J. Gen. Appl. Microbiol., **54**, 267–276 (2008)

Full Paper

Evaluation of the probiotic potential of *Lactobacillus paracasei* KW3110 based on in vitro tests and oral administration tests in healthy adults

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(Received February 28, 2008; Accepted July 1, 2008)

***Lactobacillus paracasei* KW3110 is a strain which has been reported to possess a strong anti-allergic activity. Since many lactic acid bacteria have shown to be useful as the probiotics, the possibility of this strain for use as a probiotic was studied. An in vitro test showed that this strain is strong enough to survive in the gastric juice. In addition, this strain showed exceptionally strong adherence to human intestinal epithelial cells, Caco-2 and HT29 compared to normal *L. paracasei* strains. Human oral administration testing of *L. paracasei* KW3110 showed that this strain survived in the human gut and increased the number of bifidobacteria and lactobacilli in the fecal samples of the subjects. In addition, KW 3110 was detected in 50% of the subjects up to 1 week after the end of the administration test. These results showed that *L. paracasei* KW3110 has a strong ability to survive in and colonize the human gut and improve the human intestinal microflora.**

Key Words——colonization; intestinal microflora; *Lactobacillus paracasei*; oral administration; probiotics

Introduction

From the time of birth, humans have more than 100 trillion microorganisms residing in their intestines. This gut community is composed of more than 100 different species and has been designated intestinal flora for a decade. Recently, it has been proven that the intestinal flora is profoundly involved in host health conditions. Thus, attention has focused on the consumption of probiotics and prebiotics which can improve the conditions of the intestinal flora.

"Probiotics" were initially defined in 1989 as live microbes that can provide host animals with beneficial effects on their health conditions by improving the balance of intestinal flora (Fuller, 1989). Then, probiotics were redefined as food products that contain live microbes that can beneficially affect the host health conditions (Salminen et al., 1998). On the other hand, "prebiotics" are defined as indigestible food contents that can provide the host animals with beneficial impact and improve the host health conditions by selectively influencing the proliferation and activity of certain bacteria in the large intestine (Gibson and Roberfroid, 1995).

There are many reports that demonstrate the improving effects of probiotics on intestinal flora. For example, it has been reported that an oral intake of probiotics can improve the balance of intestinal flora through an increase in the number of bifidobacteria and lactobacilli, beneficial bacteria to the host, and a decrease in the number of non-beneficial bacteria, such as bacteroides, *Clostridium perfringens*, and en-

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terobacteriaceae in the intestinal flora (Matsumoto et al., 2006; Ogata et al., 1997; Yaeshima et al., 1997). There are also some reports that oral intake of probiotics can improve bowel movement disturbance such as diarrhea (Gismondo et al., 1999) and coprostasis (Fukushima et al., 2004; Yaeshima et al., 1997). In addition, probiotics have been reported to have a prophylactic effect against infectious diseases (Namba et al., 2003) and an ameliorating effect on inflammatory bowel diseases (Fukushima et al., 2004; Gionchetti et al., 2000).

Whereas the main mechanism of action of the probiotics is to improve the conditions of intestinal flora as described above, when bacteria by themselves or substances produced by bacteria directly affect the host, this is defined as "biogenics." A representative of the biogenics is a dipeptide derived from *Lactobacillus helveticus* that has an improving effect on blood pressure (Aihara et al., 2005). In recent reports, the biogenics have been shown to alleviate various diseases in non-digestive organs; the effects include immune modulatory effects such as anti-allergic activity and immune stimulation (Björkstén et al., 1999; Kalliomaki et al., 2001), improvement effects on hyperpiesia and hyperlipidemia which are classified into lifestyle-related diseases (Nakajima et al., 1995; Schaafsma et al., 1998), and anti-tumor effects (Roos and Katan, 2000).

