Growth during the first 6 months of life in infants using formula enriched with *Lactobacillus rhamnosus* GG: double-blind, randomized trial

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**Abstract**

**Background** Probiotic bacteria have beneficial effects on the immune system and gastrointestinal tract, but the impacts of their long-term consumption on health and growth in early infancy are not well documented. The aim of this study was to evaluate the influence of *Lactobacillus rhamnosus* GG (LGG)-enriched formula on growth and faecal microflora during the first 6 months of life in normal healthy infants.

**Materials and methods** One hundred and twenty healthy infants (up to 2 months) received LGG-supplemented formula or regular formula in a double-blind, randomized manner until the age of 6 months. Weight, length and head circumference were measured monthly and transformed into standard deviation scores (SDS). Faecal samples were obtained from a random sample of infants (*n* = 25) at entry and at the end of the study.

**Results** One hundred and five infants (51 in the LGG group) completed the study. Children receiving LGG-supplemented formula grew better: their changes in their length and weight SDS (ΔSDS) at the end of the study were significantly higher than those receiving regular formula (0.44 ± 0.37 versus 0.07 ± 0.06, *P* < 0.01 and 0.44 ± 0.19 versus 0.07 ± 0.06, *P* < 0.005, respectively). The LGG group had a significant, higher defecation frequency 9.1 ± 2.6 versus 8.0 ± 2.8 (*P* < 0.05). More frequent colonization with lactobacilli was found in the LGG group, 91% versus 76% (*P* < 0.05) at the end of the study.

**Conclusions** Infants fed with LGG-enriched formula grew better than those fed with regular formula. Further studies are necessary to clarify the mechanism of LGG in infant growth.
Introduction

Over the first year of life, nutrition is the principal regulator of growth with a minimal contribution from growth hormone. Breast feeding is a natural and preferred method of feeding (American Academy of Pediatrics, 1997). When breast feeding is not possible, formula can be used as a sole source or as a supplement to breast milk.

After birth, gastrointestinal flora changes rapidly and an infant’s diet has an important impact on that (Salminen et al., 1998; Savage, 1999). The probiotic Lactobacillus rhamnosus GG ATCC 53103 (LGG) has been shown to have a positive impact on the immune system by stimulating antibody production (Kaila et al., 1992; Saxelin, 1997) as well as by enhancing phagocytic activity of leucocytes (Pelto et al., 1998). It also promotes recovery from rotavirus diarrhoea (Isolauri et al., 1991; Kaila et al., 1992) and reduces the incidence of antibiotic-associated diarrhoea in children (Vanderhoof et al., 1999). Lactobacillus rhamnosus GG has been shown to reduce allergic inflammation (Isolauri et al., 2000) and food allergy (Majamaa & Isolauri, 1997). It also prevents atopic disease in children at high risk (Kalliomäki et al., 2003). No adverse effects have thus far been reported. The supplement of infant formula with probiotic bacteria is currently under consideration in the EU legislation. However, the data regarding the impact of LGG on growth are not published (ESPGHAN Committee on Nutrition, 2004).

In this double-blind study, we investigated the growth and faecal flora in healthy infants receiving formula with or without LGG during the first 6 months of life.

Materials and methods

This prospective, randomized, double-blind, placebo-controlled clinical study was conducted between February and December 2002 at Tartu University Children’s Clinic. One hundred and twenty healthy term infants (60 boys) aged from 0 to 2 months were recruited through four child healthcare centres. The infants had to be on formula for at least half of their daily feedings. Prior to the recruitment, a specialist nurse advised all the mothers on how to facilitate breast feeding. An infant was recruited into the study only then when the amount of breast milk was still insufficient after a week of trying. All the infants were being cared for at home during the study. Subjects were randomized into two treatments groups: active \((n = 60)\) and placebo \((n = 60)\). The study was approved by the local ethics committee and informed consent was obtained from the parents.

Study formulas

The placebo formula was Tutteli® Infant Formula (Valio Ltd, Helsinki, Finland), dairy milk-based spray-dried powder, from birth onwards. The probiotic formula was the same, but enriched with L. rhamnosus GG (LGG, ATCC 53103). Dry bacteria were mixed to the final dry product in Valio Ltd. At the end of shelf life of formula the concentration of bacteria in the dry powder was \(10^7\) colony-forming units per gram \(\text{(CFU g}^{-1})\). The formulas were delivered to the parents in a randomized manner. All packages looked equal (consecutively numbered with the codes of study subjects) and the powders looked, tasted and smelled the same.

