P < 0.01$) of the ingested strain in the probiotic group than in the placebo group (Table 4).

In addition, no statistically significant differences were found in the abundances or ratios of target microbial groups in the faecal samples of infants who had IGSQ > 30 (four, eight weeks and six months after enrolment) or IGSQ < 30 (data not shown).

Microbiome data analysis of the bacterial community between the probiotic and placebo groups

Permutation analysis of variance (PERMANOVA) revealed that the bacterial community structure differed significantly between the two follow-up assessments, i.e. at six weeks and one year after enrolment, explaining 11.8% of the interindividual variability (PERMANOVA, $R^2 = 0.118$, $P = 0.001$; Supplementary Table S2). In addition, the type of feeding (breastfeeding vs formula feeding) 8 weeks after enrolment, obesity assessment (body mass index > 2Z score) after one year of follow-up and mode of delivery showed weak correlations and could prove significant given the larger sample size but will not be discussed further (Supplementary Table S2). However, there were no significant differences in bacterial community characteristics between the study groups (AMOVA, $P = 0.789$ and 0.404 at six weeks and one year after enrolment, respectively; Figure 2A). Additionally, neither community richness nor Shannon diversity differed between the study groups in terms of alpha diversity (Figure 2B). The abundance of *Lactobacillus* (ZOTU306) was significantly greater in the probiotic group than in the placebo group at six weeks after the beginning of the intervention, which was consistent with the qPCR results (LEfSe, LDA = 2.430, FDR < 0.05; Figure 2C). The other seven ZOTUs listed in Fig-

TABLE 1 Demographic and clinical characteristics of the study population.[1]

Variables	Probiotic (n = 44)	Placebo (n = 45)	*P*-value
Gender male, n (%)	24 (55)	33 (73)	0.065[b]
Gestational age (weeks), mean (SD)	39.5 (1.2)	39.5 (1.0)	0.887[a]
Birth weight (g), mean (SD)	3,410 (390)	3,680 (540)	**0.007**[a]
Birth head circumference (cm), mean (SD)	34.5 (1.14)	35.2 (1.38)	**0.018**[a]
Body mass index, mean (SD)	13.0 (1.19)	13.3 (1.48)	0.352[a]
Apgar 1 min, mean (SD)	8.6 (1.1)	8.7 (1.1)	0.542[a]
Apgar 5 min, mean (SD)	9.2 (0.8)	9.3 (0.8)	0.716[a]
Mode of birth Caesarean section, n (%)	7 (15.9)	7 (15.6)	0.963[b]
Age at enrolment (days), mean (SD)	3.9 (5.1)	4.5 (5.9)	0.620[a]
Mode of feeding at enrolment			
Exclusively breastfed, n (%)	26 (59.1)	23 (51.1)	0.751[b]
Exclusively formula fed, n (%)	5 (11.4)	6 (13.3)	
Feeding probiotic enriched formula at enrolment, n (%)	0 (0)	0 (0)	
Mode of feeding four weeks[2]			
Exclusively breastfed, n (%)	24 (54.5)	20 (45.5)	0.423[b]
Exclusively formula fed, n (%)	7 (15.9)	12 (27.2)	
Feeding probiotic enriched formula four weeks, n (%)	4 (21.1)	3 (13)	0.682[b]
Mode of feeding eight weeks			
Exclusively breastfed, n (%)	22 (50)	19 (42.2)	0.183[b]
Exclusively formula fed, n (%)	9 (20.5)	17 (37.8)	
Feeding probiotic enriched formula eight weeks, n (%)	6 (27.3)	4 (16.7)	0.484[b]
Breastfed at six months, n (%)	26 (59.1)	20 (44.4)	0.167[b]
Feeding probiotic enriched formula at six months, n (%)	0 (0)	4 (21.1)	0.105[b]
Introduction of solid foods at six months, n (%)	40 (90.1)	42 (93.3)	0.714[b]
Breastfed at one year of age[3], n (%)	17 (41.5)	8 (19.5)	**0.031**[b]
Mothers' age, mean (SD)	31.5 (4.6)	30.8 (5.2)	0.546[a]
Siblings, Yes, n (%)	24 (54.5)	24 (53.3)	0.909[b]
Allergy at one year of age[3], Yes, n (%)	1 (2.4)	3 (7.5)	0.359[c]
Additional antibiotic treatment, Yes			
In first six months, n (%)	5 (11.4)	8 (17.8)	0.392[b]
From six months to one year[4], n (%)	13 (31.7)	13 (32.5)	0.939[b]
Compliance with the study product four weeks[2]			
Every day, n (%)	38 (88.4)	34 (79.1)	0.243[b]
Almost every day, n (%)	5 (11.6)	9 (20.9)	
Compliance with the study product eight weeks[5]			
Every day, n (%)	31 (73.8)	29 (70.7)	0.754[b]
Almost every day, n (%)	11 (26.2)	12 (29.3)	