Lactobacillus paracasei KW3110 is a strain that was found to possess such biogenic activities in our laboratory (Fujiwara et al., 2004). A screening of lactic acid bacteria for anti-allergic activity was performed based on the activity suppressing a Th2-polarized response of Th1/Th2 balance in an in vitro culture of mouse splenocytes. We found that the anti-allergic activity of these 100 lactic acid bacteria was dependent on the strains but not the species of lactic acid bacteria and that *Lactobacillus paracasei* KW3110 is the most effective among more than 100 strains. Oral administration of *L. paracasei* KW3110 to ovalbumin-sensitized BALB/c mice resulted in induction of IL-12 secretion as a Th1 parameter and repression of IL-4 secretion as a Th2 parameter from splenocytes, and suppression of serum IgE elevation, compared with control animals that did not receive the cells of *L. paracasei* KW3110 (Fujiwara et al., 2004). In addition, administration of *L. paracasei* KW3110 to patients with allergic symptoms to cedar pollen appeared to improve their Th1/Th2 balance and suppress ECP elevation (Fujiwara et al., 2005). Furthermore, it has been suggested that oral intake of *L. paracasei* KW3110 suppressed the symptoms of dermatitis and production of IgE in atopic dermatitis model animals induced by applying picryl chloride onto the skin of NC/Ng mice (Wakabayashi et al., 2007). Since heat-killed cells of *L. paracasei* KW3110 were used in these studies, it was suggested that cell components of this strain acted directly on the hosts as biogenics.

On the other hand, several reports suggested the involvement of intestinal flora in allergic responses. Bjorksten et al. reported that the conditions of intestinal flora of children with allergies were different from those of healthy ones (Björkstén et al., 1999). Wickens et al. suggested that the use of antibiotics in infants was a risk factor in the later development of allergic diseases and speculated that the diseases were caused by the changes in the conditions of intestinal flora due to the use of antibiotics (Wickens et al., 1999). Taken together, it is suggested that the intestinal flora are related to allergies and thereby probiotics which can improve the conditions of the intestinal flora have the ability to alleviate allergies. In addition, it has been reported that probiotics could suppress the apoptosis of intestinal epithelial cells caused by inflammatory bowel ranging behavior or diarrhea and that killed bacterial cells did not have such suppressive effects (Yan and Polk, 2002). Thus, if *L. paracasei* KW3110 strain possesses activities as a probiotic, it is expected that the strain can exert anti-allergic activities not only as a biogenic but also through improvement of the conditions of intestinal flora.

In the present study, we tried to address the mechanisms of the anti-allergic effect of *L. paracasei* KW3110 by examining whether the strain can improve the intestinal flora as a probiotic. We firstly examined acid and bile acid resistance, and adherence property to gut epithelial cells of *L. paracasei* KW3110. Then, we carried out an oral administration study of *L. paracasei* KW3110 in healthy Japanese adult volunteers; by analyzing intestinal flora with time, we have studied the in vivo disposition of *L. paracasei* KW3110 in the gastrointestinal tract and the effect of the KW3110-intake on the conditions of intestinal flora.

Materials and Methods

Bacterial strains. Twenty strains of *Lactobacillus paracasei* and 7 strains of *L. acidophilus*, 4 strains of *L. jonsonii*, 3 strains of *L. gaserri*, and 1 strain each of

L. delbrueckii subsp. *bulgaricus*, *L. casei*, *L. brevis*, *L. helveticus*, *L. hilgardii*, *L. plantarum*, *Leuconostoc mesenteroides*, and *Streptococcus thermophilus* were collected from our own laboratory collection. The lactic acid bacteria were grown in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Adelaide, Austraria) at 30 or 37°C.

Survival of lactic acid bacteria at low pH. Cultures were grown by measuring optical density at 600 nm (OD_{600}) to logarithmic phase （OD_{600}=0.5−1.0）and were harvested by centrifugation. The harvested cells were adjusted to OD_{600}=0.5 with phosphate-saline-buffer (PBS, pH 6.5). Cell suspension was diluted 10 times with acidic MRS medium which was adjusted to pH 3.0 with HCl. The mixtures were incubated at 37°C for 3 h. pH tolerance of the cells was determined by counting the viable cells enumerated on MRS medium agar plates after 48 h incubation.

Tolerance against bile. Pre cultures were grown overnight in MRS media, then inoculated to 10^6 cells/ml to the new MRS media containing 2.0% bile salts (Oxoid No. L55, Oxoid). The inoculated cultures were grown for 24 h and cell growth was monitored with Bioplotter（Oriental Instrument, Tokyo）by measuring optical density at 630 nm (OD_{630}) every 1 h. Tolerance to the bile acids was determined according to the final OD_{630}.

Adherence to intestinal cells. Enterocyte-like Caco-2 cell line was cultured as previously described (Coconnier et al., 2000). Briefly, cells were seeded on the slide glass in the 6-well plates at the concentration of 1.4×10^4 cells/cm^2, and grown in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with 20% (Caco-2) or 10% (HT29) inactivated fetal calf serum, and 1% nonessential amino acids. Cells were maintained at 37°C in a 10% CO_2−90% air atmosphere.

