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Title page

Probiotics promoted head growth in extremely low birth weight infants in a double-blind placebo-controlled trial

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Short title: Probiotics for extremely low birth weight infants

Abbreviations:
ANOVA: analysis of variance
BPD: bronchopulmonary dysplasia
CI: confidence interval
ELBW: extremely low birth weight
ICH-GCP: International Conference on Harmonisation guidelines for Good Clinical Practice
IVH: intraventricular hemorrhage
IQR: interquartile range
NEC: necrotising enterocolitis
SD: standard deviation

Abstract

Aim:

This study evaluated if oral supplementation with the probiotic bacterium *Lactobacillus reuteri* DSM 17938 improved enteral feeding tolerance and growth rates in extremely low birth weight infants.

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Method:

A randomised, double-blind, placebo-controlled trial comprising 134 extremely low birth weight (<1,000g) infants born before gestational week 28+0. Daily supplementation of *L. reuteri* (1.25 x 10^8 bacteria/day) or placebo started within 3 days and continued until gestational week 36+0. Primary outcome was feeding tolerance and secondary outcome growth rate calculated as z-score development.

Results:

Feeding tolerance was similar in the probiotic and placebo group. Time to full enteral feeds was 15 days in both groups. The z-score of the head circumference decreased in both groups from birth to day 28 of life, but it decreased less in the *L. reuteri* group compared to the placebo group: -1.2 SD (95% CI -1.4 to -1.0) versus -1.7 SD (95% CI, -2.0 to -1.5; p=0.001). Other growth parameters were similar in the study groups.

Conclusion:

Lactobacillus reuteri did not reduce time to reach full enteral feeds in extremely low birth weight infants. The *L. reuteri* supplemented infants, however, had a better growth rate of the head during the first month of life.

Keywords: Feeding tolerance; Growth; Necrotising enterocolitis; Premature infants; Probiotics;

Key notes

- Probiotics seem to reduce feeding intolerance and necrotizing enterocolitis in infants with a birth weight above 1,000g, but the effect on infants with extremely low birth weight is still questioned.

- *Lactobacillus reuteri* did not reduce time to reach full enteral feeds in extremely low birth weight infants in the present trial.
- The *L. reuteri* supplemented infants had a better growth rate of the head during the first month of life.

INTRODUCTION

Nutrition is essential for growth and long-term neurological development in extremely low birth weight infants under 1,000g (ELBW) (1). A common cause of poor initial nutrition in this patient group is food intolerance, i.e. the premature neonate cannot be fully fed by the enteral route. Feeding intolerance and gastrointestinal tract dysfunction also result in problems with abdominal distension, pain and a need for the prolonged use of intravenous catheters. Meta-analyses of prospective randomised controlled trials evaluating probiotics as a measure to prevent feeding intolerance and necrotizing enterocolitis (NEC) in premature infants have provided encouraging results (2). However, despite promising results in infants with a birth weight above 1000g, the effect on ELBW infants is still questioned (3). Thus, further trials in ELBW infants are needed before probiotics can be recommended in this patient group. *Lactobacillus reuteri* DSM 17938 is an interesting candidate for such studies, since it has been shown to increase intestinal peristalsis in animal models (4) and increase gastric emptying and reduce food intolerance in human trials in preterm infants (5).

Growth during the first weeks after extremely preterm birth is inferior to intrauterine growth despite efforts to optimise nutrition (6). While weight development usually improves during the last part of neonatal hospitalisation, the growth of length and head circumference does not show the same recovery, leaving infants growth-restricted at term age (7). Indeed, poor neonatal growth – in particular inadequate growth of the head circumference – of the preterm