1 SD = standard deviation; bold values are significant; [a] t test, [b] chi-square test, [c] Fisher's exact test.
2 Data for 86 participants, 43 in the placebo and 43 in the probiotic group.
3 Data for 82 participants, 41 in the placebo and 41 in the probiotic group.
4 Data for 81 participants, 40 in the placebo and 41 in the probiotic group.
5 Data for 83 participants, 41 in the placebo and 42 in the probiotic group.

ure 2C were significantly differentially abundant only before false discovery rate (FDR) correction, and a larger sample size would be needed to confirm their association with probiotic consumption.

4 Discussion and conclusions

Our study is the first RCT to assess the effects of probiotics on the occurrence of FGIDs, crying and sleep duration in infants exposed to antibiotics in the neona-

TABLE 2 Comparison of gastrointestinal symptoms between the probiotic and placebo groups at three time points during the observation period.[1,2]

Variables	Probiotic (n = 44)	Placebo (n = 45)	*P*-value
IGSQ score at four weeks, mean (SD)[3]	26.9 (7.8)	29.7 (8.1)	0.112[b]
IGSQ score at eight weeks, mean (SD)	24.2 (7.6)	25.8 (8.2)	0.368[b]
IGSQ score at six months, mean (SD)	17.6 (3.3)	19.8 (6.4)	
Median (IQR)	17 (7)	18.5 (5)	0.591[a]
IGSQ > 30 at four weeks, n (%)[3]	12 (27.2)	18 (41.9)[4]	0.152[c]
IGSQ > 30 at eight weeks, n (%)	7 (15.9)	10 (22.7)[5]	0.418[c]
IGSQ > 30 at six months, n (%)	0	6 (13.3)	**0.026**[d]

1 Infant gastrointestinal symptom questionnaire score and proportion of infants with infant gastrointestinal symptom questionnaire score > 30.

2 IGSQ = Infant Gastrointestinal Symptom Questionnaire, SD = standard deviation, IQR = interquartile range, bold values are significant, [a] nonparametric test, [b] t – test, [c] chi-square test, [d] Fisher's exact test.

3 Data point for 88 participants, 44 in the placebo and 44 in the probiotic group.

4 n = 18/43.

5 n = 10/44.

TABLE 3 Comparison of daily crying time at three time points during the observation period and of daily sleep duration at four time points during the observation period between the probiotic and placebo groups.[1]

Variables	Probiotic (n = 44)	Placebo (n = 45)	*P*-value
Daily crying duration at four weeks (min), mean (SD)[1]	27 (40)	32 (42)	
Median (IQR)	15 (20)	15 (40)	0.856[a]
Daily crying duration at eight weeks (min), mean (SD)	22 (39)	33 (42)	
Median (IQR)	12 (10)	15 (41)	0.232[a]
Daily crying duration at six months (min), mean (SD)	11 (10)	15 (21)	
Median (IQR)	10 (12)	5 (13)	0.932[a]
Daily sleep duration at four weeks (h), mean (SD)[1]	16.2 (2.4)	15.3 (3.8)	0.191[b]
Daily sleep duration at eight weeks (h), mean (SD)	15.1 (2.7)	14.6 (3.2)	0.549[b]
Daily sleep duration at six months (h), mean (SD)	13.6 (2.4)	13.3 (2.5)	0.547[b]
Daily sleep duration at twelve months (h), mean (SD)	12.9 (1.1)	12.7 (1.1)	0.386[b]