The adherence assay of bacteria to Caco-2 cells was examined as followings. Caco-2 cells were washed three times with the DMEM medium. Bacteria cells were cultivated for 48 h and washed with phosphate-buffered saline (PBS, without Ca) and suspended into the same buffer at the concentration of OD_{600}=0.7. This suspension (2 ml) was added to each well of the tissue culture plate, and the plates were incubated at 37°C in 10% CO_2−90% air. After 1 h of incubation, the layers were washed three times with DMEM media, fixed with methanol at 4°C for 30 min, and stained with Gram stain. The number of adherent bacteria was counted in four independent areas.

Human oral administration test of L. paracasei KW3110. The following human oral administration test *L. paracasei* KW3110 was conducted in accordance with the Declaration of Helsinki and was approved by Ethics Committee of Kirin Brewery Co., Ltd. and the Ethics Committee of Showa Women's University.

The yogurt-style test food (KW-yogurt) was manufactured so as to contain 90.1 kcal energy, 3.5 g protein, 3.1 g lipid, 12.2 g carbohydrate, 51.0 mg sodium, and $10^{10.6}$ cells of *L. paracasei* KW3110 strain per 100 g KW-yogurt.

Nine healthy subjects (2 male and 7 female, mean age 27.1±6.8 years, mean BMI 21.6±1.9) participated in this study. The inclusion criterion was general good health. Exclusion criteria were treatment with a prescribed medicine, intolerance of milk products, heavy constipation, and diarrhea.

The experimental design is illustrated in Fig. 1. The total test period was 28 days and the subjects were prohibited to take other dairy foods, oligosaccharides, and dietary fibers during the test period. After a 1-week observation period (from Day −7 to −1), subjects started to consume 100 g KW-yogurt every day for a week (100 g-intake period, from Day 0 to 6). Subjects were then asked to stop taking KW-yogurt for a week (Rest period, from Day 7 to Day 13) and restarted the 10 g yogurt intake every day for a further week (10 g-intake period, from Day 14 to 20). A daily questionnaire on the following matters was used to monitor the condi-

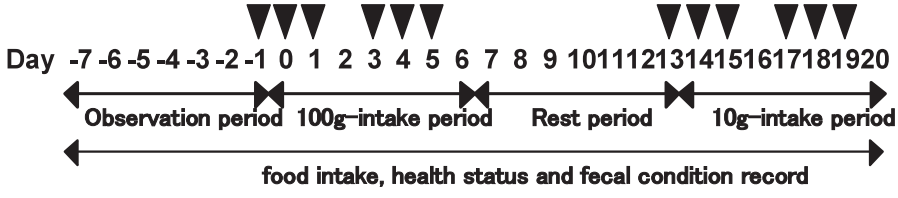

Fig. 1. Experimental procedure of oral administration test of *L. paracasei* KW3110. Each period is indicated by an arrow. Filled triangles indicate the day of sampling.

tion of the subjects: their daily diet, the frequency and the amount of defecation, the need for clinical medicine, and their general health conditions. All subjects had a clinical interview before and after the test. No subject failed to finish the test schedule and no harmful effects were observed during the test period.

Microflora analysis. All subjects underwent fecal sampling once a day, if they were able to produce a sample. Fecal samples were anaerobically stored using Anaero Packs (Mitsubishi Gas Chemical Co., Tokyo) at 4°C, and subjected to microflora analysis within 24 h according to the previously described method (Nakamura et al., 2001) with the following modification.

Fecal samples were thoroughly mixed and suspended in saline containing 0.09% agar. Samples were stepwise diluted and plated onto the plates. EG agar plates and BL agar plates were used for enumerating total anaerobic bacteria. TS agar plates, TOS-propionate agar plates, and LBS agar plates were used for enumerating total aerobic bacteria, bifidobacteria, and lactobacilli. The enumerated colonies after 48 h cultivation were counted and were represented in colony forming units (cfu). The number of total bacteria was indicated as the sum of total anaerobic bacteria and total aerobic bacteria. The presence of a *L. paracasei* colony on an LBS plate was firstly determined by the colony morphology and was confirmed by sequencing the 16S rRNA gene.