Intervention

At the beginning of the study and monthly thereafter up to 6 months of age, the infants were clinically examined and their weight, length and occipitofrontal head circumference (OFC) were measured. Two faecal samples, at entry and at the end of the study, were obtained in a cohort of 25 randomly selected infants. Defecation frequency per 24 h, faecal consistency and an average crying time per 24 h were reported during every visit i.e. monthly. The mothers were instructed to estimate the faecal consistency of the infant. Every day the mother recorded the daily consumption of formula and defecation frequency, stool consistency [on a 3-point scale of loose \((=1)\), not lose or consistent \((=2)\), consistent \((=3)\)] and the hours the infant was crying. In addition, if the supplementary food was assigned to the infant menu, mothers were asked to keep a 5-day food diary before every visit. Illness and medication (yes or no) were registered monthly. Symptoms (for
example, rash) were graded (mild = 1, moderate = 2, severe = 3). The summative indexes during the whole study period were formed for the main parameters of tolerance (crying, consistency of faeces and rash at different visits).

Microbial analyses of faeces

The faecal samples were kept at −80 °C until analyses (maximum frozen time was 225 days). For the isolation of lactobacilli, bacteriological plate count (Bac) and molecular methods were used. The samples were weighed and homogenized before dilution and cultivation on de Man-Rogosa-Sharpe (MRS) agar medium and Rogosa agar medium (Oxoid Ltd, Basingstoke, Hampshire, UK). The plates were incubated at 37 °C for 2 days in microaerobic atmosphere (incubator IG 150, Jouan, St. Herblain Cedex, France; 10% CO₂) and identified according to biochemical properties. Putative Lactobacillus colonies were selected on the basis of colony morphology, cell microscopy and Gram staining. The Lactobacillus sp. isolates were identified according to biochemical properties: carbohydrate fermentation patterns, gas formation from glucose, hydrolysis of arginine and growth at 15 °C. Putative L. rhamnosus isolates were distinguished on the basis of rhamnose fermentation (Lencner et al., 1984) and identified by polymerase chain reaction (PCR) at Valio R&D research laboratory (Halme et al., 2002). Fluorescent in situ hybridization (FISH) analyses were performed on the slides using Ribo Technologies FISH Kits (Microscreen, Groningen, the Netherlands). For estimation of bifidobacteria the Bif 164 probe (Harmsen et al., 2000), for lactobacilli and enterococci the Lab 158 probe (Harmsen et al., 2000) were used. For estimation of clostridia ‘Clostridium mixture’ (Alm et al., 1996; Franks et al., 1998; Harmsen et al., 2000) were used, which contains oligonucleotide for groups of C. coccoïdes, C. lituseburensense and C. butyricum, and it could ascertain approximately all species and subspecies of clostridia in gut (Franks et al., 1998). The counts of micro-organisms were given in log CFU g⁻¹ faeces and the detection limit of bacteriological method was 3 log CFU g⁻¹ and of FISH analysis 7 log CFU g⁻¹.

Statistical analysis

Statistical analyses were performed by statistical program BMDP SOLO (version 4.0) and a ‘SIGMASTAT’ (Jandel Scientific, San Rafael, CA, USA). Mean values with standard deviation were used for growth parameters, median and range for microbiological variables. Weight, length and OFC were also expressed in age-adjusted standard deviation scores (SDS) calculated from the data of normal Estonian infants, at entry (SDS1), at 3 (SDS2) and at 6 (SDS3) months of study. Change in length, weight and OFC SDS (ΔSDS) were calculated at 3 months (SDS2-SDS1) and 6 months (SDS3-SDS1). Auxiliary data between the two treatment groups were compared using two-sample t-test and Mann–Whitney rank sum test. Frequencies of the positive microbiological samples were compared using Fisher’s exact test. P-value of <0.05 was considered statistically significant.

Results

One hundred and five infants completed the study (LGG group n = 51, placebo group n = 54). Final analyses of growth are based on these infants. Reasons for discontinuation of the study were similar in both groups (LGG/placebo): colic pain (n = 1/3), cow’s milk protein intolerance (n = 2/1), constipation (n = 1/1) or diarrhoea (n = 2/0) and excessive breast feeding (n = 2/2).

