Improvement of the human intestinal flora by ingestion of the probiotic strain *Lactobacillus johnsonii* La1

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To exert beneficial effects for the host, for example, improving the intestinal microflora, a probiotic must reach the intestine as a viable strain. These properties must be demonstrated by *in vitro* as well as *in vivo* methods. However, only a few well-designed human clinical studies have shown these properties. *Lactobacillus johnsonii* La1 has been shown to give many beneficial effects for the host, but it is unclear whether a viable strain of *L. johnsonii* La1 has the effect of improving host intestinal microflora. In the present study, a randomised double-blind placebo-controlled cross-over trial was conducted to elucidate the effect of *L. johnsonii* La1 on human intestinal microflora. Twenty-two young healthy Japanese women were randomly divided into two groups, and either received fermented milk with *L. johnsonii* La1 or a fermented milk without *L. johnsonii* La1 (placebo) daily for 21 d. Consumption of the fermented milk: (a) increased total *Bifidobacterium* and *Lactobacillus*, and decreased lecithinase-positive *Clostridium* in the faeces; (b) increased the faecal lactic acid concentrations; (c) decreased the faecal pH; (d) increased the defecation frequency. These changes were stronger than those observed with the placebo. *L. johnsonii* La1 was identified in all subjects only after the consumption of the fermented milk. These results suggest that *L. johnsonii* La1 can contribute to improve intestinal microflora with probiotic properties.

Double-blind placebo-controlled trial: *Lactobacillus johnsonii* La1: Probiotics: Intestinal microflora: Viable strains

In order to protect against invasion of pathogens and maintain body homeostasis, man has developed body defence systems. Since the intestine is continuously exposed to potential pathogens (partially taken up with the diet), which can invade the host, natural defence systems have been developed in the intestine, such as mucosal mucins and lysozyme. The intestinal microflora also plays an important role to contribute to the overall defence system in the intestine. It is known that germ-free animals have a higher incidence of infection than conventional animals (Collins & Carter, 1978), and intestinal intra-epithelial lymphocytes of axenic animals increase after bacterial colonisation (Imaoka *et al.* 1996). The intestinal microflora is significantly related to the health and diseases of the host (Benner *et al.* 1981; Sudo *et al.* 1997; Cebra *et al.* 1998); in particular, *Bifidobacterium* and *Lactobacillus* contribute to beneficial defensive effects of the host. They exert antimicrobial effects against pathogens by releasing antimicrobial substances (Corthier *et al.* 1985; Gopal *et al.* 2001) or prevent adhesion of pathogenic bacteria onto epithelial cells (Chan *et al.* 1985). They activate natural killer cells (Haller *et al.* 2000*a*; Nagao *et al.* 2000), enhance IgA synthesis (Fukushima *et al.* 1998; Ibnou-Zekri *et al.* 2003), modulate cytokine production (Marin *et al.* 1997; Haller *et al.* 2000*b*)

and contribute to the maintenance of body homeostasis (Hata *et al.* 1996; Kiessling *et al.* 2002). They also aid nutritional absorption (McDonough *et al.* 1983) and vitamin synthesis (Gibson & Roberfroid, 1995). On the other hand, harmful bacteria such as *Clostridium perfringens* are associated with various diseases and inflammatory responses (Delneste *et al.* 1998; Matzinger, 1998; Haller *et al.* 2000*b*). Considering these facts, it is quite useful to improve the intestinal microflora; that is, to increase the indigenous beneficial bacteria and, by this, decrease harmful bacteria, for both host defence and nutritional benefits.

Recently, probiotic products containing certain strains of *Bifidobacterium* and *Lactobacillus* (Fuller, 1989; Lee & Salminen, 1995), which are normal inhabitants in the human adult gastrointestinal tract (GIT; Dubos *et al.* 1965; Lidbeck & Nord, 1993), have been shown to give beneficial effects on the host by improving the intestinal microflora. However, most studies, which show the impact of probiotics on the intestinal microflora, were performed without placebo or were non-blinded. Only a few studies show that either the strain(s) in the product or the product components have an effect to improve the intestinal microflora by means of double-blind placebo-controlled design.

Lactobacillus johnsonii La1 (NCC533; Nestlé Culture Collection, Lausanne, Switzerland), which formerly belonged to the *L. acidophilus* group (Johnson *et al.* 1980; Fujisawa *et al.* 1992), was selected as a candidate of probiotics under the hypothesis that bacteria with adherence properties on intestinal epithelial cells (IEC) would more efficiently strengthen the host defence system than non-adherent strains. Since the intestinal epithelium is a target for pathogenic encounter and start site of infection, some well-adherent probiotic strains have the potential to prevent infection by competitive exclusion of pathogens *in vitro*. In addition, the modulation of the host immune system through the induction of strain-specific signals by *L. johnsonii* La1 to the IEC has been shown using the IEC–peripheral blood mononuclear cells co-culture model. It was also shown that *L. johnsonii* La1 had stronger adherence on the human Caco-2 and HT-29 cells than other *Lactobacillus* strains *in vitro* (Johnson *et al.* 1980; Bernet *et al.* 1994; Gopal *et al.* 2001). Finally *L. johnsonii* La1 was also shown to contribute to the reinforcement of the host defence systems. Antimicrobial activities against pathogens (Bernet-Camard *et al.* 1997; Blum *et al.* 1999), anti-pylori effects (Tuomola *et al.* 1999; Felley *et al.* 2001) and activation of both natural and specific immunity by the fermented milk containing *L. johnsonii* La1 have been reported (Link-Amster *et al.* 1994; Marteau *et al.* 1997; Donnet-Huges *et al.* 1999; Michetti *et al.* 1999). However, the effect of *L. johnsonii* La1 on the intestinal microflora composition remains unclear.

