Improvement of the human intestinal flora by ingestion of the probiotic strain \textit{Lactobacillus johnsonii} La1

Toshihiko Yamano\textsuperscript{1}, Hisakazu Iino\textsuperscript{2}, Mamiko Takada\textsuperscript{2}, Stephanie Blum\textsuperscript{3}, Florence Rochat\textsuperscript{3} and Yoichi Fukushima\textsuperscript{1}\textdagger

\textsuperscript{1}Nestlé Japan Ltd, Nutrition Business Group, NYK Tennoz Building, 2-2-20, Higashi-Shinagawa, Shinagawa-ku, Tokyo, 140-0002, Japan
\textsuperscript{2}Showa Women’s University, 1–7 Taishidou, Setagayaku, Tokyo, 154-8533, Japan
\textsuperscript{3}Nestlé Research Centre, Route du Jorat 57, Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland

(Received 30 May 2004 – Revised 6 April 2005 – Accepted 21 April 2005)

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 \textit{in vitro} as well as \textit{in vivo} methods. However, only a few well-designed human clinical studies have shown these properties. \textit{Lactobacillus johnsonii} La1 has been shown to give many beneficial effects for the host, but it is unclear whether a viable strain of \textit{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 \textit{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 \textit{L. johnsonii} La1 or a fermented milk without \textit{L. johnsonii} La1 (placebo) daily for 21 d. Consumption of the fermented milk: (a) increased total \textit{Bifidobacterium} and \textit{Lactobacillus}, and decreased lactic acid concentrations; (c) decreased the faecal pH; (d) increased the defecation frequency. These changes were stronger than those observed with the placebo. \textit{L. johnsonii} La1 was identified in all subjects only after the consumption of the fermented milk. These results suggest that \textit{L. johnsonii} La1 can contribute to improve intestinal microflora with probiotic properties.

Double-blind placebo-controlled trial: \textit{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 mucus 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, \textit{Bifidobacterium} and \textit{Lactobacillus} contribute to beneficial defensive effects of the host. They exert antimicrobial effects against pathogens by releasing anti-microbial 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. 2000a; Nagao et al. 2000), enhance IgA synthesis (Fukushima et al. 1998; Iinou-Zekri et al. 2003), modulate cytokine production (Marin et al. 1997; Haller et al. 2000b) 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 \textit{Clostridium perfringens} are associated with various diseases and inflammatory responses (Del-neste et al. 1998; Matzinger, 1998; Haller et al. 2000b). 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 \textit{Bifidobacterium} and \textit{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.

\textbf{Abbreviations}: cfu, colony-forming units; GIT, gastrointestinal tract; IEC, intestinal epithelial cells; MRS, de Man, Rogosa and Sharpe.
\textbf{Corresponding author}: Dr Yoichi Fukushima, fax +81 3 5769 6290, email Yoichi.Fukushima@jp.nestle.com
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 of 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-Cau thard 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. 2000a) 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 × 10⁴ cells per cm². Cells were maintained at 37°C in a CO₂–air atmosphere (10:90, v/v). Lactobacilli (1 ml; 4 × 10⁸ 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 CO₂-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 × 10⁸ 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 × 10⁸ 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 × 10⁷ 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 × 10⁸ cfu/ml) with (test) or without (placebo) L. johnsonii La1 (1 × 10⁹ cfu/ml). The composition of both fermented products was as follows: energy, 427 kcal; 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.
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 administered 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, 50μl 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-Gagnost, blood liver, trycase sulphite 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 phosphomycin (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, 1μl bacterial pellet, 18μl sterilised water and 5μl 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 (1μl) were added into the tubes premixed with 1μl 50 mM- DNA primer 5′(GGTGGGGTAGAATTGCAGC)3′ (Microsynth GmbH, Balgach, Switzerland), 1·25μl 10 mM-deoxynucleotide triphosphate (Amersham Biosciences Corp.), 0·75μl 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 μl plasmid 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>
<thead>
<tr>
<th>Media</th>
<th>Bacteria mainly identified</th>
<th>Incubation condition (48 h at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG</td>
<td>Anaerobic bacteria</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>BL</td>
<td>Anaerobic bacteria</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>TS</td>
<td>Aerobic bacteria</td>
<td>Aerobic</td>
</tr>
<tr>
<td>BS</td>
<td>Bifidobacteria</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>ES</td>
<td>Eubacteria</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>NBGT</td>
<td>Bacteroidaceae</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>CW</td>
<td>Clostridia (lecithinase +)</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Crostridia agar</td>
<td>Clostridia (lecithinase −)</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>LBS</td>
<td>Lactobacilli</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>TATAC</td>
<td>Enterococci</td>
<td>Aerobic</td>
</tr>
<tr>
<td>PEEES</td>
<td>Staphylococci</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>Yeast</td>
<td>Aerobic</td>
</tr>
</tbody>
</table>

EG, Eggerth-Gagnost; BL, blood liver; TS, trycase sulphite; BS, bifidobacteria-selective; ES, eubacterium selective; NBGT, neomycin-brilliant green-taurocholate; CW, Clostridium welchii; LBS, lactobacilli-selective; TATAC, triphenyltetrazolium chloride-acridine; PEEES, 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.

