

# Effects of feeding premature infants with *Lactobacillus GG* on gut fermentation

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## Abstract

The study aimed to find out whether gut colonisation of premature babies with a probiotic, *Lactobacillus GG*, modified enteric carbohydrate fermentation. Twenty preterm infants were randomised to receive *Lactobacillus GG* 10<sup>8</sup> colony forming units twice a day for two weeks or to a control group. Faecal short chain fatty acids (SCFAs), ethanol, and urinary 2,3-butanediol, were measured in parallel with microbiological studies. *Lactobacillus GG* colonised nine babies. From 1-28 days of age faecal SCFAs did not differ significantly from controls. Median and ranges were (treated and controls, respectively): acetic acid: 173 (trace-799), 166 (trace-700); propionic acid: 44 (trace-169), 37 (11-229); butyric acid: 31 (5-107), 37 (2-118)  $\mu\text{mol/g}$  dry weight. Ethanol was detected in more faecal samples from treated babies (65% v 37%), and at higher concentration (6.3 (trace-40) v 3.3 (0.6-8.8; one 229)  $\mu\text{mol/g}$ ). 2,3-Butanediol was found in 66% of urine samples from treated babies and 58% from controls. On 83% of these occasions *Klebsiella* sp, *Enterobacter* sp, or *Serratia* sp were cultured from faeces.

*Lactobacillus GG* had no obvious adverse effects on nutritionally important SCFAs. The small increase in ethanol excretion is unlikely to have clinical significance.

(Arch Dis Child 1993; 69: 488-492)

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Accepted 28 July 1993

Normally, the colon is sterile at birth, but is colonised rapidly. In healthy term babies this follows a well-defined sequence, facultative anaerobes appearing first, followed by strict anaerobes which predominate by the end of the first two weeks.<sup>1-3</sup> Many facets of intensive care may alter the normal acquisition of flora by preterm newborns, particularly the use of broad spectrum antibiotics.<sup>3-5</sup> Colonisation with anaerobes is delayed and there is often overgrowth of organisms, such as coliforms, acquired from the environment. These sometimes cause serious systemic infections.<sup>2,5</sup> With the aim of preventing such episodes, high risk premature babies have been fed non-pathogenic bacteria (probiotics) of species normally resident in the gut in healthy term babies.<sup>6-8</sup> Successful colonisation would prevent overgrowth by pathogens. The metabolic consequences of such treatment should be monitored. Some gut bacteria ferment carbohydrates in the colon to short chain fatty acids

(SCFAs) with chain lengths C1 to C6, particularly acetate, propionate, and butyrate. Altogether 80-90% are absorbed and provide energy for the host.<sup>9,10</sup> It is believed that enteric fermentation conserves substantial amounts of energy from unabsorbed lactose in babies born before 34 weeks' gestation.<sup>11-13</sup> SCFAs also have important beneficial effects on the colonic mucosa.<sup>9,10</sup> Successful colonisation with a probiotic organism might disturb this important symbiosis, and thereby have adverse, or beneficial, consequences.

In a randomised double blind trial, we investigated the consequences of feeding *Lactobacillus GG*, a non-pathogenic strain of *Lactobacillus casei*, to premature babies.<sup>14</sup> The biochemical findings are presented here. The main emphasis was on faecal SCFAs. We also measured urinary 2,3-butanediol, a fermentation product of certain enterobacteriaceae, notably *Klebsiella* sp. We previously found this metabolite in urine of premature babies, and suggested that it may indicate abnormal gut colonisation.<sup>15</sup> The aims were to obtain baseline data for faecal SCFAs and urinary 2,3-butanediol excretion during normal neonatal care, to observe the effects of colonisation with *Lactobacillus GG* on these metabolites, and to relate their excretion to the predominant faecal micro-organisms. For the study we developed a new automated analysis for faecal SCFAs, using head space gas chromatography, which is capable of analysing batches of small samples. This method also detects faecal alcohols, including ethanol.