The following criteria for probiotics have been suggested (Lee & Salminen, 1995; Schiffrin *et al.* 1995; Salminen *et al.* 1996): human origin; safe for consumption; adhesive property onto human intestinal epithelium; survival in the GIT, transient colonisation of the intestine; production of antimicrobial substances; antagonism against pathogenic bacteria; demonstrated beneficial effects on human health. Especially, survival ability in the GIT is considered to be of great importance for exerting probiotic effects on the heart. Although *L. johnsonii* La1 is highly resistant against gastric juice and bile acid *in vitro* (Prasad *et al.* 1998), this does not mimic exactly the conditions *in situ*.

In the present study, a randomised double-blind placebo-controlled cross-over trial was conducted to elucidate the strain-specific effect of *L. johnsonii* La1 on the intestinal microflora in young healthy Japanese women. We also determined the ability of *L. johnsonii* La1 to adhere to Caco-2 cells and survive under GIT conditions *in vitro*.

Material and methods

In vitro *test*

Adhesion test. *L. johnsonii* La1 and *L. acidophilus* La3, La4, La5, La7, La10 and La18 (Nestlé Culture Collection) were used. *L. johnsonii* La1 and *L. acidophilus* La5 and La7 are able to colonise gnotobiotic mice associated with human faecal flora, while other strains (*L. acidophilus* La3; Benner *et al.* 1981; Gibson & Roberfriod, 1995; Haller *et al.* 2000*a*) do not (, unpublished results). The adherence of bacteria onto Caco-2 cells was examined as described previously (Bernet *et al.* 1994). Briefly, Caco-2 cell monolayers were prepared on glass coverslips placed in six-well tissue culture plates (Corning Glass Works, Corning, NY, USA). Cells were seeded at 1.4×10^4 cells per cm^2. Cells were maintained at

37°C in a CO_2–air atmosphere (10:90, v/v). Lactobacilli (1 ml; 4×10^8 bacteria/ml) in the bacterial supernatant fraction or fresh de Man, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) were added to 1 ml of the cell line culture medium. This suspension (2 ml) was added to each well of the tissue culture plate. Plates were incubated at 37°C in CO_2–air (10:90, v/v) for 1 h. After the incubation, monolayers were washed with sterile PBS, fixed with methanol, stained with Gram stain, and examined with a microscope. Each adhesion assay was conducted in triplicate over three successive passages of intestinal cells. For each monolayer on a glass coverslip, the number of adherent bacteria was evaluated in twenty random microscopic areas. Two different technicians evaluated adhesions to eliminate bias.

Resistance against simulated gastric juice

L. johnsonii La1, *L. reuteri*, *L. gasseri*, *L. amylovorus*, *L. plantarum*, *Bifidobacterium longum* and *B. breve* (Nestlé Culture Collection) were used for *in vitro* GIT transit tolerance test. To simulate gastric juice, 0·3 % (w/v) pepsin in 0·5 % (w/v) sterile saline at pH 2·0 was prepared and bacteria (1·0 ml; 4×10^8 colony-forming units (cfu)/ml) were suspended into the simulated gastric juice. They were gently mixed and incubated at 37°C for 3 h. Samples of 0·1 ml were removed 0, 1 and 3 h after anaerobic incubation and decimal dilution series with saline were prepared. From an appropriate dilution, 50 μl of samples were plated onto MRS agar media. After anaerobic incubation at 37°C for 48 h, total viable bacteria were counted.

Resistance against bile acid

L. johnsonii La1, *L. reuteri*, *L. gasseri*, *L. amylovorus*, *L. plantarum*, *B. longum* and *B. breve* (1·0 ml; 4×10^8 cfu/ml) were suspended in 1 % inoculum in MRS broth with 0·1 % bile acid (Becton, Dickinson and Company) at 37°C for 15 h. Samples of 0·1 ml were removed 0 and 15 h after anaerobic incubation, and total viable bacteria were counted in the same manner as described earlier.

### Resistance against simulated gastrointestinal tract conditions

L. johnsonii La1 (1 ml; 1×10^7 cfu) was incubated with simulated gastric juice for 30 min and 0·1 % bile acid for 60 min. Samples of 0·1 ml were removed before and after the two-step incubation, total viable bacteria were counted in the same manner as described earlier.

Fermented milks

Fermented milks (120 g) contained *Streptococcus thermophilus* (1×10^8 cfu) with (test) or without (placebo) *L. johnsonii* La1 (1×10^9 cfu). The composition of both fermented products was as follows: energy, 427 kJ; protein, 4·6 g; lipids, 3·6 g; carbohydrates, 12·9 g; Na, 60 mg; Ca, 128 mg. Final pH was below 4·45. Their taste and texture were maximally adapted at the factory level.

Subjects and study design

Twenty-two young Japanese women from 20 to 22 years of age were selected as subjects under the declaration of Helsinki.
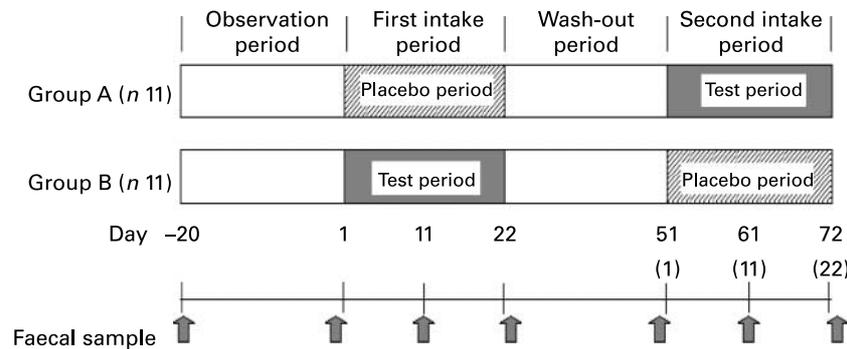
Fig. 1. Study design. A randomised double-blind placebo-controlled cross-over trial was conducted. After 21 d of the non-intake period, either fermented milk containing *Lactobacillus johnsonii* La1 (1×10^9 colony-forming units/120 g; test fermented milk) or placebo fermented milk was administered daily for 21 d.