1 SD = standard deviation, IQR = interquartile range,[a] nonparametric test, [b] t-test, [1] data point for 88 participants, 44 in the placebo and 44 in the probiotic group.

tal period. To assess infants' behaviour related to the gastrointestinal tract and detect FGIDs, we used the practical, reliable, and validated IGSQ (Riley *et al.*, 2015) as well as reported crying and sleep duration. Furthermore, we collected data on variables that could influence the gut microbiota or incidence of FGIDs (e.g. mode of delivery, maternal and infant diet, intake of antibiotics/probiotics, allergies, and socioeconomic variables). The comparison of these data revealed no difference between the two groups, except for breastfeeding at one year of age. This is a significant strength of our study, as by equalising the factors that could influence the outcome between the groups, we ensured that the observed differences in outcomes were more likely to be attributed to the intervention. Importantly, the infants were enrolled in three different hospitals, which helped reduce the risk of hospital- and region-specific results being presented as general results.

We found that the administration of a single probiotic strain had a limited effect on the development of FGIDs, sleep duration and duration of daily crying, while the overall diversity of the gut microbiota was not affected. Compared with the placebo group, the probiotic group presented a lower proportion of FGIDs, longer sleep durations and shorter crying durations. However, the differences in FGIDs were statistically significant only at six months after enrolment.

TABLE 4 Real-time PCR quantification of bacteria in faeces collected six weeks and one year after enrolment

Bacteria	log No. of copies[1]/g: median (IQR) Samples below LOD: n (%)	log No. of copies[1]/g: median (IQR) Samples below LOD: n (%)	*P*-value
Six weeks after enrolment	Probiotic (n = 43)	Placebo (n = 42)	
All bacteria	12.46 (0.37)	12.41 (0.53)	0.916[a]
	0	0	
Limosilactobacillus reuteri	6.93 (0.67)	6.93 (0.11)	0.065[a]
DSM 17938[2]	31 (72.1)	41 (97.6)	**0.001**[b]
Lactobacilli[3]	9.63 (1.33)	9.03 (1.62)	0.793[a]
	19 (44.2)	24 (57.1)	0.232[b]
Bifidobacterium	10.67 (2.14)	10.65 (1.39)	0.535[a]
	3 (7)	5 (11.9)	0.437[b]
Enterobacteriaceae	10.65 (2.91)	10.72 (2.53)	0.137[a]
	11 (25.6)	7 (16.7)	0.315[b]
One year after enrolment	Probiotic (n=38)	Placebo (n=38)	
All bacteria	12.51 (0.39)	12.58 (0.35)	0.500[a]
	0	0	
L. reuteri DSM 17938[2]	6.81 (0.3)	6.81 (0.3)	0.461[a]
	37 (97.4)	37 (97.4)	0.753[b]
Lactobacilli[3]	8.95 (0.39)	9.95 (0.39)	0.867[a]
	32 (84.2)	30 (78.9)	0.554[c]
Bifidobacterium	10.33 (0.65)	10.37 (0.47)	0.473[a]
	2 (5.3)	0	0.493[c]
Enterobacteriaceae	9.83 (1.85)	10.18 (1.17)	0.211[a]
	4 (10.5)	3 (7.9)	0.692[b]

1 IQR – interquartile range, LOD – limit of detection, bold values are significant, [a] Mann-Whitney U test, [b] chi-square test, [c] Fisher's exact test.

2 Different real-time PCRs were set for the microbial 16S rDNA target region, except for *Limosilactobacillus reuteri* DSM 17938, for which a strain-specific single-copy gene for the extracellular protein *lr*1694 was targeted.

3 Lactobacilli designation applies here to all members of the former *Lactobacillus* genus, which was reclassified in 2020 into 25 genera (Zheng *et al.*, 2021), including *Leuconostoc*, *Pediococcus* and *Weissella*.

Notably, no adverse effects related to the intervention were observed during the observation period.