Growth data are shown in Table 1. At the beginning of the study, children in the LGG group were significantly smaller than those in the placebo group. No differences in birth weight (3416 ± 507 and 3582 ± 464 g) and length (498 ± 20 and 504 ± 19 mm) were observed between the LGG and placebo group. All three parameters of growth, i.e. length, weight and OFC showed some trend upward in both groups over the study period (Fig. 1). The biggest increment over the study period was seen in OFC SDS which increased: 1.1 ± 0.06 SDS and 0.83 ± 0.12 SDS in the LGG and in the placebo group respectively. Growth in length, expressed in ΔSDS, at 3 months and growth both in length and weight at 6 months were significantly higher in the LGG group compared with the placebo.
group (Table 1). Daily consumption of formula increased steadily throughout the study period except in the placebo group where it remained the same at 3 and 6 months. Therefore, the daily amount of formula at 6 months was significantly higher in the LGG group compared with the placebo group (933 ± 368 mL versus 789 ± 277 mL, \( P < 0.05 \)).

After the first month of intervention, 15 (five in the LGG group) of the infants were partly fed with breast milk, six (three in the LGG group) after 3 months and only three (zero in the LGG group) at the end of the study. Thirty-eight infants (19 in the LGG group) received some form of supplementary food, 19 (nine in the LGG group) at 4 months and further, 19 (10 in the LGG group) at 5 months. The main foods were banana, apple, potato or carrot. The effect of the supplementary food on the total energy intake was minimal, only 7% in the LGG and 8% in the placebo group. During the intervention there was no differences between the groups regarding crying behaviour (sum of hours in study period 6.2 ± 1.8 in the LGG group versus 6.1 ± 1.4 in the placebo group). There were 0.90 ± 0.70 infectious episodes in the LGG group and 0.75 ± 0.68 cases in the placebo group. The LGG group had a significantly higher defecation frequency 9.1 ± 2.6 versus 8.0 ± 2.8 (\( P < 0.05 \)) and greater summative indexes of loose stools 9.5 ± 1.2 versus 10.2 ± 1.7 (\( P < 0.05 \)) than the placebo group.

Faecal samples were obtained from a cohort of 25 infants (12 in the LGG group). At the beginning of the study six of 12 (50%) in the LGG group and six of 13 (46%) in the placebo group harboured lactobacilli in their microflora detected by bacteriological method (Table 2). During the study the number of subjects with lactobacilli colonization increased from 50% to 91% (11 of 12) in the LGG group (\( P < 0.05 \)), and from 46% to 77% (10 of 13) in the placebo group.

However, colonization with LGG confirmed by bacteriological and PCR method showed significantly lower numbers than bacteriological or molecular FISH analyses alone (Table 2). At the end of the study LGG was isolated and confirmed by PCR in nine of 12 (75%) infants in the LGG group and in three of 13 (23%) infants in the placebo group (\( P < 0.05 \) between the groups). The colonization frequency and counts of total lactobacilli together with enterococci, bifidobacteria and clostridia analysed by the FISH method did not show significant difference between the groups (Table 2).

### Discussion

This is the first longitudinal study to evaluate the effect of LGG-enriched formula on growth and gut microflora in normal healthy term infants. Our unexpected finding was that the LGG-enriched formula increased growth more than the

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**Table 1** The characteristics (mean ± SD) of the study population