Informed consent was obtained from all volunteers before starting the study.

The study was conducted with a double-blind placebo-controlled cross-over design shown in Fig. 1. Subjects were randomly divided into two groups. Following the observation period without any fermented milks administration for 21 d, either the test or the placebo-fermented milk was administrated daily for 21 d from day 1 to day 21. All subjects were requested to stop the consumption of other fermented milks and diets that might have effects on the intestinal microflora, such as oligosaccharide or dietary fibre. Antibiotics and cathartics were restricted from being prescribed except at the time of necessity. Subjects recorded a daily questionnaire on the number of defecations, diet, prescription and health remarks during the entire study period.

Faecal flora analysis

Faecal samples were collected at day -20, -7, 0, 11, 22, 50, 61, and 72. Whole faecal samples were stored anaerobically (Anaero Pack® Kenki; Mitsubishi Gas Chemical Company Inc, Tokyo, Japan) at 4°C and provided for faecal flora analysis within 24 h after defecation. Decimal dilution series of faecal samples into the saline were prepared. From an appropriate dilution, 50 μl of samples were plated onto the media and bacteria were analysed according to the Mitsuoka's method (Mitsuoka *et al.* 1976). Three non-selective media (Eggerth-Gagnon, blood liver, trypcase sulfite agar media) and nine selective media (bifidobacteria-selective, enhanced selectivity, neomycin brilliant green taurocholate, *Clostridium welchii*, Clostridia, lactobacilli-selective, triphenyltetrazolium chloride-acridine, potato dextrose, phenylalcohol–egg yolk suspension agar media) were used (Table 1). Bacterial species were identified by colonisation, Gram staining, morphology, lecithinase reaction, aerobic growth and sporulation. The ratio of bifidobacteria was calculated by dividing the number of bifidobacteria by the number of total bacteria.

Identification of L. johnsonii La1

The *Lactobacillus* colony isolated from lactobacilli-selective agar media was transferred onto MRS agar media with antibiotics phosphomycine (0·8 mg/ml; Sigma-Aldrich Co. Ltd, St Louis, MO, USA), sulfamethoxazole (0·93 mg/ml; Wako Pure Chemical Industries Ltd, Osaka, Japan) and tripetho-

prime (0·05 mg/ml; Wako Pure Chemical Industries Ltd), and anaerobically incubated at 37°C for 48 h. Grown bacteria were washed with 1 ml Ringer buffer with 0·5 g L-cysteine hydrochloride/l (Wako Pure Chemical Industries Ltd) in the Eppendorf tube and centrifuged at 4000 rpm for 5 min. The supernatant fraction was removed and the pellet was frozen at -20°C until use. For DNA amplification, 1 μl bacterial pellet, 18 μl sterilised water and 5 μl proteinase K (10 mg/ml; Amersham Biosciences Corp., Piscataway, NJ, USA) were mixed and heated at 55°C for 30 min with Gene Amp PCR System 9600-R (Perkin Elmer Co., Ltd, Tokyo, Japan). Heated bacteria (1 μl) were added into the tubes premixed with 1 μl 50 mM-DNA primer 5′(GGTTGGGTGAGAATTG-CACG)3′ (Microsynth GmbH, Balgach, Switzerland), 1·25 μl 10 mM-deoxynucleotide triphosphate (Amersham Biosciences Corp.), 0·75 μl 50 mM-MgCl₂ (Amersham Biosciences Corp.), 1·25 μl PCR buffer (Invitrogen Co. Ltd, Tokyo, Japan) and 8·25 μl sterilised water, and incubated at 98°C for 10 min, then 1·25 μl PCR buffer, 1 U plantinum Taq DNA polymerase (Invitrogen Co. Ltd, Tokyo, Japan) and 11 μl sterilised water were added (reaction mix). Following the initial step of incubation at 94°C for 5 min, reaction mix

Table 1. Media and conditions used for faecal bacteria identification*

Media	Bacteria mainly identified	Incubation contidion (48 h at 37°C)
Non-selective media		
EG	Anaerobic bacteria	Anaerobic
BL	Anaerobic bacteria	Anaerobic
TS	Aerobic bacteria	Aerobic
Selective media		
BS	Bifidobacteria	Anaerobic
ES	Eubacteria	Anaerobic
NBGT	Bacteroidaceae	Anaerobic
CW	Clostridia (lecithinase +)	Anaerobic
Crostridia agar	Clostridia (lecithinase −)	Anaerobic
LBS	Lactobacilli	Anaerobic
TATAC	Enterococci	Aerobic
PEES	Staphylococci	Aerobic
Potato dextrose agar	Yeast	Aerobic

EG, Eggerth-Gagnon; BL, blood liver; TS, trypcase sulfite; BS, bifidobacteria-selective; ES, eubacterium selective; NBGT, neomycine-brilliant green-taurocholate; CW, *Clostridium welchii*; LBS, lactobacilli-selective; TATAC, triphenyltetrazolium chloride-acridine; PEES, phenylalcohol–egg yolk suspension.
* Mitsuoka's method was used for faecal bacteria identification (Mitsuoka *et al.* 1976).

was provided to the amplification reaction for thirty cycles: denaturation (1 min at 95°C), annealing (1 min at 40°C), and extension (4 min at 72°C). Amplification products were electrophoresed at 70 V for 2 h on 0·8 % (w/v) agarose gel with ethidium bromide (0·3 μg /ml) and visualised under UV illumination.