Several studies have evaluated the prophylactic and therapeutic effects of *L. reuteri* on infantile colic, and a recent meta-analysis revealed that *L. reuteri* DSM 179389 was effective in reducing crying and/or fussing time in breastfed infants with colic (Sung *et al.*, 2017). A systematic review and meta-analysis, which also included 10 RCTs evaluating the effect of *L. reuteri* DSM 17938, reported a reduced duration of crying regardless of the infant's feeding type. However, the probiotic effect was not demonstrated to be influenced by modulation of the gut microbiota or immune system (Skonieczna-Żydecka *et al.*, 2020). Similarly, in our study, subgroup analysis of the gut microbiota in infants with an IGSQ > 30 revealed no differences in the abundance or ratios of bacteria, suggesting that the beneficial effect of *L. reuteri* DSM 17938 could be mediated by characteristic properties of this probiotic, such as its effect on inflammatory cytokines (Dos Reis Buzzo Zermiani *et al.*, 2021), and not by modulation of the gut microbiota at the taxonomic level. The development of a healthy gut microbiota has also been associated with versatile microbial fermentation and degradation capacity (Schwab *et al.*, 2022). The metabolic network of the microbiota and its functional substrate cross-feeding capacity complement compositional and diversity data. Microbes belonging to different taxonomic groups can perform analogous functions by digesting the same food source and producing comparable metabolites. However, the surveillance of specific functional groups of bacteria involved in lactate metabolism during initial colonisation of the gut was beyond the scope of this study. The lactate-utilising bacterial community and the imbalanced production and utilisation of its gas metabolite (hydrogen) have been reported to be involved in infant wellbeing (Pham *et*