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>At entry</th>
<th>Placebo group (n = 54)</th>
<th>After 3 months LGG group (n = 51)</th>
<th>Placebo group (n = 54)</th>
<th>6 months of age LGG group (n = 51)</th>
<th>Placebo group (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (days)</td>
<td>37.4 ± 10.3</td>
<td>42.2 ± 10.0</td>
<td>37.4 ± 10.3</td>
<td>42.2 ± 10.0</td>
<td>180.9 ± 8.6</td>
<td>181.4 ± 5.4</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>546 ± 4.7(^a)</td>
<td>564 ± 4.4(^a)</td>
<td>642 ± 3.6(^c)</td>
<td>654 ± 3.8(^c)</td>
<td>678 ± 2.9</td>
<td>685 ± 3.2</td>
</tr>
<tr>
<td>SDS</td>
<td>-0.34 ± 1.06(^a)</td>
<td>0.29 ± 1.02(^a)</td>
<td>0.24 ± 0.66</td>
<td>0.60 ± 1.04</td>
<td>0.09 ± 0.68</td>
<td>0.36 ± 0.95</td>
</tr>
<tr>
<td>ΔSDS</td>
<td>0.58 ± 0.39(^b)</td>
<td>0.31 ± 0.02(^b)</td>
<td>0.58 ± 0.27(^b)</td>
<td>0.25 ± 0.16(^b)</td>
<td>0.44 ± 0.37(^b)</td>
<td>0.07 ± 0.06(^b)</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>4424 ± 125(^c)</td>
<td>4864 ± 123(^c)</td>
<td>7100 ± 134</td>
<td>7423 ± 135</td>
<td>8140 ± 140</td>
<td>8290 ± 144</td>
</tr>
<tr>
<td>SDS</td>
<td>-0.28 ± 1.32(^c)</td>
<td>0.36 ± 1.32(^c)</td>
<td>0.30 ± 1.04</td>
<td>0.60 ± 1.16</td>
<td>0.16 ± 1.13</td>
<td>0.36 ± 1.32</td>
</tr>
<tr>
<td>ΔSDS</td>
<td>0.58 ± 0.27(^b)</td>
<td>0.25 ± 0.16(^b)</td>
<td>0.58 ± 0.27(^b)</td>
<td>0.25 ± 0.16(^b)</td>
<td>0.44 ± 0.19(^a)</td>
<td>0.00 ± 0.01(^a)</td>
</tr>
<tr>
<td>Head circumference (mm)</td>
<td>378 ± 2.6</td>
<td>383 ± 2.5</td>
<td>420 ± 2.4</td>
<td>423 ± 2.0</td>
<td>439 ± 2.3</td>
<td>439 ± 1.9</td>
</tr>
<tr>
<td>SDS</td>
<td>-0.69 ± 1.08</td>
<td>-0.38 ± 1.08</td>
<td>0.32 ± 1.01</td>
<td>0.45 ± 0.90</td>
<td>0.40 ± 1.02</td>
<td>0.44 ± 0.96</td>
</tr>
<tr>
<td>ΔSDS</td>
<td>1.02 ± 0.08</td>
<td>0.83 ± 0.18</td>
<td>1.02 ± 0.08</td>
<td>0.83 ± 0.18</td>
<td>1.10 ± 0.06</td>
<td>0.83 ± 0.12</td>
</tr>
<tr>
<td>Formula intake daily (mL)</td>
<td>623 ± 48</td>
<td>597 ± 48</td>
<td>803 ± 172</td>
<td>805 ± 280</td>
<td>933 ± 368(^c)</td>
<td>789 ± 277(^c)</td>
</tr>
</tbody>
</table>

\( ^{a} \text{SDS change in SDS at 3 and 6 months are also given. Two-sample t-test was used for comparison between LGG and placebo group: (a) } P < 0.005; (b) P < 0.01; (c) P < 0.05. \)
non-LGG-enriched formula. The LGG-enriched formula was well tolerated, only the frequency of defecation was slightly higher than in the placebo group.

There have been many studies indicating a positive effects of the use of probiotics (including different species of lactobacilli) on weight gain in animals (Lan et al., 2003; Chiofalo et al., 2004; Timmerman et al., 2005). However, to our knowledge, not such an effect has been shown in humans. It is known that nutrition is the main determinant of childhood growth during the first 3 years. Alternations in dietary intake have an impact on growth not only during that time, but also in the long term. In the majority of children born small for the gestational age (SGA) catch-up growth occurs during the first 2 years. This catch-up has been shown during 9 months to be dependent on extra protein intake and not so much on extra energy intake (Fewtrell et al., 2001). A recent study by Di Caro et al. (2005) showed that LGG increased or decreased gene expression in human small bowel mucosa of more than 300 different genes including many involved in cell growth and apoptosis, such as MAPkinas, caspases-3, -6 and -8. Banaszak et al. (2002) showed that LGG increase proliferation of villus cell and this may increase absorption of nutrient and promote growth. As LGG has also a direct effect on the cellular immune system in humans (Kaila et al., 1992; Saxelin, 1997; Pelto et al., 1998), the other possible mechanism for better growth may by through ghrelin – a potent growth hormone stimulator from stomach. Ghrelin and its receptor, growth hormone secretagogue receptor, are both expressed in human T lymphocytes and monocytes (Dixit et al., 2004).

It has been shown that orally supplemented LGG can improve the balance of the gut flora (Isolauri et al., 1991; Saxelin, 1997; Macfarlane & Cummings, 1999), reduce the incidence of gastrointestinal and respiratory infections in children (Isolauri et al., 1991; Hatakka et al., 2001), modulate immune response (Majamaa & Isolauri, 1997; Kalliomäki et al., 2003), and it may have an antagonistic effect on intestinal pathogens (Isolauri et al., 1991; Vanderhoof et al., 1999).