Faecal organic acid concentrations and pH

Faecal samples were homogenised and centrifuged. Obtained supernatant fractions were filtrated through chromatodisc and provided to a SCFA analysis set (Shodex OA; Shouwa-denkou K.K., Tokyo, Japan). Formic acid, acetic acid, lactic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid were identified.

Faecal pH was measured with a pH meter (Twin; Horiba Ltd, Kyoto, Japan) by touching the pH meter to faeces directly.

Statistic analysis

The results are expressed as mean values and standard deviations.

Data were analysed with software SPSS 11.0 J (SPSS Japan, Tokyo, Japan). Statistical differences between the test and placebo groups, and between pre- and post-administration were examined with the Wilcoxon rank sum test. Statistical difference on the appearance ratio of faecal bacteria was examined with the χ^2 test. The difference between means was considered significant at $P < 0.05$.

Results

Adhesion ability onto Caco-2 cell *in vitro*

Fig. 2 shows the adhesion ability of *L. johnsonii* La1, *L. acidophilus* La3, La4, La5, La7, La10 and La18 onto Caco-2 cells. Bacteria with the property of colonisation (*L. johnsonii* La1, *L. acidophilus* La5, La7) showed better adhesion ability onto Caco-2 cells than bacteria with poor colonisation property (*L. acidophilus* La3, La4, La10, La18). *L. johnsonii* La1 showed the best adhesion ability among the tested bacteria.
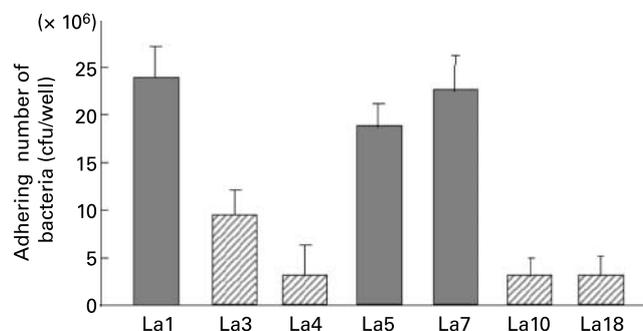
Fig. 2. Adhesion property of lactobacilli. The number of adhering bacteria onto Caco-2 cell is shown. *Lactobacillus johnsonii* La1, *L. acidophilus* La3, La4, La5, La7, La10 and La18 were used. (■), Bacteria with a property to colonise in gnotobiotic mice associated with human faecal flora; (▨), bacteria with a property to colonise in gnotobiotic mice associated with human faecal flora; cfu, colony-forming units.

Survival ability in the gastrointestinal tract *in vitro*

Table 2 shows the survival ability of seven strains of *Bifidobacterium* and *Lactobacillus* in bile acid and the simulated gastric juice. *L. johnsonii* La1, *L. reuteri*, *L. amylovorus*, *L. plantarum*, and *B. breve* showed survival ability after 15 h incubation in 0·1 % bile acids. *L. johnsonii* La1 showed the best survival ability against the simulated gastric juice among the test bacteria and more than 10 % of tested *L. johnsonii* La1 survived after 3 h incubation. In the two-step incubation with simulated gastric juice for 30 min and bile acid for 60 min, *L. johnsonii* La1 was added at 1×10^7 cfu and about 4×10^6 cfu of *L. johnsonii* La1 showed survival after the incubation.

Intestinal microflora

Table 3 shows the effect of the fermented milks on the intestinal microflora. During the test period, the average number of *Bifidobacterium* in the faeces increased from $10^{9·3}$ (SD $10^{0·2}$) cfu/g (day 0) to $10^{9·4}$ (SD $10^{0·2}$) cfu/g (day 11; $P < 0.05$) and $10^{9·7}$ (SD $10^{0·8}$) cfu/g (day 22; $P < 0.001$). The ratio of *Bifidobacterium* in the faeces increased from 11·0 (SD 4·3) % (day 0) to 16·6 (SD 4·9) % (day 11; $P < 0.005$) and 18·0 (SD 7·4) % (day 22; $P < 0.005$) (Fig. 3). Both the number and the ratio of *Bifidobacterium* at day 22 were high compared with those during the placebo period ($P < 0.001$ and $P < 0.01$, respectively). The average number of *Lactobacillus* increased from $10^{5·3}$ (SD $10^{1·1}$) cfu/g (day 0) to $10^{6·5}$ (SD $10^{1·3}$) cfu/g (day 11; $P < 0.001$) and $10^{7·1}$ (SD $10^{0·2}$) cfu/g (day 22; $P < 0.001$) and they were high compared with those during the placebo period ($P < 0.005$ and $P < 0.001$, respectively). The average number of lecithinase-positive *Clostridium* decreased from $10^{5·5}$ (SD $10^{1·8}$) cfu/g (day 0) to $10^{3·2}$ (SD $10^{0·7}$) cfu/g (day 22; $P < 0.005$) and the number at day 22 was low compared with that during the placebo period ($P < 0.05$). The appearance ratio of lecithinase-positive *Clostridium* decreased from 55 % (day 0) to 14 % (day 22; $P < 0.005$).