infant has been related to worse neurodevelopmental outcome at preschool follow-up (1, 8). The microbiota-gut-brain axis hypothesis suggests that gut microbiota is important for brain development (9). Animal models with germ-free and conventional mice indicate that the gut microbiota early in life is important for neurological functions and cortex myelination (10). Interestingly, supplementation of the commensal bacteria *L. reuteri* corrected oxytocin levels, synaptic potentiation in the ventral tegmental area of the brain and social deficit in a mouse model (11). Also, the commensal *Bacteroides fragilis* corrected gut permeability, altered microbial composition, and ameliorated autism-like symptoms in mice (12). In human trials *L. reuteri* has been shown to increase growth in infants in low-socioeconomic communities (13) and reduce abnormal neurological outcome in infants born moderately preterm (14). We hypothesized that oral supplementation of *Lactobacillus reuteri* DSM 17938 would improve tolerance to enteral feeding, nutrition and, consequently, growth in ELBW infants, and designed a randomized double-blind placebo-controlled trial to test this hypothesis. We limited the inclusion to infants with a birth weight below 1,000g, since it is particularly in this patient group evidence for efficacy and safety of probiotics supplementation is still lacking.

PATIENTS AND METHODS

Study design and participants

The PROPEL trial (Prophylactic probiotics to extremely low birth weight preterm infants was a prospective, multi-centred, double-blind, placebo-controlled, randomized trial in which 134 ELBW infants were supplemented either with the probiotic bacterial strain *Lactobacillus reuteri* DSM 17938 or placebo. The trial was conducted in 10 neonatal units in Sweden from June 2012 to November 2015 in the regions of Stockholm and Linköping and approved by the Ethics Committee for Human Research at Linköping University (Dnr 2012/28-31, Dnr

2012/433-32). Written informed consent was obtained from parents within three days after delivery. Infants between gestational week 23+0 and 27+6 and a birth weight less than 1000 g were eligible for enrolment within three days after delivery. The exclusion criteria were major congenital or chromosomal anomalies, death was considered likely within three days after birth, or the infant were included in another intervention trial on growth, feeding intolerance or severe morbidity. The study subjects were enrolled at the two level III neonatal intensive care units, Astrid Lindgren Children´s Hospital, Stockholm, and Linköping University Hospital, and transferred to their home clinic in the catchment area when they were clinically stable. Due to the 100% coverage of breast milk donor banks all infants were fed exclusively with breast milk until they have reached a weight of at least 2,000g. Protein and lipid fortification was based individually on analyses of the macronutrient and energy content of the breast milk given to each infant. Oral feeding started during the first day of life and increased gradually at a rate specified in clinical guidelines. Breast milk fortification with bovine protein fortifier started when the enteral feeds had reached 100 mL/kg/day. All aspects of management, including whether to interrupt the intervention if the infant was unwell, were at the discretion of the attending clinical staff. The infants were characterized using comprehensive clinical data including perinatal data, growth, feeding intolerance, treatment, antibiotics and mild and severe morbidities collected daily in a study specific case report form until gestational week 36+0. The conduct complied with the principles of the International Conference on Harmonisation guidelines for Good Clinical Practice (ICH-GCP). The study is registered at ClinicalTrials.gov (ID NCT01603368).

Study intervention

The active intervention, once daily *L. reuteri* DSM 17938, was provided in oil drops consisting of sunflower oil, medium chain triglyceride oil and silicon dioxide. The placebo

was maltodextrin provided in an identical oil suspension and was not possible to differentiate from the active product by smell, taste or visual appearance. The daily supplementation 1.25 x 10^8 bacteria (0.2 mL drops) in the *L. reuteri* group was similar to the effective dose in previous trials with *L. reuteri* (5, 15). It started within three days after birth and continued until gestational week 36+0. The study product was administered in the gastric tube or by mouth (when the nasogastric tube had been removed) but was withheld during periods when infants were nil orally. The drops were flushed down by at least 0.3 mL breast milk after the administration in the gastric tube. The study product was manufactured and provided by Biogaia AB, Stockholm, Sweden. The manufacturer checked the quality of the study product regularly, and the concentration of *L. reuteri* was within the stipulated limits in all batches used in the trial. The product was stored in a refrigerator in the pharmaceutical room at the neonatal ward and handled by nurses as a pharmaceutical product in compliance with the instructions of the manufacturer.