## Patients and methods

Twenty preterm infants with a gestational age of 33 weeks or less who were admitted to the neonatal unit of Princess Anne Hospital, Southampton between 1 September 1991 and 31 January 1992 were studied. With informed parental consent, they were randomised to two groups: group A received standard milk feeds with *Lactobacillus GG* (Valio Finnish Co-operative Dairies Association, Finland) added in a dose of 10<sup>8</sup> colony forming units (cfu) twice daily for 14 days, starting with the first feed. Group B received standard milk feeds. Other care, including antibiotic treatment, followed routine procedures. Details of the babies, and their clinical and microbiological monitoring, are reported in the companion paper.<sup>14</sup> Oral feeding and *Lactobacillus GG* supplements started on days 1 to 3. Seventeen study babies (eight group A, nine group B) received breast milk initially until 3 to 10 days of age (median 6 days), when formula milk

feeds were supplemented. Three were fed a standard formula milk initially. A low birth-weight formula milk (SMA, Wyeth) was introduced from age 5 to 19 days (median 10) in 16 cases, eight in each group. Antibiotics given were: none (two group A, three group B); cefotaxime only, aged 1–5 days (four group A, five group B); flucloxacillin with netilmicin (four group A); erythromycin (one group B); and multiple (one group B).

Faecal samples were collected daily for microbiological studies up to 35 days from the start of oral feeds. Quantitative microbiology was done on samples on, or within 48 hours of, the first day of feeding and 7, 14, 21, 28, and 35 days from this. Samples from these, or adjacent days, were collected for biochemical analyses, together with a random, bag, sample of urine. Additional faecal samples were also available for study. Samples were frozen immediately in airtight containers and stored at  $-20^{\circ}\text{C}$ , without preservative.

SCFAs and ethanol were measured by automated head space analysis with gas chromatography, using a Hewlett Packard automated head space sampler HP19395A and gas chromatograph (HP5890 Series II) with flame ionisation detection (Hewlett Packard, Bracknell) and a megabore fused silica column (25 m $\times$ 0.53 mm internal diameter) coated with polyethylene glycol, BP-20 (SGE Ltd, Milton Keynes).<sup>16</sup> Quantification was by standard addition. According to availability, 1–10 g wet weight faeces was homogenised in 10 ml distilled water; 2 ml was dried and weighed. Two 4 ml aliquots were placed in vials with 1 g of lithium sulphate and 0.5 ml of Aristar formic acid. One ml of 2-ethylbutyric acid (15.62 mmol/l in water) was added as internal standard to one vial and 1 ml of an aqueous standard mixture containing internal standard, ethanol, and nine other SCFAs found in faeces (6.76–35.72 mmol/l) to the other. After incubation at  $90^{\circ}\text{C}$  for 30 minutes, the vials were pressurised with helium at 1 bar for 40 seconds, and gases from above the faecal mixture transferred to the gas chromatograph and injected. A vial containing only 5 ml water, 0.5 ml formic acid, and 1 g lithium sulphate was analysed before each pair of sample vials to minimise errors from ghosting.

Detection limits were 3.2, 7.8, and 26.5  $\mu\text{mol/g}$  dry weight of faeces for butyric, propionic, and acetic acids, respectively, and 1.0–1.6  $\mu\text{mol/g}$  for ethanol and the other six SCFAs. Recoveries ranged from 96–133% and

between batch precision (coefficient of variation) from 3.0–12.0%. Urinary 2,3-butanediol was measured in solvent extracted urine using gas liquid chromatography and urinary D-lactate with an enzymatic method.<sup>15</sup>

The  $\chi^2$  and Mann-Whitney U tests were used for statistical comparisons. The study was approved by the local ethics committee.

## Results

The clinical and microbiological findings are reported in the companion paper.<sup>14</sup> Clinically, there were no adverse effects, but no obvious benefits either. Nine of the 10 group A babies were colonised by *Lactobacillus GG*. There were no statistically significant differences from group B in colonisation by other bacterial species.

### FAECAL SCFAS AND ETHANOL

One hundred and eighty two faecal samples were analysed, 83 from group A and 99 from group B. The results up to 28 days are presented. The SCFAs detected were acetic, propionic, and butyric acids and, rarely, the branched chain SCFAs, isobutyric, isovaleric, and isocaproic acids. No other SCFAs were found. The only alcohol detected was ethanol.