During the placebo period, no significant changes were observed in either number or the ratio of *Bifidobacterium* and *Lactobacillus*. The average number of lecithinase-positive *Clostridium* decreased from $10^{5·3}$ (SD $10^{0·7}$) cfu/g (day 0) to $10^{4·3}$ (SD $10^{1·4}$) cfu/g (day 14; $P < 0.05$), and the appearance ratio of lecithinase-positive *Clostridium* decreased from 55 % (day 0) to 14 % (day 11; $P < 0.01$) and 23 % (day 22; $P < 0.05$). No significant changes were observed in the count of total bacteria, *Bacteroidaceae* or *Eubacterium* during both the test and placebo period.

**Table 2.** Survival ability against bile acid (0·1 % for 15 h) and simulated gastric juice

		Gastric juice	
Strain	Bile acid	1 h	3 h
LactobacillusL. Johnsonii La1	+	++	++
L. reuteri	+	+	−
L. gasseri	−	+	−
L. amylovorus	+	++	+
L. plantarum	+	+	−
BifidobacteriumB. longum	−	+	−
B. breve	+	+	−

+ , More than 1/1000 of the tested bacteria survived; ++, more than 1/10 of the tested bacteria survived; −, no bacterial growth.

Table 3. Effect of fermented milk containing *Lactobacillus johnsonii* La1 on faecal flora

(Mean values and standard deviations)

	Placebo period (n 22)									Test period (n 22)								
	Day 0			Day 11			Day 22			Day 0			Day 11			Day 22		
	BC			BC			BC			BC			BC			BC		
	Mean	SD	AR	Mean	SD	AR	Mean	SD	AR	Mean	SD	AR	Mean	SD	AR	Mean	SD	AR
Total	10·3	0·3	100	10·3	0·1	100	10·3	0·1	100	10·3	0·2	100	10·3	0·1	100	10·4	0·2	100
Total anaerobes	10·3	0·3	100	10·3	0·1	100	10·3	0·1	100	10·3	0·2	100	10·3	0·1	100	10·4	0·2	100
Total aerobes	8·1	0·1	100	8·2	0·4	100	8·2	0·3	100	8·2	0·3	100	8·4	1·3	100	8·4	0·9	100
Bacteroidaceae	9·8	1·1	100	9·6	0·4	100	9·5	0·2	100	9·4	0·2	100	9·4	0·2	100	9·6	0·6	100
Bifidobacteria	9·3	0·4	100	9·4	0·3	100	9·3	0·2	100	9·3	0·2	100	9·4*	0·2	100	9·7****††††	0·8	100
Eubacteria	9·6	1·0	100	9·6	0·5	100	9·8	0·9	100	9·4	0·4	100	9·4	0·2	100	9·7	0·9	100
Clostridia (lecithinase-positive)	5·3	0·7	55	4·3*	1·4	14**	5·3	1·3	23*	5·5	0·8	55	5·1	0·4	41	10·3***†	0·7	14***
Clostridia (lecithinase-negative)	8·1	0·2	100	8·2	0·3	100	8·1	0·2	100	7·3	0·5	100	7·8	1·7	100	8·1		100
Lactobacilli	5·7	1·2	91	6·3	1·4	86	5·3	5·3	86	5·3	1·1	100	6·5****†††	1·3	100	7·1****††††	0·2	100
L. johnsonii La1	ND		0	ND		0	ND		0	ND		0	6·3****††††	0·5	100****††††	6·9****††††	1·4	100****††††
Streptococci	6·5	1·1	100	7·1	0·3	100	6·5**	0·6	100	7·3	1·1	100	7·2	0·4	100	8·2	0·9	100
Staphylococci	6·6	1·2	100	5·2****	0·4	91	5·3****	0·9	95	6·1	0·3	100	5·6	1·3	95	7·2*	0·9	91
Enterobacteriaceae	7·4	0·5	100	8·0*	0·3	100	7·6	1·1	100	7·3	0·5	100	7·4	0·5	100	7·4	0·4	100
Bacilli	7·8	1·1	100	7·9	2·1	100	8·1	0·2	100	8·1	0·2	100	7·9	1·0	100	7·9	1·2	100
Yeasts	3·5	1·1	64	3·7	1·3	59	6·7	2·2	41	3·6	1·4	100	4·2	1·7	41	3·6	1·2	45

BC, bacterial count ($\log_{10}$ colony-forming units/g); AR, appearance rate (%); ND, not detected ($<10^3$ colony-forming units/g).

Mean values were significantly different from those before intake (day 0): *$P<0.05$, **$P<0.01$, ***$P<0.005$, ***$P<0.001$.

Mean values were significantly different between placebo and test period: †$P<0.05$, ††$P<0.01$, †††$P<0.005$, ††††$P<0.001$.
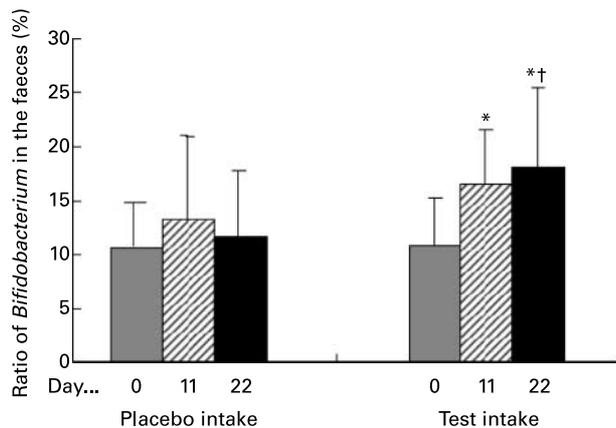
Fig. 3. Effect of fermented milk on faecal *Bifidobacterium* ratio. Data are expressed as mean values (*n* 22), with standard deviations represented by vertical bars. * The ratio of *Bifidobacterium* in the faeces was significantly increased compared with day 0 ($P < 0.005$). † The ratio of *Bifidobacterium* at day 22 was significantly higher compared with that during the placebo period ($P < 0.01$).