Randomisation and masking

The Linköping Academic Research Centre, Linköping University, Sweden, which was independent of the study investigators, constructed the randomisation sequence using Stata v12 statistical software (StataCorp LLC, College Station, Texas, USA). Randomization was stratified by study centre and allocation was 1:1 with random block sizes of 2, 4, 6 and 8. Infants from multiple births were allocated to the same randomisation number and treatment group to avoid problems with cross contamination of *L. reuteri* between twins. The randomisation schedule was provided to the independent contract research organisation (Inpac Pharma, Lund, Sweden), which made up individual bottles for each randomized infant, coded by sequential study number. Included infants were assigned consecutive study numbers and bottles.

Participants were enrolled by the attending physician at the neonatal unit. All staff, investigators, parents and outcome assessors were masked to the treatment allocation.

Primary and secondary outcomes

The primary outcome was feeding tolerance defined as the time to reach full enteral feeds (≥ 150 mL/kg/day). Measures of feeding intolerance also included days of interrupted feeding due to vomiting, distended abdomen or clinical suspicion of NEC. Gastric residuals exceeding the volume that had been given during the last two hours in case of continuous feeding, or the volume given during the last meal in case of bolus feeding, as well as the time until the first passing of stools and the number of daily stools, were also analyzed as an approximation for gastrointestinal motility. Secondary outcomes were growth rate and severe morbidity as outlined below. Weight, length and head circumference were recorded at birth, at 14 and 28 days, and at gestational week 36+0. The standard deviation score (z-score) for each measurement was calculated using Niklasson's growth chart, which is based on information of normal deliveries from gestational week 24 to full term in the Swedish medical birth registry, 1990-1999 (16). Growth rate was calculated using the difference in z-score between the later measurements and birth. All-cause mortality during the study and the cause of death was recorded. Necrotizing enterocolitis was staged according to Bell's criteria (17), and all cases of stage II or greater were recorded. A sepsis diagnosis required positive blood and/or cerebral spinal fluid culture, clinical deterioration and laboratory inflammatory response. In case of positive culture of coagulase negative straphylococci, the sepsis diagnosis required two separate blood cultures with the same antibiotic resistance pattern and, or, central venous/arterial line prior onset. The culture method used at the hospitals was sensitive for lactobacilli. Bronchopulmonary dysplasia (BPD) was defined as oxygen supplementation at 36 completed gestational weeks, and patent ductus arteriosus diagnosis

only when requiring medical or surgical treatment. Intraventricular haemorrhage, retinopathy of prematurity and periventricular leukomalacia were defined according to international classification (18-20).

Sample size and statistical analyses

In a previous study at one of our study centers, the time to reach full enteral feeds was 20 days (21). A 25% reduction from 20 to 15 days was deemed clinically important in the present trial. With at least 47 subjects in each group, a 25% reduction in days from 20 (SD 10) days in the placebo to 15 (SD 7.5) days in the *L. reuteri* group could be detected at a 5% level of significance with 80% power. With a drop out frequency of 30%, a total sample size of 134 infants was sufficient. The primary outcome and other continuous variables with skewed distributions were analysed with Mann-Whitney U test, while Student's t-test were employed for continuous variables with normal distributions. Pearson's chi-square test were used for categorical outcome variables. Fisher's exact test was used when the observed frequency for any cell was less than five. Baseline characteristics were summarized by means and standard deviations (SDs) for continuous data and counts and percentages for categorical data. Primary and secondary outcome variables were summarized by means with 95% confidence interval (CI) or medians with interquartile range (IQR) for continuous data and counts and percentages for categorical data. Main analysis was by intention to treat. Factorial analysis of variance (ANOVA) was made for adjusting continuous variables for the influence from possible confounders. Repeated measures ANOVA was used in analyses of multiple longitudinal measures. A probability level of <0.05 was considered statistically significant. No adjustments for multiple comparisons was made for those outcomes, for which there were separate hypotheses, such as time to reach full enteral feeds as a measure of feeding tolerance and growth rate parameters. All statistical analyses were performed using IBM SPSS

Statistics software, version 23 (IBM Corp, NY, USA). The trial was overseen by an independent data and safety monitoring board.

RESULTS

Between June 2012 and November 2015 in total 134 ELBW infants were enrolled in the study, 68 in the *L. reuteri* and 66 in the placebo group (Fig. 1). Baseline characteristics are displayed in Table 1. There were more caesarean sections and maternal chorioamnionitis in the *L. reuteri* group. Male gender tended to be less common in the *L. reuteri* than in placebo group.