DNA extraction and quantitative PCR analysis. Fecal samples were suspended in 2 ml extraction buffer (100 mM Tris-HCl, 50 mM EDTA, pH 9.0) and washed three times with this buffer. DNA extraction was performed using a Fast DNA SPIN Kit for soil (Qbiogen, California, USA) according to the manufacturer's instructions. The purified DNA samples were subjected to quantitative PCR analysis (Qt-PCR) to determine the number of bacteria using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplifications of the 16S rRNA gene were performed with the SYBR *Premix Ex Taq*™ (TaKaRa, Tokyo) containing 0.2 μM of each primer and 0.1 μg/μl BSA.

For bifidobacteria quantification, g-Bifid-F: 5′-CTCCTGGAAACGGGTGG-3′ and g-Bifid-R: 5′-GGTGTTCTTCCCGAATCTAA-3′ (Matsuki et al., 2002) were used as primers and *Bifidobacterium longum* JCM1217 was used as the standard strain. For lactobacilli quantification, LactoF: 5′-TGGAAACAGRTGCTAATACCG-3′ and LactoR: 5′-CCATTGTGGAAGATTCCC-3′ (Jackson et al., 2002) were used as primers and *L. gasseri* JCM1131 was used as the standard strain. For *L. paracasei* quantification, PARA: 5′-CACCGAGATTCAACATGG-3′ and Y2: 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ (Ward and Timmins, 1999) were used as primers and *L. paracasei* KW3110 was used as the standard strain. The standard strains were cultivated to the stationary phase and harvested. Cells were washed twice with PBS. The cell-mixtures were stained with DAPI to determine the cell numbers by microscopic observation. Chromosomal DNA extracted from the dilution series of the standard strains were used to quantify the number of cells in fecal samples.

Statistical analysis. The number of total bacteria, total anaeroba, total aeroba, bifidobacteria, lactobacilli, *L. paracasei*, the counts of defecation, and the amount of defecation level were statistically analyzed. All the data during the 100 g-intake period and 10 g-intake period were respectively compared to those of observation period (Day −1) and that of rest period (Day 13) using the Wilcoxon Matched-Pairs Signed-Ranks Test. $p<0.05$ was considered to be significant in all tests.

Results

In vitro evaluation of resistance property of KW3110 to acid and bile acid

We performed in vitro experiments to evaluate probiotic activities of *L. paracasei* KW3110. Previous reports have described in vitro studies of probiotics (Jacobsen et al., 1999; Liong and Shah, 2005; Prasad et al., 1998). Figure 2 shows the results of acid resistance test of the *L. paracasei* KW3110 and 14 type strains of lactic acid bacteria. The most acid-resistant strain (*L. paracasei*) showed a viability of more than 85% after 3 h exposure. The *L. paracasei* KW3110 also showed high viability (approximately 70%). In contrast, the viability at 3 h was less than 0.01% for bacterial species such as *L. delbrueckii* subs. *bulgaricus* and *Streptococcus thermophilus* which are regularly used in making dairy products.

Then, we evaluated the resistance to 2.0% bile acid of the *L. paracasei* KW3110, a further 17 strains of *L. paracasei*, 6 strains of *L. acidophilus*, 4 strains of *L. jonsonii*, and 3 strains of *L. gasseri* (Fig. 3). Among 17 strains of *L. paracasei* tested in the present study, 13 strains were able to grow during 24 h incubation ($OD_{630}>0.1$) in the presence of bile acid, and 6 strains

showed bile acid resistance equal or superior to lactic acid bacteria such as *L. gasseri*, *L. jonsonii*, and *L. acidophilus* which can be found in the intestine in high numbers. Among the bile acid-resistant lactic acid bacteria, the *L. paracasei* KW3110 was one of the most resistant strains to bile acid. This suggested that this strain was highly capable of growing in the enteric environment.

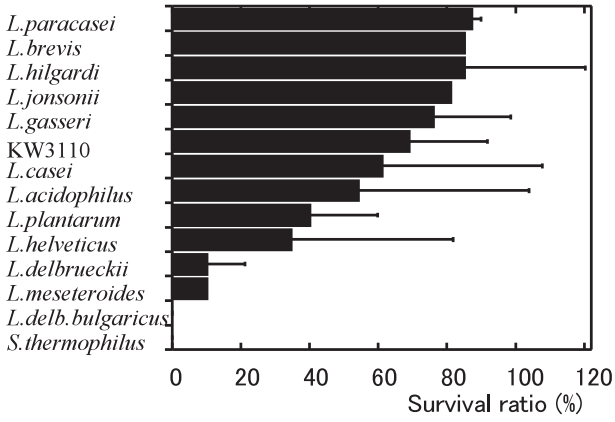

Fig. 2. Acid tolerance of type strain of lactic acid bacteria and *L. paracasei* KW3110.