#### *Faecal SCFA and ethanol concentrations of control babies (group B)*

The microbiological studies showed that treatment with the antibiotic cefotaxime during the first five days of life did not cause significant changes in faecal bacterial counts after 6 days of age, when compared with those of babies who did not receive antibiotics. There was no demonstrable effect on faecal SCFAs. Data for SCFAs (table 1) and ethanol (table 2) was, therefore, pooled for three babies who received no antibiotics and five who had cefotaxime only. Results for the two other babies, treated, respectively, with erythromycin (days 16–28) and multiple antibiotics (days 1–28) have been excluded as these antibiotic regimens were associated with decreased counts of faecal bacteria. During the first week, SCFAs were often undetectable, acetic acid being found in only seven of the 26 samples, butyric acid in two, and propionic acid in none. Thereafter, they were detected more often (table 1). Acetic acid was always the most abundant SCFA. Only five samples, from two babies, contained

Table 1 Faecal SCFAs ( $\mu\text{mol/g}$  dry weight) in six babies colonised with *Lactobacillus GG* (group A) and eight controls (group B) who received no antibiotics or cefotaxime only during the first five days

Age (days)	Acetic		Propionic		Butyric							
	Group A		Group B		Group A		Group B					
	No†	Median (range)‡	No†	Median (range)‡	No†	Median (range)‡	No†	Median (range)‡				
1–7	5/13	134 (trace–534)	7/26	19 (29–510)	1/13	37	0/26	0/13	2/26	28; 118		
8–14	19/25	167 (28–501)	25/35	160 (trace–405)	4/25	36 (6–169)	11/35	29 (14–122)	4/25	19 (5–34)	11/35	40 (7–106)
15–21	11/11	248 (trace–799)	9/15	136 (59–246)	6/11	32 (trace–51)	4/15	56 (21–68)	4/11	69 (7–107)	3/15	8 (2–59)
22–28	3/3	524 (153–684)	6/7	266 (31–700)	3/3	80 (58–87)	7/7	81 (11–229)	1/3	31	2/7	33; 48
1–28	38/52	173 (trace–799)	47/83	166 (trace–700)	14/52	44 (trace–169)	22/83	37 (11–229)	9/52	31 (5–107)	18/83	37 (2–118)

†Number of all samples analysed that were positive. ‡Median (range) of positive samples only.

isobutyric, isovaleric, and isocaproic acids, at low concentration (5–28  $\mu\text{mol/g}$ ).

*Effect of Lactobacillus GG colonisation on faecal SCFAs and ethanol (group A)*

Results were pooled for two babies who received no antibiotics and four treated with cefotaxime only aged 1–5 days. They were all colonised with *Lactobacillus GG*. Colonisation was not associated with statistically significant changes in faecal SCFAs (table 1). Branched chain SCFAs were present at very low concentration (8–14  $\mu\text{mol/g}$ ) in only one sample. The only statistically significant differences from controls, were a higher proportion of samples that contained ethanol (34 of 52 group A, 31 of 83 group B;  $p < 0.01$ , 1–28 days) and a higher concentration of ethanol in positive samples (median (range) for group A 6.3 (trace–40), for group B 3.3 (0.6–8.8; one 229);  $p < 0.01$ ). These differences were greatest during the first two weeks of life: for days 1–14, both the ethanol concentrations ( $p < 0.05$ ) and frequency ( $p < 0.01$ ) of positive samples were significantly increased.