The primary outcome of the trial was feeding tolerance. The median time to reach full enteral feeds (150 mL/kg/day) was 15 days in both groups (p=0.74) (Table 2). Three infants in the *L. reuteri* and two in the placebo group never reached full enteral feeds until the end of the follow-up at gestational week 36+0. There were no differences in the other measures of feeding tolerance such as gastric residuals and number of days of feeding interruption. Neither were there any differences in stool frequency between the two study groups, although the infants in the *L. reuteri* group passed their first stool earlier than the ones in the placebo group (Table 2).

Growth parameters were assessed as secondary outcomes of the trial. In order to adjust for different gestational age the crude measurements were transformed to standard deviation score (z-score) employing Niklasson's growth chart for premature infants (16). The z-score of the head circumference decreased in both groups from birth to day 28 of life, but it decreased less in the *L. reuteri* group compared to the placebo group: -1.2 SD (95% CI -1.4 to -1.0) versus -1.7 SD (95% CI: -2.0 to -1.5), (p=0.001) (Fig. 2). In absolute numbers, the head circumference increased with 2.3 cm (95% CI: 2.0 – 2.5) in the *L. reuteri*-group and

with 1.8 cm (95 % CI: 1.5 – 2.1) in the control group (p=0.01) from birth to day 28. Repeated measures ANOVA including all three time points of assessment also showed significant differences between the *L. reuteri* and the placebo group (p=0.02). The other growth parameters (Fig. 2) as well as severe morbidities such as NEC, sepsis and mortality (Table 3) were similar in the two study groups. Thus, there were not more severe adverse events in the *L. reuteri* than the placebo group. Neither were there any reports of infections caused by *L. reuteri.*

As described above, the distribution of caesarean section and chorioamnionitis differed in the two study groups, and there was also a close to significant less number of boys in the *L. reuteri* group. However, only gender was significantly associated with head growth. Girls had a better development of head circumference than boys between birth and 28 days of life (-1.2 SD; 95% CI -1.4 to -1.0) versus -1.7 SD; 95% CI -1.9 to -1.5; p<0.001). Adjusting the effect of *L. reuteri* on head growth for gender, delivery mode and maternal chorioamnionitis using factorial ANOVA did not remove the significant effect of *L. reuteri* on head growth (p=0.009).

DISCUSSION

This randomised double-blind placebo-controlled trial in ELBW infants did not show an effect of the probiotic bacterium *L. reuteri* on the primary outcome feeding intolerance, which is in consistence with most reports on probiotic interventions in infants with a birth weight <1,000g (22, 23) but in contrast with the positive results in a previous trial using *L. reuteri* by Oncel *et al.* (15). Our trial was specifically designed to study the effect in ELBW infants. A different case-mix with more immature infants in our trial might explain the

different findings. Approximately two-thirds of the infants in the present study were born in gestational weeks 23-25. Other factors such as a reduced mucosal barrier function, hypoxic episodes and impaired microvascular circulation (24) might be more important than microbial composition in these the most immature infants. The fact that all infants exclusively received breast milk until they weighed 2,000g, could also have affected the outcome.

Our study had several strengths. The double-blind design was crucial since the primary outcome was based on the judgment of attending staff. The formulation with oil drops facilitated the dosing and blinding and made administration easy even when the oral feeds still were small. The study product was checked regularly by the manufacturer and handled by nurses as a pharmaceutical product in compliance with the instructions of the manufacturer. There was a low dropout rate and therefore sufficient power to detect a relevant difference in the primary outcome. A higher number of enrolled infants are unlikely to have changed the results of the primary outcome, since the number of days was highly similar in the two treatment groups. The intention to treat approach also makes it possible to generalize the results to all ELBW infants in affluent countries that have survived the first days of life. A limitation in the study might have been the definition of the primary outcome feeding tolerance. All feasible measures of feeding tolerance are elusive and merely a proxy for gut motility. They depend on the discretion of the attending physician and not necessarily on the status of the infants. Thus, if the attending physicians were prone to follow older routines and increased the feeding more slowly than the infants could have tolerated, it might have introduced a false negative result in the trial.