Identification of Lactobacillus johnsonii *La1*

Fig. 4 shows the changes of *L. johnsonii* La1 in the faeces. *L. johnsonii* La1 was identified in all subjects after the administration of the test fermented milk only during the test period but not at day 0. *L. johnsonii* La1 was identified neither during the observation nor placebo period. The number of *L. johnsonii* La1 was $10^{6.3}$ (SD $10^{0.5}$) cfu/g (day11) and $10^{6.9}$ (SD $10^{1.4}$) cfu/g (day 22) during the test period. *L. johnsonii* La1 occupied more than 90 % of identified total lactobacillus at day 22. The maximum number of identified *L. johnsonii* La1 was 7.5×10^{9} cfu/d. More than the ingested number of *L. johnsonii* La1 (1×10^{9} cfu/d) was identified from nine out of twenty-two subjects. No more *L. johnsonii* La1 was identified 4 d after stopping the test fermented milk in all subjects.

Faecal organic acid concentrations and faecal pH

Fig. 5 shows the effect of the fermented milks on faecal lactic acid and SCFA concentrations. During the test period, lactic
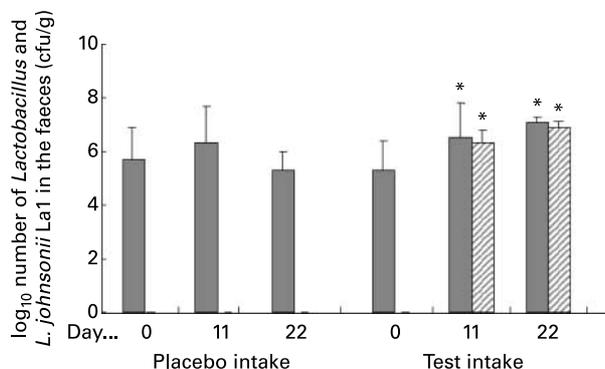
Fig. 4. Effect of the fermented milks on faecal total *Lactobacillus* (■) and *Lactobacillus johnsonii* La1 (▨). Data are expressed as mean values (*n* 20), with standard deviations represented by vertical bars. *L. johnsonii* La1 was identified neither during the observation nor placebo period. * The number of *L. johnsonii* La1 was significantly higher during the test period compared with that during the placebo period ($P < 0.001$). cfu, Colony-forming units.

acid increased from 0.031 (SD 0.063) mg/g (day 0) to 0.208 (SD 0.300) mg/g (day 22; $P < 0.05$) on average. Faecal pH decreased from 7.66 (SD 0.71) (day 0) to 7.17 (SD 0.62) (day 11; $P < 0.05$) and 7.28 (SD 0.61) (day 22; $P < 0.05$) (Fig. 6). During the placebo period, average lactic acid concentration increased from 0.025 (SD 0.048) mg/g (day 0) to 0.113 (SD 0.234) mg/g (day 22; $P < 0.05$). No significant changes were observed on faecal pH during the placebo period.

Defecation frequency

Fig. 7 shows the effect of the fermented milks on defecation frequency in the subjects with mild constipation at less than seven defecations/week during the observation period. Twenty out of twenty-two subjects had mild constipation. During the test period, defecation frequency in the subjects with mild constipation increased from 3.8 (SD 1.4) times/week (days −6 to 0) to 4.9 (SD 1.9) times/week (days 1−7; $P < 0.05$), to 5.2 (SD 2.8) times/week (days 8−14; $P < 0.05$) and to 5.7 (SD 2.7) times/week (days 15−21; $P < 0.05$). During the placebo period, weekly defecation frequency tended to increase but not significantly. No changes were observed in two subjects with normal defecation frequency (more than seven defecations/week during the observation period) during both the test and placebo periods.