Thirteen type-strains of lactic acid bacteria and *L. paracasei* KW3110 were subjected to an acid tolerance test. Cells were suspended in MRS medium adjusted to pH 3.0 and were incubated at 37°C for 3 h. Tolerance to pH was determined by counting the viable cells enumerated on MRS agar plates after 48 h incubation.

To further study the survival properties of *L. paracasei* KW3110 under conditions similar to the gastrointestinal tract, we examined whether the strain was viable in sequential exposure to artificial stomach fluid and artificial intestinal fluid (data not shown). MRS medium (pH 3.0 and 2.5) containing 0.04% pepsin was used as artificial stomach fluid. *L. paracasei* KW3110 was highly viable in the artificial stomach fluid of pH 3.0. In contrast, the number of the bacteria decreased to a level of 10^4 cells in the artificial stomach fluid at pH 2.5. Thus, *L. paracasei* KW3110 that was treated with the artificial stomach fluid at pH 3.0 for 2 h was inoculated to artificial intestinal fluid containing 0.01% trypsin, 0.01% pancreatin, and then cultivated in MRS medium containing 0.1–2.0% bile acid to monitor the growth. As a result, the *L. paracasei* KW3110 strain was viable in the fluid at any tested concentration of bile acid. This further suggests that *L. paracasei* KW3110 is viable in the enteric environment.

In vitro adherence activity of L. paracasei KW3110 to the intestinal epithelial cells

Table 1 shows the results of adhesion studies of the *L. paracasei* KW3110, *L. acidophilus*, *L. gasseri*, *L. jonsonii*, and *L. paracasei* onto human large intestine-derived cell lines, Caco-2 and HT-29. It has been suggested that the adhesive activity to the epithelial cells may help bacterial colonization in the gut. Our results showed that the average of adhesive activity of enteric

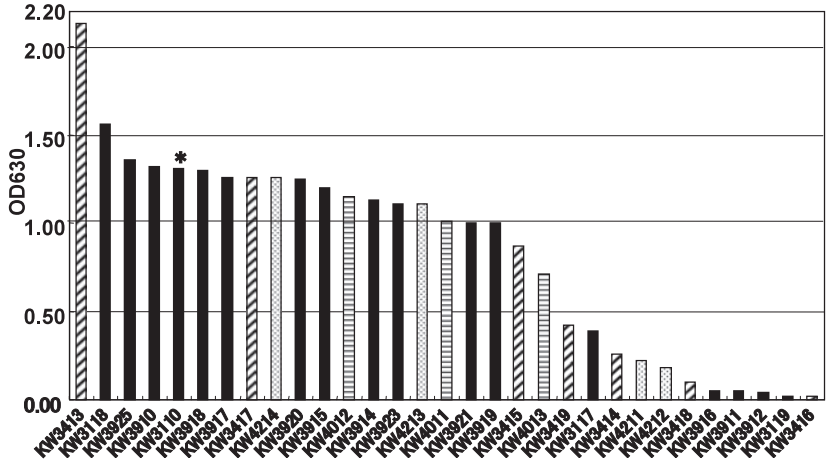

Fig. 3. Bile tolerance of lactic acid bacteria.

Sixteen *L. paracasei* strains (solid columns), 7 *L. acidophilus* strains (hatched columns), 4 *L. jonsonii* strains (dotted columns), and 3 *L. gasseri* strains (horizontal lined columns) were subjected to the bile tolerance test. Cells were cultured in MRS medium containing 2.0% oxygall (Oxoid). The tolerance was determined by measuring OD_{630} after 24 h cultivation. **L. paracasei* KW3110.