*Faecal SCFAs and ethanol of babies receiving antibiotics other than cefotaxime*

Table 3 presents data for one group B baby who was treated with erythromycin aged 16–28 days, and for four group A babies who all had courses of flucloxacillin with netilmicin. Two of the four group A babies did not colonise with *Lactobacillus GG*, one colonised initially but lost the organism during antibiotic treatment and, in the fourth, *Lactobacillus GG* counts were decreased transiently. In three

Table 2 Faecal ethanol ( $\mu\text{mol/l g dry weight}$ ) in six babies colonised with *Lactobacillus GG* (group A) and eight controls (group B) who received no antibiotics or cefotaxime only during the first five days

Age (days)	Group A		Group B	
	No†	Median (range)‡	No†	Median (range)‡
1–7	7/13	6.4 (1.6–13.0)	8/26	4.9 (0.6–8.8)
8–14	18/25*	7.8 (1.2–24.0)	14/35	3.7 (1.3–7.9; one 229)
15–21	6/11	4.8 (trace–8.9)	6/15	3.2 (2.1–4.8)
22–28	3/3	18.0 (4.6–40.0)	3/7	3.3 (0.8–5.5)
1–28	34/52**	6.3** (trace–40)	31/83	3.3 (0.6–8.8; one 229)

†Number of all samples analysed that were positive.

‡Median (range) of positive samples only.

\* $p < 0.05$ ; \*\* $p < 0.01$  groups A and B compared.

Table 3 Faecal SCFAs and ethanol in five babies (four group A and one group B) who received antibiotics other than cefotaxime

Age (days)	SCFAs ( $\mu\text{mol/g dry weight}$ )							
	Acetic		Propionic		Butyric		Ethanol ( $\mu\text{mol/g dry weight}$ )	
	No†	Median (range)‡	No†	Median (range)‡	No†	Median (range)‡	No†	Median (range)‡
1–7	0/4		0/4		0/4		0/4	
8–14	8/11	81 (47–620)	1/11	7	0/11		7/11	3.8 (0.4–9.0)
15–21	6/8	217 (57–571)	2/8	8; 81	0/8		3/8	6.3 (2.0–23.0)
22–28	9/12	218 (66–495)	4/12	79 (10–158)	2/12	5; 12	6/12	5.9 (1.4–35.0)
1–28	23/35	175 (47–620)	7/35	69 (7–158)	2/35*	5; 12	16/35	5.3 (0.4–35.0)

†Number of all samples analysed that were positive. ‡Median (range) of positive samples only.

\* $p < 0.05$  when compared with group B controls who had no antibiotics or cefotaxime only during the first five days.

cases, antibiotic treatment was associated with decreased faecal anaerobes. Faecal butyric acid was found significantly less often ( $p < 0.05$ ) than for control (group B) babies who received no antibiotics, or only an early course of cefotaxime. There were no other differences. A sixth baby (from group A) has been excluded from table 3. He was born at 24 weeks' gestation, developed staphylococcal septicaemia and chronic lung disease and received almost continuous antibiotic treatment up to 38 days of age, followed by a further course of netilmicin and flucloxacillin aged 48–53 days. By chance, his monitoring was extended. Up to 42 days of life, the only organisms grown from faecal samples were enterococci and coagulase negative staphylococci (all six samples) and enterobacteriaceae (one only). Strict anaerobes were not isolated. He was milk fed but, from day 3 up to 55 days of age, concentrations of faecal acetate were low and no propionate or butyrate was detectable.

BACTERIAL SOURCES OF SCFAS

Propionic and butyric acids are produced by strict anaerobes. Paired microbiological data was available for 43 occasions when propionate was excreted in faeces. On 39, there was colonisation with propionate-producing bacterial species (*Veillonella*, *Clostridium*, and (six samples only) *Bacteroides*). The distribution was similar for groups A and B. *Clostridium* sp were a likely source of butyrate in 23 samples (no apparent source in three others) and of branched chain SCFAs on five of six occasions. Sources of acetic acid may have included *Lactobacillus GG*, *Escherichia coli* and species of *Enterobacter*, *Klebsiella*, *Veillonella*, *Bacteroides*, and *Bifidobacterium*. Positive ethanol samples were associated with yeast colonisation on eight occasions, including one with an extremely high faecal ethanol (229  $\mu\text{mol/g}$ ).