Discussion

Since most of the probiotic strains exert their effects in the gut, it is very important for the evaluation of probiotics to show the capability of the given strain to reach the intestine viable and in high numbers. In the *in vitro* test, we evaluated the capability of *L. johnsonii* La1 to attach onto Caco-2 cells and survive in simulated GIT conditions. In the adhesion assay using Caco-2 cells, *L. johnsonii* La1 showed the strongest adhesion among seven other bacteria tested. It was recently shown that the lipoteichoic acid of *L. johnsonii* La1 and the gangliotri- and gangliotetra-osylceramides (asialo-GM1) of Caco-2 cells are involved in the mediation of adhesion (Granato *et al.* 1999; Neeser *et al.* 2000). We also found a positive correlation between adhesion and colonisation. Bacteria with ability to colonise in axenic mice associated with human faecal flora demonstrated a better adhesion property on Caco-2 cells, suggesting that strains with colonisation properties *in vitro* are likely to exert adhesion *in situ*. Although there is no direct evidence that probiotic bacteria with adhesion properties *in vitro* can also attach to the intestinal epithelium *in vivo*, the fact that *L. johnsonii* La1 has properties to adhere to other human intestinal cell-lines (HT-29 and HT-29MTX) suggests a potential of *L. johnsonii* La1 to colonise the human intestine (Bernet *et al.* 1994; Blum *et al.* 1999). In the GIT survival test, 0.1 % bile acid is likely in human GIT, pH of human gastric juice is approximately 2.0 and salt content is not less than 0.5 % (w/v). These concentrations of bile acid and gastric juice were used to simulate human GIT conditions. *L. johnsonii* La1 showed survival in both simulated gastric juice and bile acid. Moreover, *L. johnsonii* La1 showed the best survival ability among seven tested bacteria, which have been reported to have beneficial effects on health (Mogensen *et al.* 2002). Although these long-time incubation tests are often used to evaluate the potential of probiotic
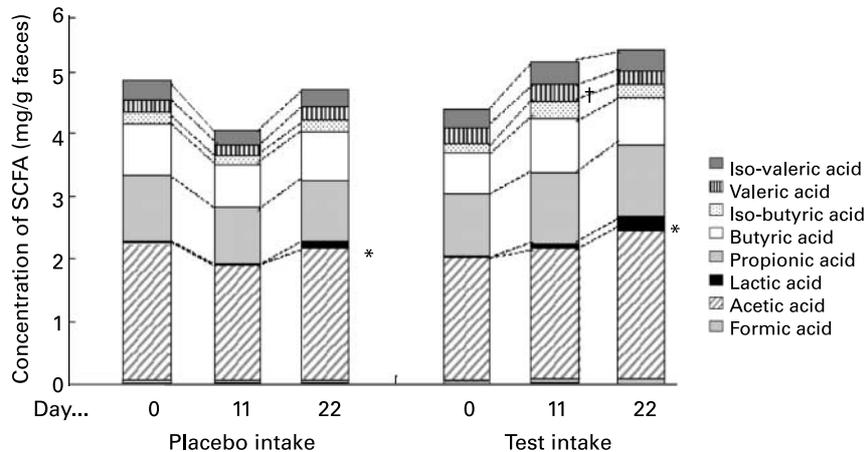
Fig. 5. Effect of the fermented milk on faecal SCFA concentrations. Data are expressed as mean values (n 22), with standard deviations represented by vertical bars. * Lactic acid significantly increased from day 0 to day 22 during the test period and during the placebo period ($P < 0.05$). † Valeric acid significantly increased from day 0 to day 11 during the test period ($P < 0.05$).

strains (Goldin *et al.* 1992; Charteris *et al.* 1998; Prasad *et al.* 1998), these situations probably do not occur in the body, and no studies have reported the survival ability of bacteria by simulating real human GIT conditions for bacteria (temperature, pH, salts, passage-time and speed, etc). We mimicked human GIT conditions by a two-step incubation with simulated gastric juice for 30 min and bile acid for 60 min, and more than 40 % of tested *L. johnsonii* La1 survived in these conditions.

The presented *in vitro* data suggest that ingested *L. johnsonii* La1 can survive in the GIT and reach the intestine in a viable state. To confirm these results *in vivo*, a randomised double-blind placebo-controlled cross-over trial was conducted. By this the effect of *L. johnsonii* La1 on the intestinal microflora composition was investigated.

As seen in Table 3 and Fig. 3, increases in the number and the ratio of *Bifidobacterium* in the faeces were observed after the ingestion of the test fermented milk. It is reported that ingestion of the fermented milk containing *Bifidobacterium* and *Lactobacillus* increases the indigenous *Bifidobacterium* (Gilliland *et al.* 1978; Fukushima *et al.* 1997; Fuller &

Gibson, 1997; Yaeshima *et al.* 1997). Since more than 90 % of identified *Lactobacillus* were *L. johnsonii* La1 after the test fermented milk ingestion, the increase of *Bifidobacterium* observed in the present study is possibly due to the intestinal pH decrease induced by lactic acid or other fermented products produced by *L. johnsonii* La1. However, as the volume of lactic acid in the faeces is too small, it is doubtful that such a small volume of lactic acid can decrease the intestinal pH. There are bacteria that utilise lactic acid such as *Megasphaera* spp., *Deslfolvibrio* spp. and *Propionibacterium* spp. (Stewart & Bryant, 1988). They might convert lactic acid to other SCFA and induce faecal pH decrease. Although we did not examine the changes in bacterial counts utilising lactic acid in the present study, we did observe a tendency of the other SCFA to increase after ingestion of the test fermented milk. *L. johnsonii* La1 secretes non-bacteriocin antimicrobial substances, which have a broad anti-pathogenic spectrum (*Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, etc), but are not effective against *Bifidobacteria* and *Lactobacillus* (Bernet-Camard *et al.* 1997). Although we did not demonstrate the production of antimicrobial substances *in vivo*, it might be speculated that these antimicrobial substances also contribute to the change in the intestinal environment advantageous for the growth of *Bifidobacterium*. Intestinal pH decrease inhibits the growth of harmful bacteria (Eklund, 1983). In accordance with this, we observed decreases in the number and the appearance ratio of lecithinase-positive *Clostridium,* which is associated with carcinogenic enzyme production. It is also known that ingestion of *L. johnsonii* La1 decreases the carcinogenic enzymes nitroreductase and azoreductase in human subjects (F. Rochat, unpublished results).