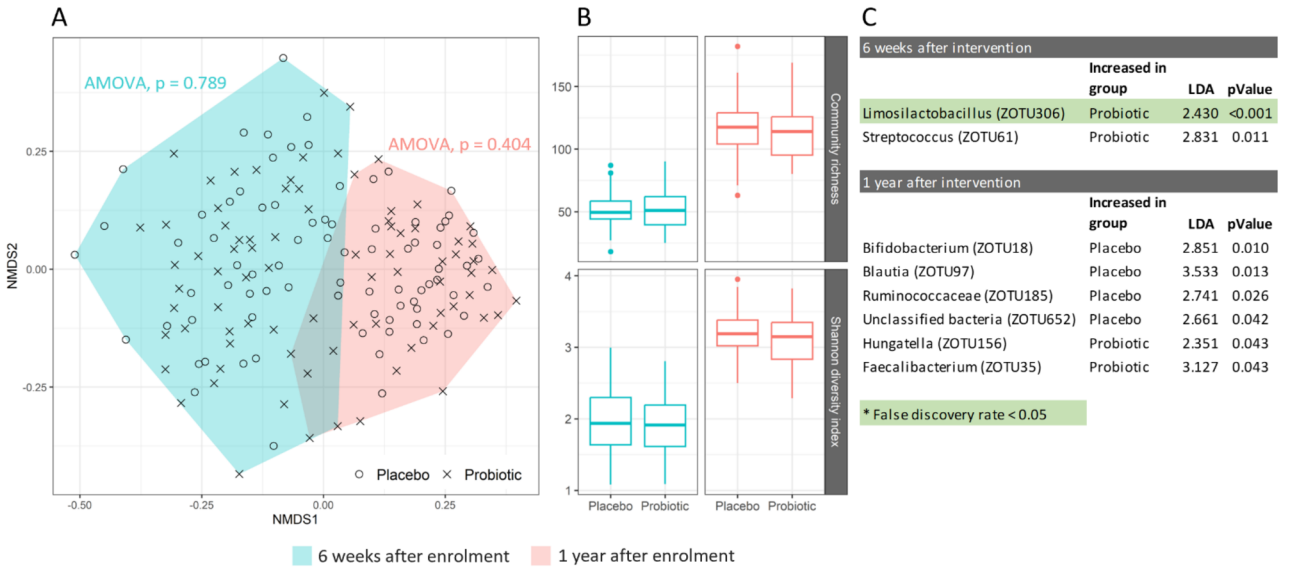


FIGURE 2 Differences in the gut microbiota between the probiotic and placebo groups. The nonmetric multidimensional scaling (NMDS) plot visually depicts the distribution of subjects in the placebo (represented by circles) and probiotic groups (indicated by crosses), colour-coded according to different time points (A); the alpha diversity analysis included the observed bacterial taxa (indicative of community richness) and the Shannon diversity index (B); differentially abundant taxa identified through the LEfSe test are presented separately for each time point, with ZOTUs highlighted in green after false discovery rate (FDR) correction (C). AMOVA = analysis of molecular variance; ZOTU = zero-radius operational taxonomic unit; LDA = linear discriminant analysis.

al., 2016) and may also explain the lack of association between the microbiota composition and FGIDs phenomena observed in our study.

We found only two RCTs in which the effects of probiotics administered concurrently with antibiotics on the neonatal gut microbiota were investigated. Similar to our results in the study of Zhong *et al.* (2021), the use of multiple probiotic species (*Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*) had no effect on the overall diversity of the gut microbiota. However, they reported that probiotics are beneficial in increasing the abundance of the genus *Bifidobacterium*. Furthermore, they reported that administering probiotics concurrently with antibiotics was more beneficial for the gut microbiota than postponing the introduction of probiotics until after antibiotic treatment (Zhong *et al.*, 2021). Importantly, in our study, the probiotic was introduced during antibiotic treatment, which was continued for a total of six weeks. Korpela *et al.* (2018) investigated the effects of probiotics on the microbiota of infants born by caesarean delivery or treated with antibiotics. The multispecies probiotic supplementation prevented or corrected the antibiotic-associated increase in the families *Bacteroidaceae*, *Enterococcaceae* and *Enterobacteriaceae* and the decrease in *Bifidobacteriaceae* observed in the antibiotic-treated control group (Korpela *et al.*, 2018). RCTs investigating the management of gastrointestinal disorders in adults suggest that combinations of probiotic strains may be more effective than single strains. This is attributed to synergistic and additive effects observed among the various strains (Kwoji *et al.*, 2021). The limited effect of single-strain supplementation observed in our study may be more pronounced when it is used in conjunction with multi-strain probiotics, a research topic that warrants further investigation.

In the present study, the detection of the supplemented probiotic in faecal samples was rather low; it was confirmed in less than 30% of the subjects in the probiotic group. Marti *et al.* (2021) reported a greater prevalence of the *L. reuteri* DSM 17938 strain in the faecal samples of extremely preterm infants, although the dosage of the probiotic strain used was similar to that used in our study. However, they reported that nonspecific amplification was not observed when the *lr*1694 gene was targeted and that the upper quantification cycle (Cq) for data analysis subsequently increased (Cq 35). In our study, the detection resolution was lower because nonspecific amplicons were observed in some of the negative samples (Cq greater than 30) following the melting analysis. In addition, high antibiotic pressure reportedly does not affect the overall intestinal colonisation of *L. reuteri* DSM 17938 unless specific antibiotics are applied (Spreckels *et al.*, 2021). Recently, on the basis of phenotypic characterisation and whole-genome sequence analysis, *L. reuteri* DSM 17938 was

reported to be susceptible to gentamicin (but not ampicillin) (Rozman *et al.*, 2022). In our study, antibiotics were administered only at the beginning of the supplementation. Therefore, their use is less likely to be associated with reduced probiotic detection. In addition to the adherence factor of probiotic consumption, which was very good based on the parental reports in our study, the timing of faecal sampling after the last probiotic intake may have influenced the detection of the probiotic strain, which could ultimately indicate its transient nature in the gut.

In our study, the proportion of breastfed infants in the probiotic group at one year of age was greater than that in the placebo group, while it was similar at enrolment. The duration of breastfeeding is influenced mainly by breastfeeding support, level of education, maternal age, obesity and socioeconomic status (Bürger *et al.*, 2022; Haas *et al.*, 2022; Lechosa-Muñiz *et al.*, 2020), which are comparable in the studied groups. Parental concern about the quantity of breast milk, often inferred from infant fussing, has previously been described as one of the factors leading to cessation of breastfeeding (Tracz *et al.*, 2020). In addition, mothers of infants diagnosed with colic are at risk for shorter full breastfeeding durations (Howard *et al.*, 2006). The aforementioned unexpected observation can be attributed to the positive influence of probiotics on the duration of crying, sleeping and gastrointestinal function, which ultimately leads to a more positive breastfeeding experience.