**Statistics 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 χ² 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.

**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 × 10⁷ cfu and about 4 × 10⁶ 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 10⁵ (SD 10²) cfu/g (day 0) to 10⁶ (SD 10³) cfu/g (day 11; P<0.001) and 10⁷ (SD 10⁸) 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 10⁵ (SD 10³) cfu/g (day 0) to 10⁶ (SD 10³) cfu/g (day 11; P<0.001) and 10⁷ (SD 10³) 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 10⁵ (SD 10³) cfu/g (day 0) to 10⁴ (SD 10²) cfu/g (day 11; P<0.001) and 10³ (SD 10²) 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 10⁵ (SD 10³) cfu/g (day 0) to 10⁴ (SD 10²) cfu/g (day 14; P<0.001), and the appearance ratio of lecithinase-positive *Clostridium* decreased from 55 % (day 0) to 14 % (day 11; P<0.001) and 23 % (day 22; P<0.005). 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 15h) and simulated gastric juice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bile acid</th>
<th>Gastric juice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Lactobacillus L. Johnsonii La1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>L. amylovorus</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bifidobacterium B. longum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. breve</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+++, 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)

<table>
<thead>
<tr>
<th></th>
<th>Placebo period (n 22)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 11</td>
<td>Day 22</td>
<td>Day 0</td>
<td>Day 11</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>AR</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Total 10·3 0·3 100</td>
<td>10·3</td>
<td>0·3</td>
<td>100</td>
<td>10·3</td>
<td>0·1</td>
</tr>
<tr>
<td>Total anaerobes 10·3 0·3 100</td>
<td>10·3</td>
<td>0·1</td>
<td>100</td>
<td>10·3</td>
<td>0·3</td>
</tr>
<tr>
<td>Total aerobes 8·1 0·1 100</td>
<td>8·2</td>
<td>0·4</td>
<td>100</td>
<td>8·2</td>
<td>0·3</td>
</tr>
<tr>
<td><em>Bacteroidaceae</em> 9·8 1·1 100</td>
<td>9·6</td>
<td>0·4</td>
<td>100</td>
<td>9·6</td>
<td>0·2</td>
</tr>
<tr>
<td><em>Bifidobacteria</em> 9·3 0·4 100</td>
<td>9·4</td>
<td>0·3</td>
<td>100</td>
<td>9·2</td>
<td>0·1</td>
</tr>
<tr>
<td><em>Eubacteria</em> 9·6 1·0 100</td>
<td>9·6</td>
<td>0·5</td>
<td>100</td>
<td>9·8</td>
<td>0·9</td>
</tr>
<tr>
<td><em>Clostridia</em> (lecithinase-positive) 5·3 0·7 100</td>
<td>5·3</td>
<td>0·3</td>
<td>100</td>
<td>5·6</td>
<td>0·9</td>
</tr>
<tr>
<td><em>Clostridia</em> (lecithinase-negative) 8·1 0·2 100</td>
<td>8·1</td>
<td>0·3</td>
<td>100</td>
<td>7·3</td>
<td>0·5</td>
</tr>
<tr>
<td><em>Lactobacilli</em> 5·7 1·2 91</td>
<td>5·3</td>
<td>1·4</td>
<td>86</td>
<td>5·3</td>
<td>1·1</td>
</tr>
<tr>
<td><em>L. johnsonii</em> La1 ND 0 ND 0</td>
<td>ND 0</td>
<td>ND 0</td>
<td>ND 0</td>
<td>ND 0</td>
<td>ND 0</td>
</tr>
<tr>
<td><em>Streptococci</em> 6·5 1·1 100</td>
<td>7·1</td>
<td>0·3</td>
<td>100</td>
<td>6·5</td>
<td>0·6</td>
</tr>
<tr>
<td><em>Staphylococci</em> 6·6 1·2 100</td>
<td>5·2</td>
<td>0·4</td>
<td>91</td>
<td>5·3</td>
<td>0·9</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em> 7·4 0·5 100</td>
<td>8·0</td>
<td>0·3</td>
<td>100</td>
<td>7·6</td>
<td>1·1</td>
</tr>
<tr>
<td><em>Bacilli</em> 7·8 1·1 100</td>
<td>7·9</td>
<td>0·2</td>
<td>100</td>
<td>8·1</td>
<td>0·2</td>
</tr>
<tr>
<td>Yeasts 3·5 1·1 64</td>
<td>3·7</td>
<td>1·3</td>
<td>59</td>
<td>6·7</td>
<td>2·2</td>
</tr>
</tbody>
</table>

BC, bacterial count (log₁₀ colony-forming units/g); AR, appearance rate (%); ND, not detected (≤10³ 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.
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 significantly higher during the test period 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 10⁶·3 (SD 10⁰·5) cfu/g (day 11) and 10⁶·9 (SD 10⁰·9) 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 x 10⁹ cfu/d. More than the ingested number of L. johnsonii La1 (1 x 10⁹ 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 acid increased from 0·031 (SD 0·063) mg/g (day 0) to 0·208 (SD 0·300) mg/g (day 22; P<0·005) 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<0·005) (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<0·005). 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

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.
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, 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., Desulfovibrio 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...
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 × 10⁸ 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.

**References**


![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).](image-url)