SCFA in the intestine, especially butyric acid, are utilised for energy by the intestine and promote peristaltic movements of the intestine (Yokokura *et al.* 1977; Young & Gibson, 1995). Poor peristalsis causes defecation frequency decrease, so-called constipation. Chronic constipation is related to various diseases such as colon cancer. Thus, it is important to prevent constipation to improve quality of life. As seen in Fig. 7, increase in defecation frequency was observed in the subjects
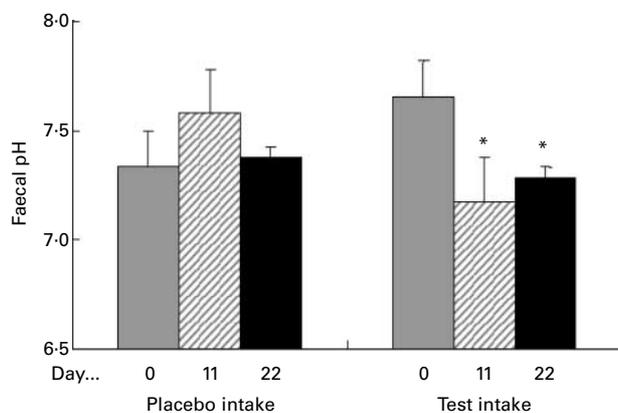
Fig. 6. Effect of the fermented milk on faecal pH. Data are expressed as mean values (n 22), with standard deviations represented by vertical bars. * Faecal pH was significantly decreased compared with day 0 ($P < 0.05$).
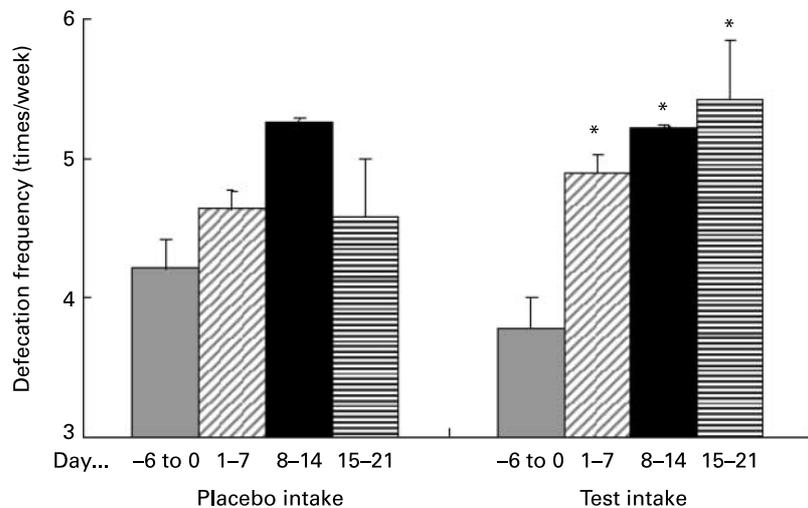
Fig. 7. Effect of the fermented milks on the defecation frequency in the subjects with mild constipation (less than seven defecations/week during the observation period). Data are expressed as mean values (*n* 20), with standard deviations represented by vertical bars. * Weekly defecation frequency in the subjects with mild constipation was significantly higher compared with at days − 6 to 0 ($P < 0.05$).

with mild constipation (less than seven times defecation/week during the observation period) after the ingestion of the test fermented milk. A tendency of increased butyric acid and total organic acid concentration was also observed. Hence, increased organic acids might be utilised for energy by the intestine and promote peristaltic movements, and then increase defecation frequency. These data suggest that the fermented milk containing *L. johnsonii* La1 has beneficial effects to improve the intestinal microflora and defecation frequency.

During the placebo period, a decrease in the number of lecithinase-positive *Clostridium* number and tendency of increased defecation frequency were observed. Although the placebo-fermented milk does not contain *L. johnsonii* La1, it contains lactose and fermented products that have intrinsic effects to improve the intestinal microflora. It is likely that the placebo-fermented milk used in the present study may have impact on the intestinal microflora. However, no changes were observed on *Bifidobacterium*, *Lactobacillus* and faecal pH. These findings indicate that the observed improvements of intestinal microflora and defecation frequency by the test fermented milk are apparently stronger than those by the placebo fermented milk. According to the subjects' questionnaires, the amounts of ingested nutrients, such as dietary fibre, did not change so much during the study period and the nutritional components were the same between the test and placebo fermented milk. The only difference between the test and placebo fermented milk is the presence of *L. johnsonii* La1, suggesting that *L. johnsonii* La1 has an essential role for intestinal microflora improvement.

In order to exert the beneficial effects on health, it is important for probiotics to access the intestine as a viable strain, i.e. survive the human GIT, but no reports have shown the survival ability of *L. johnsonii* La1 in human GIT using a strain-specific DNA primer for *L. johnsonii* La1. In the present study, we examined the survival ability of *L. johnsonii* La1 in the human GIT by identifying *L. johnsonii* La1 in the faeces with a RAPD-PCR method (Welsh & McClelland, 1991; Johansson *et al.* 1995) using a specific primer for *L. johnsonii* La1 (Ventura & Zink, 2002). *L. johnsonii* La1 was

identified in all subjects who ingested the test fermented milk only during the test period. More than the ingested number of *L. johnsonii* La1 (1×10^9 cfu/d) was identified from nine out of twenty-two subjects. *L. johnsonii* La1 was identified within 3 d after stopping the test fermented milk. Since the turnover cycle of IEC is 3–4 d, colonised *L. johnsonii* La1 in the intestine might disappear followed by the IEC turnover. These results suggest that *L. johnsonii* La1 has an ability to reach the intestine as a viable bacterium, and to proliferate and colonise temporarily in the intestine. The fact that *L. johnsonii* La1 disappeared within 4 d after stopping the test fermented milk is also important to show the safety of *L. johnsonii* La1. Thus, the probiotic properties of *L. johnsonii* La1 to reach the intestine as a viable strain and colonise in the intestine seems to work favourably in the improvement of the intestinal microflora.

In conclusion, the strain-specific effect of *L. johnsonii* La1 to improve the intestinal microflora as a viable strain was elucidated in the present study. *L. johnsonii* La1 is of human origin, safe for consumption (Shu *et al.* 1999), adheres to human intestinal cell lines, survives in the human GIT and colonises temporarily in the intestine, produces antimicrobial substances, antagonises the pathogenic bacteria, and has beneficial effects on human health. Therefore, *L. johnsonii* La1 can be a useful tool to promote human health as a probiotic.

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