Although our subjects were randomized at the beginning of the study, the infants in the LGG group were smaller in length and weight than those in the placebo group. They were also shorter than children of the same age in the normal population. The growth difference between groups persisted after multiple regression analyses including age, birth weight and length. It is known that children born SGA grow more rapidly during the
first year than those of normal birth weight (Fewtrell et al., 2001). However, our subjects in the LGG group were not born SGA, but were just slightly below the average ($0.36 \pm 1.01$ SDS). According to our knowledge, there is no data indicating that infants with lower birth weight, which still is appropriate for gestational age, i.e. not SGA, would result in better growth during the first year.

Up to 6 months supplementary food should not influence growth significantly; breast milk or infant formula is the main energy resource. Formula intake did not differ between the study groups at the beginning and after 3 months of the study, a period when length increased more in the LGG than in the placebo group. However, at the end of the study the difference was significant. Supplementary food was added into the diet in some infants after 3 months of study, but the amount of that was small and therefore was considered not to be a significant contributor to growth. The early introduction of solid food (weaning), i.e. before 12 weeks has no effect on future growth compared with so-called late weaning, i.e. after 12 weeks (Morgan et al., 2004).

As expected, LGG-enriched formula increased significantly the number of infants colonized with lactobacilli. At the end of the study, 91% of children in the LGG group harboured lactobacilli, compared with 76% in the placebo group, when measured by bacteriological methods. However, specification for the form, colonization with LGG, confirmed by PCR was slightly lower, 75% in the LGG group. There were also three infants in the placebo group who harboured LGG. It is very likely that these infants obtained the bacteria most likely through family (Alvarez-Olmos & Oberhelman, 2001). Milk products enriched with LGG is commonly used in Estonia. These infants could get LGG from colonized family member by direct contact.

With regard to the other bacteria, counts of bifidobacteria were steady throughout the study and at the level seen in normal healthy children (Sepp et al., 2000). However, counts and colonization rate of clostridia in this study was a little higher, compared with those from an earlier Estonian study (Sepp et al., 2000; Björksten et al., 2001). The reason for discrepancies might lie in methodological differences, because the earlier study counted only the spore-formed clostridia, now all clostridia were counted.

In our study the incidence of infections was low in both groups with no differences between the groups. Infants in the LGG group had slightly greater defecation frequency and number of loose stools, which was likely to help in prevention of constipation. The LGG may stimulate gut peristaltic by balancing gut microflora (Isolauri et al., 1991; Saxelin, 1997) and mucus production (Mack et al., 2004).

### Table 2 Faecal micro-organisms in the infants presented as the prevalence of colonization (number of positive samples) and counts (log CFU g$^{-1}$, range and median)

<table>
<thead>
<tr>
<th>Method</th>
<th>Micro-organisms</th>
<th>LGG group ($n = 12$)</th>
<th>Placebo group ($n = 13$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive samples</td>
<td>Counts (log CFU g$^{-1}$)</td>
<td>Positive samples</td>
</tr>
<tr>
<td>Bacteriological</td>
<td>Lactobacillus</td>
<td>2$^a$ 6.0 (5.0–7.0)</td>
<td>9$^a$ 6.3 (2.0–8.0)</td>
</tr>
<tr>
<td>method</td>
<td>rhamnosus</td>
<td>by Bac and PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>6$^b$ 6.6 (4.0–9.8)</td>
<td>11$^b$ 7.9 (5.3–8.3)</td>
</tr>
<tr>
<td>method</td>
<td>by Bac only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular</td>
<td>Lactobacilli</td>
<td>8 9.1 (8.1–9.7)</td>
<td>11 9.1 (8.2–9.8)</td>
</tr>
<tr>
<td>FISH analyses</td>
<td>enterococci</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bifidobacteria</td>
<td>12 9.9 (9.1–10.1)</td>
<td>11 9.7 (8.0–10.1)</td>
</tr>
<tr>
<td></td>
<td>Clostridia</td>
<td>12 9.5 (8.3–10.1)</td>
<td>12 9.3 (9.2–10.1)</td>
</tr>
</tbody>
</table>

Fisher’s exact test was used in comparisons: (a) $P < 0.001$; (b) $P < 0.05$. 

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The more frequent and loose stools did not result in diarrhoea but were in the range of normal infant stool consistency. No other side-effect was reported in the LGG group or placebo group.

Results of this randomized, double-blind controlled trial showed that LGG-enriched formula were well tolerated. Infants fed with LGG-enriched formula grew normally better than those on regular formula. Further studies are necessary to clarify the mechanism of LGG on infants’ growth.

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