The study was not designed and powered to detect any significant effect on NEC or mortality. The better head growth in the *L. reuteri* group during the first month of life,

however, is interesting, especially in the light of the microbiota-gut-brain axis hypothesis (9), although it should be interpreted with caution as it was a secondary outcome. The effect was seen during the first month of life when the brain should develop the most. The clinical importance of this finding is yet unknown, but possibly important since inadequate growth of the head circumference of preterm infants has been related to worse neurodevelopmental outcome (1, 8). In the 6.5-year follow-up of the Swedish EXPRESS trial 34% of the children born extremely preterm had moderate to severe neurological disability (25), which emphasizes the importance of finding measures to improve brain development. Animal models with germ-free and conventional mice support the hypothesis that the gut microbiota early in life is important for the function and development of the central nervous system(10). The effect of *L. reuteri* on the brain has been tested in mouse models. Supplementation with *L. reuteri* corrected oxytocin levels and synaptic potentiation in the ventral tegmental area of the brain and social deficit in mice (11). Microbial influence on tryptophan metabolism and the serotonergic system has been suggested to be an important mechanism in the gut-brain axis (26). By catabolizing dietary tryptophan to aryl hydrocarbon receptor agonists and thereby increasing interleukin-22, *L. reuteri* reduced gut inflammation (27). Interestingly, it has been suggested that similar mechanism may protect against inflammation in the central nervous system (28). Treatment with probiotics has also been shown to prevent memory dysfunction in mice caused by severe infection.(29) In human trials *L. reuteri* has been shown to increase growth in infants in low-socioeconomic communities (13) and reduce abnormal neurological outcome in infants born moderately preterm (14).

CONCLUSION

The trial could not confirm an effect of *Lactobacillus reuteri* on feeding intolerance in ELBW infants. The *L. reuteri* supplemented infants, however, had a better head growth rate

during the first month of life and may therefore run a reduced risk of developing neurological impairment later in life. The relevance of this finding will be evaluated in a future follow-up trial focusing on neurological development.

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CONFLICT OF INTEREST

Thomas Abrahamsson, has received honoraria for lectures and a grant for the present trial from Biogaia AB. The other authors have indicated they have no potential conflict of interest to disclose.

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TABLE 1. Background and clinical characteristics

	***L. reuteri* (n=68)**		**Placebo (n=66)**		***p****
Gestational age, weeks, mean (SD)	25.5	(1.2)	25.5	(1.3)	0.95
Gestational weeks 23-25, n (%)	43	(63%)	38	(58%)	0.50
Birth weight, g, mean (SD)	731	(129)	740	(148)	0.71
Birth weight z-score, mean (SD)	-1.2	(1.2)	-1.2	(1.4)	0.91
Birth length, cm, mean (SD)	32.3	(2.4)	32.5	(2.6)	0.59
Birth length z-score, mean (SD)	-1.7	(1.7)	-1.5	(1.9)	0.56
Birth head circumference, cm, mean (SD)	22.9	(1.4)	23.0	(1.6)	0.48
Birth head circumference z-score, mean (SD)	-0.9	(0.8)	-0.7	(0.8)	0.16
Apgar at 5 minutes, mean (SD)**	6.2	(2.6)	6.4	(2.5)	0.69
Apgar at 10 minutes, mean (SD)**	7.7	(1.9)	7.9	(2.0)	0.64
Small for gestational age (weight < 2 SD), n (%)	19	(28%)	14	(21%)	0.37
Male, n (%)	32	(47%)	42	(64%)	0.054
Infants from multiple pregnancy, n (%)	28	(41%)	19	(29%)	0.15
Caesarean section, n (%)	50	(74%)	37	(56%)	0.03
Maternal smoking, n (%)	4	(6%)	6	(9%)	0.53
Preeclampsia, n (%)	7	(10%)	6	(9%)	0.81
Chorioamnionitis, n (%)	21	(31%)	10	(15%)	0.03
Preterm premature rupture of membranes, n (%)	25	(37%)	16	(24%)	0.12
Maternal antibiotics, n (%)	42	(62%)	32	(48%)	0.12
Intubated at inclusion, n (%)	54	(79%)	49	(74.2%)	0.48
Surfactant, n (%)	56	(82%)	51	(77%)	0.46
Antenatal corticosteroids, n (%)	67	(98%)	65	(98%)	1.0
Full course of antenatal corticosteroids, n (%)	50	(74%)	47	(71%)	0.76
Inclusion site - Stockholm/Linköping, n/n (%/%)	48/20	(71%/29%)	44/22	(67%/33%)	0.71
Proportion of days with continuous feeding first 28 d, % (SD)	77	(34)	71	(39)	0.38