lactic acid bacteria such as *L. acidophilus*, *L. gasseri*, and *L. jonsonii* to Caco-2 was higher than that of *L. paracasei.* However, considerable variation was observed among the strains in the same species. Some strains of *L. acidophilus* and *L. jonsonii* showed very high adhesive activities to Caco-2 cells (>1,000 cells/50 Caco-2 cells), but some showed low adhesive activities (<100 cells/50 Caco-2 cells). Interestingly, *L. paracasei* KW3110 showed exceptionally higher adhesive property (193 cells/50 Caco-2 cells) than the other strain of *L. paracasei* tested (15±10 cells/50 Caco-2 cells). We also performed an additional experiment using HT29 to compare the adhesive activity of the KW3110 strain and 8 other strains of *L. paracasei*. Again, *L. paracasei* KW3110 showed higher adhesive activity (208 cells/50 HT29 cells) than the other strains of *L. paracasei* tested (57±35 cells/50 HT29 cells). These results suggested that *L. paracasei* KW3110 would be able to strongly adhere to the epithelial cells and colonize for a longer period than the other *L. paracasei* strains.

Oral administration of L. paracasei KW3110 to human subjects

The results of the in vitro studies suggested that *L. paracasei* KW3110 can pass through the upper gastrointestinal tract and reach the lower gastrointestinal tract where it can colonize. It was also suggested that the strain had higher adhesive property to epithelial cells in the lower gastrointestinal tract than the other strains of *L. paracasei* tested. To confirm these findings, we performed an oral administration study of *L. paracasei* KW3110 on human subjects according to the scheme shown in Fig. 1. Table 2 shows the average number of *L. paracasei* in the fecal samples of the subjects, which was determined by the cultivation method. Figure 4 shows the number of bacteria in each subject, which was determined by the Qt-PCR method. The cultivation method was applied to all the 9 subjects, and the Qt-PCR method was applied to only 6 subjects.

Initial number of *L. paracasei* in the subjects was determined on the day previous to *L. paracasei* KW3110 administration (Day −1); only one subject out of 9 was detected with *L. paracasei* while the level was very low ($10^{2.6}$ cfu/g feces). Six subjects who had not been detected with *L. paracasei* were further examined by the Qt-PCR method; nevertheless, the number of the bacteria was below the detection limit (<10^3 cells/g feces).

Upon starting intake of 100 g of KW-yogurt, *L. paracasei* became detectable in the feces as early as on the first day of administration in 7 subjects out of 9. Throughout the 100 g-intake period, the level of *L. paracasei* stayed very high ($10^{4.6-6.7}$ cfu/g and $10^{8.0-9.1}$ cells/g based on the cultivation method and the Qt-PCR method, respectively).

After 6 days of the 100 g-intake period, the subjects stopped taking yogurt (rest period) to wash out the *L. paracasei* KW3110. At the end of rest period (Day 13), the number of residual *L. paracasei* was determined by the cultivation and Qt-PCR methods. The cultivation method showed that the *L. paracasei* was washed out to a level less than 10^3 cfu/g in 8 subjects out of 9, while there were still $10^{3.1}$ cfu/g detected in one subject (data not shown). This result was also supported by the Qt-PCR method. *L. paracasei* was washed out to a level below the detection limit in 3 subjects out of 6, while there was still $10^{4.4-5.3}$ cells/g detected in the other 3 subjects (Fig. 4). These results suggest that *L. paracasei* KW3110 can colonize stably in the human intestine for a week.

Table 1. Adhesion properties of lactobacilli to Caco-2 cells.

Strains	No. of tested strains	Average[a] (St.dev.)	Max[a]	Min[a]
L. acidophilus	8	564 (728)	1,868	36
L. gasseri	9	590 (271)	1,138	229
L. jonsonii	6	274 (163)	430	128
L. paracasei	19	15 (10)	39	3
L. paracasei[b]	8	57 (35)	125	19
KW3110	1	193		
KW3110[b]	1	208		

[a]Number of adhering lactobacilli to 50 epithelial cells.
[b]Adhesion properties to HT29 cells.

Table 2. Enumeration of bacteria during the oral-administration test periods.