URINARY EXCRETION OF 2,3-BUTANEDIOL AND D-LACTATE

2,3-Butanediol was found in 25 of 38 (66%) urine samples from group A and 21 of 36 (58%) from group B babies. These differences were not significant. Concentrations in the positive samples were also similar: for groups A and B respectively 23% and 33% had  $< 10 \mu\text{mol/mmol creatinine}$ ; 35% and 38%, 10–49; 23% and 10%, 50–99; 19% and 19%  $> 99 \mu\text{mol/mmol creatinine}$ . On 38 of the 46

occasions when 2,3-butanediol was excreted in urine, there was gut colonisation with diol-producing enterobacteriaceae: *Klebsiella* sp (22 episodes); *Enterobacter cloacae* (5); *Serratia* sp (1); combinations of *Klebsiella*, *Enterobacter* sp and *Serratia* sp (10). However, similar enterobacteriaceae were cultured on 15 of the 28 (54%) occasions when urine was negative for 2,3-butanediol. The assay for D- lactate was sensitive only to 200  $\mu\text{mol/l}$  of urine (for example 400  $\mu\text{mol/mmol}$  creatinine, if the creatinine was 0.5 mmol/l). D- lactate was detectable in only seven of 47 samples from group B babies, and four of 42 from group A babies colonised with *Lactobacillus GG*. The median (range) concentrations were similar: group B 200 (170–540) and group A 200 (100–400)  $\mu\text{mol/mmol}$  creatinine.

### Discussion

The SCFAs, acetic, propionic and butyric acids, are the major end products of microbial fermentation of undigested carbohydrates in the colon. They are readily absorbed from the colon: butyrate and propionate are cleared, and utilised, by the liver; acetate is also used by peripheral tissues. In addition, SCFAs are trophic to the colonic epithelium, are its preferred energy source, and stimulate sodium and water absorption from the colon.<sup>9 10 17</sup> Production of SCFAs is probably more important for preterm babies born before 34 weeks' gestation than for any other age group. Generally, lactose is a major source of their dietary carbohydrate. Although deficient in the brush border enzyme, lactase, these babies excrete no more than 15% of dietary energy from lactose in the faeces.<sup>11 13</sup> Based on findings in adults and animals, the probable explanation is that unabsorbed lactose entering the colon is fermented by the microflora to SCFAs, which are absorbed and utilised. Increased breath hydrogen is an indirect indication of this fermentation.<sup>3</sup> Alcohols, including ethanol, are also fermentation products of some gut bacteria as well as yeasts.<sup>18</sup>

Faecal SCFAs are believed to reflect production in the colon.<sup>19</sup> There are reported data for faecal concentrations of healthy term babies,<sup>20–22</sup> but we found only one report for premature babies.<sup>23</sup> This is surprising, considering the probable nutritional importance of SCFAs for very immature babies. In the study of Anyon and Clarkson, undertaken in 1971, premature babies were fed cows' milk and sucrose.<sup>23</sup> SCFAs were analysed using a complex and laborious extraction procedure. The SCFA concentrations found were higher than in our study, their mean (SD) values for the third week of life being: acetate 487 (76), propionate 147 (27), and butyrate 122 (8)  $\mu\text{mol/g}$  dry weight with, surprisingly, even higher concentrations of acetate (622 (208)  $\mu\text{mol/g}$ ) and butyrate (132 (31)  $\mu\text{mol/g}$ ) during the first week. These differences may reflect a combination of factors: firstly, maturity: gestational age in the 1971 study was stated only as below 37 weeks. Secondly, the influence of the nursing environment on the rate of anaerobe

colonisation of the gut and the predominant bacterial species. It was notable that only three propionate and butyrate producing species of anaerobes – *Veillonella*, *Clostridium* and, infrequently *Bacteroides* – were identified in our study. This sparsity contrasts strikingly with the mature colonic flora.<sup>24</sup> Thirdly, the effects of different feeds: the lactose load from cows' milk was probably higher than in this study, in which 16 of the 20 babies were graded to a low birthweight formula, supplying half of the carbohydrate energy as lactose and half as maltodextrin. Finally, differences in analytical methods. The procedure developed for this study was accurate and reproducible. Because little sample preparation was needed, losses of the volatile SCFAs during analysis were minimised. Some of the samples analysed may have been too small (less than 1 g wet weight) to detect very low concentrations of SCFAs, accounting for the relatively high proportion of negative samples. This would not, however, influence the concentrations found in positive samples, or alter the overall findings of the study. We also noted that concentrations sometimes varied among samples collected from individual babies on the same days. In future studies, analysis of pooled 24 hour collections would give a more representative profile and ensure adequate sample.