Antibiotics during first week, n (%)	67	(98%)	66	(100%)	1.0
Antibiotics during second week, n (%)	57	(85%)	51	(77%)	0.25
Days on antibiotics, mean (SD)	28.1	(14.3)	26.0	(15.0)	0.42
Continuous opioids during first week, n (%)	15	(22%)	12	(18%)	0.58
Continuous opioids during second week, n (%)	22	(33%)	19	(29%)	0.61
Days on continuous opioids, mean (SD)	9.4	(12.7)	9.8	(13.1)	0.85
Days on mechanical ventilation, mean (SD)	18.9	(18.3)	17.8	(15.4)	0.70
Patent ductus arteriosus (treated), n (%)	47	(69%)	47	(71%)	0.79
Surgical ligation of ductus arteriosus, n (%)	22	(32%)	21	(32%)	0.95

* Student´s *t*-test was used to compare means. Frequencies were compared using Pearson's X^2-test, or – when the observed frequency in any cell was five or less – Fisher's exact test.
** Apgar score was missing for one infant in each group.

TABLE 2. Feeding intolerance and passing of stools

	L. reuteri			**Placebo**			
	Median	IQR	n	Median	IQR	n	*p**
Days to full enteral feeding (>150 ml/kg/d) **	15	11 - 23	64	15	10 - 20	64	0.74
Days with interrupted feeding	5.5	3 - 9.5	68	6	4 - 10	66	0.56
NEC onset (day of life)	11	7-16	7	18	12-42	8	0.15
	Mean	95% CI	n	Mean	95% CI	n	*p**
Days with parenteral nutrition	24.1	20.3 - 27.9	68	23.1	19.9 - 26.3	66	0.69
Gastric residuals week 1-4 ***	3.0	2.4 - 3.6	68	3.8	3.1 - 4.5	66	0.06
First stools, day of life	2.6	2.1 - 3.0	66	3.3	2.7 - 4.0	64	0.04
Stools week 1-4, no	69.6	64.1 - 75.1	47	69.9	63.8 - 75.9	44	0.94

* Mann-Whitney *U*-test was used to compare distribution and Student´s *t*-test to compare means. ** Seven infants receiving *L. reuteri* and four receiving placebo did not reach full enteral feeding before the end of the study at 36 gestational weeks. Six infants – four allocated to receive *L. reuteri* and two allocated to the placebo group – died before reaching full enteral feeding and were not included in the analyses. *** A gastric residual was considered significant if it exceeded the volume of the previous meal or the volume given continuously during the last two hours. Also it should be larger than 2 ml/kg.

TABLE 3. Severe morbidity and mortality

	***L. reuteri* (n=68)**		**Placebo (n=66)**		
	n/N	%	n/N	%	*p**
NEC, Bell's stage 2-3	7/68	10%	8/66	12%	0.74
NEC, Bell's stage 3	4/68	6%	7/66	11%	0.32
Culture proven sepsis	25/68	37%	23/66	35%	0.82
Bronchopulmonary dysplasia	40/63	63%	39/61	64%	0.96
ROP, grade 3-5	10/62	16%	6/63	10%	0.27
IVH, grade 3-4	6/67	9%	6/66	9%	0.98
Posthemorrhagic hydrocephalus	2/67	3%	0/66	0%	0.50
Periventricular leukomalacia	1/67	1%	4/66	6%	0.21
Death	5/68	7%	5/66	8%	1.0
No severe morbidity or death	13/68	19%	14/66	21%	0.76

N are the number of infants that underwent the relevant diagnostic test or survived until diagnosis could be set. NEC = necrotizing enterocolitis, ROP = retinopathy of prematurity, IVH = intraventricular hemorrhage. * Pearson's X^2-test, or – when the observed frequency was five or less – Fisher's exact test.