Day	−1	0	2	3	4	5
Total Bacteria	10.49±0.24	10.52±0.24	10.67±0.13	10.67±0.22	10.49±0.26	10.47±0.39
Total Anaerobes	10.48±0.24	10.52±0.24	10.67±0.13	10.67±0.22	10.48±0.26	10.46±0.39
Total Aerobes	7.71±0.83	7.98±0.68	4.81±5.39	7.65±0.93	5.95±3.15	7.62±0.58
Bifidobacteria	9.81±0.31	9.82±0.36	10.28±0.09	10.06±0.26*	9.88±0.36	9.90±0.52
Lactobacilli	4.83±1.44	6.44±1.32*	6.80±0.28	6.74±1.11*	6.57±0.89*	6.62±0.92*
L. paracasei	0.29±0.87	4.63±3.50*	3.96±3.74*	6.74±1.11*	6.00±2.45*	6.46±1.37*

Day	13	14	15	17	18	19
Total Bacteria	10.53±0.25	10.63±0.29	10.68±0.21	10.62±0.32	10.44±0.32	10.50±0.25
Total Anaerobes	10.52±0.25	10.58±0.31	10.67±0.22	10.60±0.35	10.47±0.29	10.49±0.25
Total Aerobes	8.26±0.73	8.07±0.61	7.88±0.88	8.07±0.71	7.88±1.00	7.91±1.03
Bifidobacteria	9.80±0.37	9.86±0.36	10.11±0.23	9.93±0.51	9.90±0.24	9.93±0.11
Lactobacilli	4.09±0.99	5.33±1.08*	5.63±1.13	5.84±1.85**	6.10±1.16**	5.42±0.68
L. paracasei	0.34±0.38	1.07±1.19	3.36±3.84	3.11±2.68	3.78±4.20**	3.55±4.14

*Significantly different compared with Day −1 ($p<0.05$).
**Significantly different compared with Day 13 ($p<0.05$).

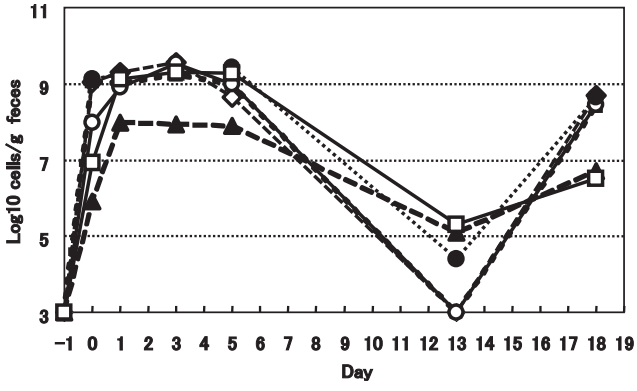

Fig. 4. Enumeration of *L. paracasei* KW3110 in fecal samples during the oral-administration test period.

Fecal samples from 6 subjects were subjected to Qt-PCR to determine the number of cells of *L. paracasei* KW3110. Subject 1: (open diamonds), subject 2: (filled squares), subject 3: (filled triangles), subject 4: (open circles), subject 5: (filled circles), subject 6: (open squares). The detection limit of the Qt-PCR method was 3.0 $\log_{10}$ cells/g feces.

Upon restarting the administration of 10 g of KW-yogurt, *L. paracasei* KW3110 was able to be detected in 8 out of 9 subjects again, though the level varied quite widely among the individual ($10^{4.0-7.7}$ cfu/g). By the Qt-PCR methods, $10^{6.5-8.7}$ cells/g of bacteria was detected in all 6 subjects, during 10 g-intake period. Interestingly, the number of *L. paracasei* KW3110 of subject 3 was 10 times less than those of the other subjects. It is likely that the number of the *L. paracasei* KW3110 in the gut was dependent on host-related factors as well as the amount of intake.

The effect of L. paracasei KW 3110 on the profiles gut flora

Table 2 shows the profiles of the average numbers of total bacteria, total anaerobes, total aerobes, bifidobacteria, and lactobacilli, which were determined by the cultivation method. Figure 5 also shows the time courses of the average numbers of bifidobacteria and lactobacilli, which were determined by the cultivation method and the Qt-PCR method using 16S rRNA-targeted primers. There were no substantial changes in the numbers of total bacteria or total anaerobes throughout the experimental period. The cultivation method showed that the average number of bifidobacteria was $10^{9.8\pm0.3}$ cfu/g before administration (Day −1 and Day 13). It was elevated to $10^{9.9\pm0.3}$ cfu/g during the 100 g-intake period (Day 0 to Day 5). Significant difference was observed between the number of bifidobacteria on Day 3 or Day 5 of the 100 g-intake period and that on Day −1. The number of bifidobacteria also increased to $10^{9.9\pm0.3}$ cfu/g cells during the 10 g-intake period (Day 14 to Day 19); however, no significant difference was observed during this period. The results obtained with the Qt-PCR method were almost the same as those obtained with the cultivation method (Fig. 5A).