The use of infant formula and the specific type of formula consumed by the infants included in the study could potentially influence the study outcome. Notably, there was no significant difference between the study and placebo groups in terms of the proportion of infants who used infant formula or the proportion of those who used infant formula with added probiotics. This study has several other limitations. It was designed to detect significant effects of *L. reuteri* DSM 17938 supplementation on the duration of crying and IGSQ scores and was not powered to detect effects on the gut microbiota composition. Data from Indrio *et al.* (2014) were used to calculate the sample size to detect differences in the duration of crying (Indrio *et al.*, 2014). Interestingly, the average daily duration of crying four weeks after enrolment of all infants was significantly shorter than that reported in the study of Indrio *et al.* (2014). In addition, our RCT included only term infants, which limits the possibility of generalising our findings to other groups, especially preterm infants. Moreover, owing to limited funding, we could not analyse faecal samples before the introduction of the probiotic. Hence, we could not analyse the effect of potential differences in the gut microbiota at enrolment on outcome measurements. In addition, the low colonisation rate of *L. reuteri* DSM 17938 in the probiotic group may reflect the low sensitivity of the assays we used. Although the detection of DNA from *L. reuteri* DSM 17938 in faeces could not be equated with successful intestinal colonisation, it is also possible that infants did not receive the dose of *L. reuteri* DSM 17938 high enough to have a detectable effect. The outcome measures were obtained through parental self-reports. This may be another limitation of the study, but it is the only feasible way of collecting this type of data. In addition, data on the timing of the introduction of solid foods, which could also influence the results, were not obtained and could therefore not be analysed. However, there was no difference between the two groups in terms of the proportion of infants who had already consumed solid food by the age of six months.

In conclusion, supplementation with *L. reuteri* DSM 17938 during and after antibiotic treatment in term neonates had a limited effect on FGIDs. Given the negligible influence of probiotic supplementation on the gut microbiota composition, the beneficial effect of probiotic supplementation on FGIDs might be mediated by other properties of the probiotic strain and not only by modulation of the gut microbiota composition. Despite confirming the limited effect on FGIDs, we believe that probiotic supplements during and after antibiotic therapy have the potential to minimise the negative impact of antibiotics on gut function during a period of human development that is particularly vulnerable. Further studies are needed to evaluate the effects of probiotics on infant well-being and the gut microbiota in neonates exposed to antibiotics in early life and to understand the mechanism of the positive effects of *L. reuteri* DSM 17938 on FGIDs.

Supplementary material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.27241542

Acknowledgements

We would like to express our gratitude to all the study participants and their parents/legal guardians for their willingness to participate in the study. We are also thankful to the staff working in the inclusion centres

who assisted in recruiting the participants and to Anja Srpčič for her contribution to achieving a high questionnaire response rate.

Authors' contribution

Conception and design: JLK, KLM, AP, EB, DPP; Data acquisition: JLK, PB, AV, TO, UŠ; Analysis and interpretation of data: JLK, AP, KLM, TO, BBM, UŠ, AM; Drafting the article: JLK, TO, AM; Revising it critically for important intellectual content: JLK, KLM, PB, AV, EB, TO, BBM, UŠ, AM, DPP; Approved final version of the manuscript: JLK, PB, AV, KLM, AP, EB, TO, BBM, UŠ, AM, DPP; Agreement to be accountable for all aspects of the work: JLK, PB, AV, KLM, EB, TO, BBM, UŠ, AM, DPP.

Conflict of interest

The authors have declared no conflict of interest.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Funding

The work was supported by the Slovenian Research and Innovation Agency (Record no. P3-0395, P4-0097), as well as internal research funding from the University Medical Centre Ljubljana, Slovenia (Record ID–20150021).

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