Legends to figures

Figure 1

Flow chart of the trial. * Study product discontinued by mistake after transfer to other hospital (n=1). ** Study product not administered again by mistake after temporarily being withheld during nil oral (n=3). *** Study product ran out temporarily at the study site (n=3).

Figure 2

Change in weight (A), length (B) and head circumference (C) in z-score from birth to 14 and 28 days and gestational week 36+0, respectively, in infants receiving *L. reuteri* (solid lines) and placebo (broken lines), using Niklassons's growth charts.(16) Error bars show 95% confidence intervals. * p=0.02 with repeated measures ANOVA. *** p=0.001 with Student´s t-test.

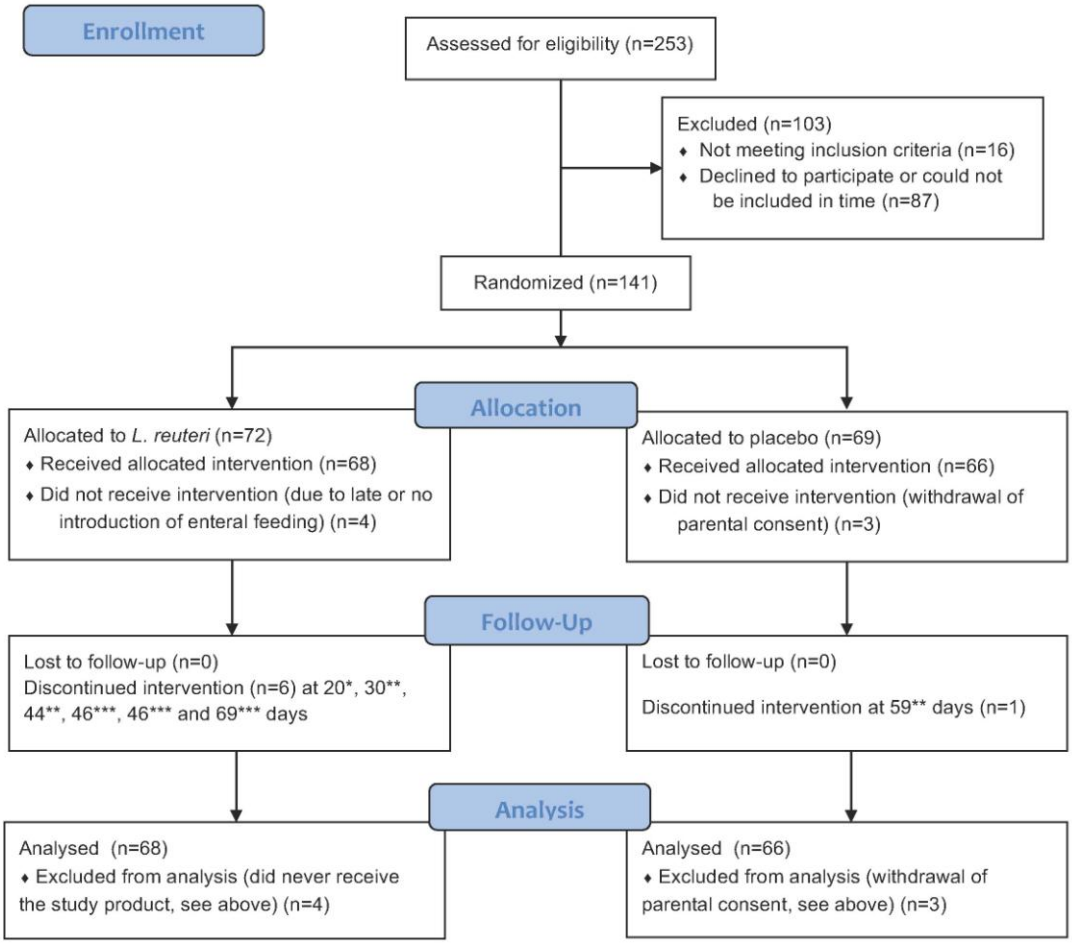
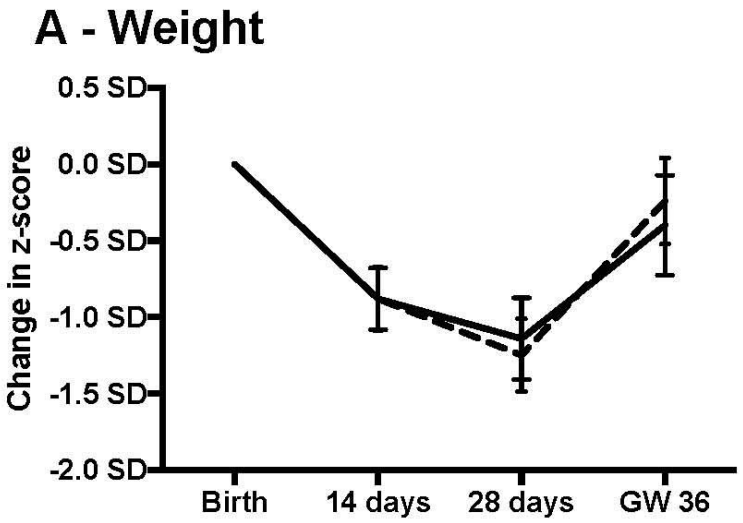
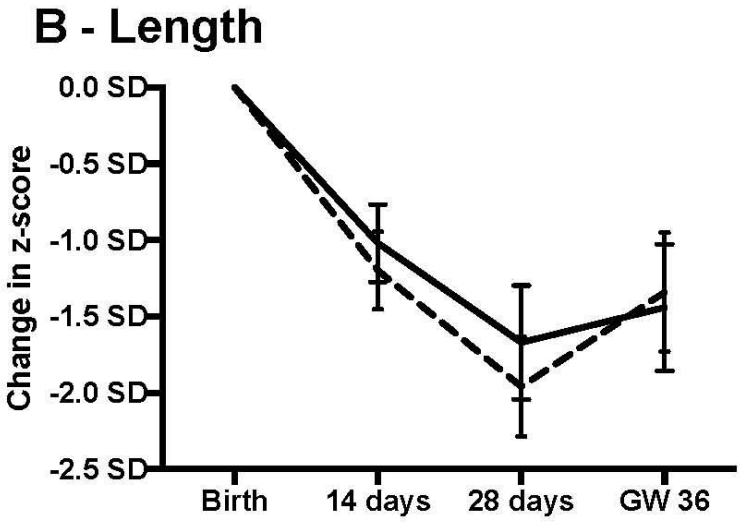