Colonisation with *Lactobacillus GG* had little impact on faecal SCFAs and, it may be assumed,<sup>19</sup> enteric production of these nutritionally important compounds. Weight gain was similar for *Lactobacillus GG* colonised babies and controls, although SCFA production would be only one of several factors contributing to this observation. It was interesting that colonisation was associated with a definite increase in the proportion of faecal samples containing ethanol and a small, but significant, increase in its concentration. The differences from controls were most marked during the first 14 days of life when faecal counts of *Lactobacillus GG* were highest. *Lactobacillus GG*, itself, does not produce ethanol (personal observation), its fermentation products being predominantly lactic acid and a small amount of acetate. It is possible that utilisation of oxygen by *Lactobacillus GG* promoted earlier, or more widespread, colonisation with anaerobes, and that these were the source of ethanol. Ethanol is absorbed readily from the intestine and metabolised in the liver. The increases found were small, however, and it seems unlikely that the amounts produced would have had significant nutritional consequences.

Colonisation with *Lactobacillus GG* had no effect on urinary excretion of 2,3-butanediol, which was found in 63% of all samples. This metabolite is produced during fermentation at pH <6.0 by certain enterobacteriaceae (*Klebsiella* sp, *Enterobacter* sp, and *Serratia* sp) which are potential pathogens.<sup>25</sup> On 83% occasions when excretion occurred, there was coincident gut colonisation with enterobacteriaceae – most often *Klebsiella* sp. The study, therefore, confirms the association of this metabolite with colonisation with these

species.<sup>15</sup> Absence of the metabolite in urine, however, does not exclude colonisation: on 54% occasions with a negative urine test, enterobacteriaceae were grown from faeces. In these cases, the colonic pH may have exceeded 6.0.

Lactic acid is an end product of carbohydrate fermentation by lactobacilli. L+ or D- lactate is produced, depending upon whether organisms have L+ or D- lactate dehydrogenase, or both. D- Lactate absorbed from the gut is metabolised poorly by the host and excreted in urine. Some patients with carbohydrate malabsorption have absorbed large amounts of D- lactic acid which has caused an acute metabolic acidosis with encephalopathy.<sup>26</sup> Urinary excretion of D- lactate was not increased by *Lactobacillus GG* supplementation. This organism is a strain of *L casei*, which produces L+ lactate.<sup>18</sup>

Treatment of newborns with antibiotics may reduce the numbers of faecal bacteria, particularly anaerobes, with recovery of counts delayed for one to five weeks.<sup>3,27</sup> Antibiotics may also decrease faecal SCFAs.<sup>28,29</sup> In this study, brief courses of cefotaxime in the first five days of life did not prevent colonisation with *Lactobacillus GG*, have an obvious effect on colonisation by other bacteria after seven days, or alter faecal SCFAs. The only SCFA reduced significantly in five babies treated with other antibiotics was butyric acid. In three cases, this was associated with depletion of faecal anaerobic bacteria, which produce this acid.<sup>18</sup> In a sixth baby, there was convincing evidence that prolonged broad spectrum antibiotic treatment decreased faecal bacteria and SCFAs. Propionate and butyrate were undetectable up to 55 days of life, reflecting the lack of anaerobes. Such prolonged depletion of SCFAs might, in theory, have adverse effects on nutrition and the colonic mucosa.

Monitoring colonic SCFA production would seem to be important when attempting to modify the gut microflora of preterm newborns. Practically, this means measuring faecal SCFAs. Despite their importance in preterm nutrition, there are surprisingly few baseline data. Automated analyses may facilitate further studies. The impact of prolonged treatment with antibiotics needs further investigation. Urinary excretion of 2,3-butanediol is associated with gut colonisation by enterobacteriaceae.

We are grateful to Scientific Hospital Supplies, Liverpool, for supplying *Lactobacillus GG* to the Wessex Regional Health Authority and Children's Research Fund for support.

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