Fig 1





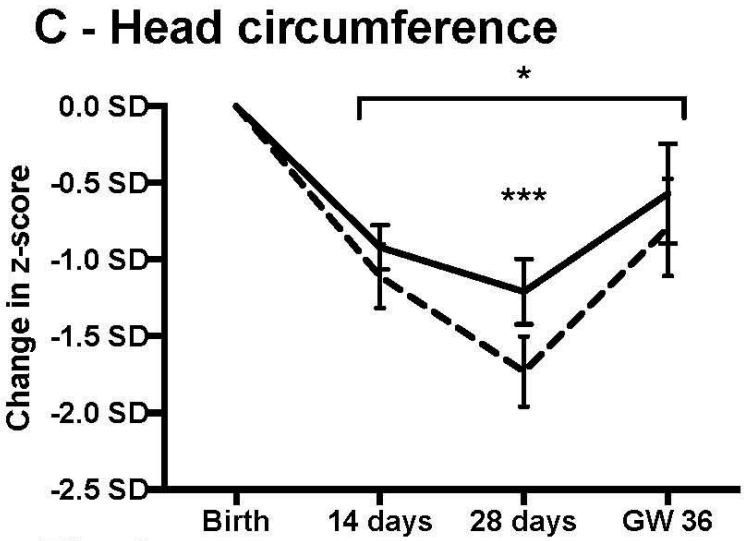


Fig 2