The cultivation method also showed that the average number of lactobacilli was greatly increased by the intake of KW-yogurt. It was $10^{4.8\pm1.4}$ cfu/g in the observation period and was elevated to around $10^{6.6\pm1.0}$ cfu/g during the 100 g-intake period. The statistical analysis suggested that the number of lactobacilli on Day 0, Day 3, Day 4, and Day 5 of the 100 g-intake period were significantly different from that on

Day −1. Then, the average number plunged to $10^{4.1\pm1.0}$ cfu/g in the rest period followed by re-elevation to around $10^{5.4\pm1.5}$ cfu/g during the subsequent 10 g-intake period. Again, the number of lactobacilli on Day 14, Day 17, and Day 18 was significantly increased compared to Day 13.

These results were also supported by the Qt-PCR method. Significant increase of lactobacilli was observed on Day 0, Day 3, and Day 5 of the 100 g-intake period and on the Day 18 of 10 g-intake period (Fig. 5B). Surprisingly, the number of lactobacilli measured by Qt-PCR was much higher than that measured by the cultivation method throughout the experimental period. The greatest differences between the results of the two methods were observed on Day −1 and Day 13 (>100-fold) and the difference during the KW-intake period was smaller (almost 10-fold).

These data suggested that most lactobacilli detected by Qt-PCR on Day −1 and Day 13 were not able to form a colony on an LBS plate. It is likely that most of the lactobacilli got damaged or died in the gut and were unable to form a colony on an acidic LBS plate (pH 5.5). Some reports have shown that the membrane-ATPase activity is important for bacteria to survive under acidic conditions, and is easily damaged by some chemical treatments (Bender and Marquis, 1987; Sturr and Marquis, 1992). Our data strongly suggested that the number of the undamaged lactobacilli in the gut was significantly increased by the intake of 10 g of KW-yogurt.

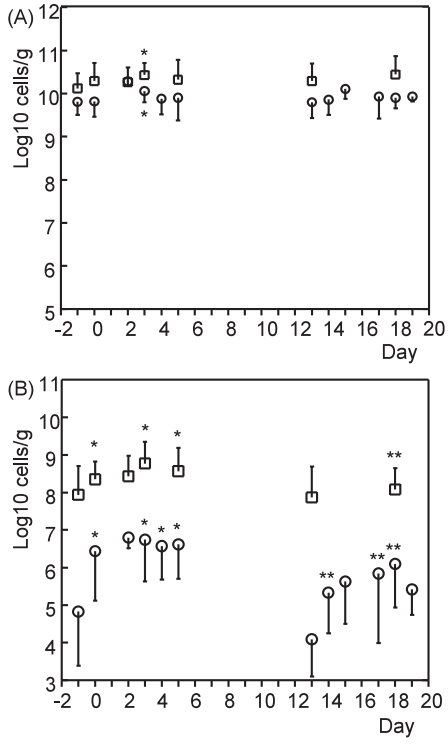

Fig. 5. Enumeration of bifidobacteria and lactobacilli in fecal samples during the oral-administration test period.

(A) Enumeration of bifidobacteria. (B) Enumeration of lactobacilli. Open circles indicate the data obtained by the cultivation method from 9 subjects (log_{10} cfu/g feces). Open rectangles indicate the data obtained by the Qt-PCR method from 7 subjects (log_{10} cells/g feces). The detection limits of the Qt-PCR method and cultivation method were 3.0 log_{10} cells/g feces. Data are shown as the mean±S.D. *$p<0.05$ compared with Day −1. **$p<0.05$ compared with Day 13.

Discussion

As shown above, the present study revealed that *L. paracasei* KW3110 has potential as a probiotic to improve the microflora. The results of in vitro studies indicated that *L. paracasei* KW3110 was resistant to acid and that it was able to grow in the presence of bile acid. Our in vivo study using human subjects strongly suggested that *L. paracasei* KW3110 was resistant to gastric juice in the upper gut-intestinal tract and reached the lower gut-intestinal tract alive. The number of undamaged lactobacilli and bifidobacteria measured by the cultivation method was significantly increased after the KW3110-intake. In addition, according to the results of the questionnaire for the participants of this study, there was a tendency, not significant for the frequency of daily defecation and the fecal amount to increase (data not shown). Taken together, consumption of *L. paracasei* KW3110 is likely to improve bowel movements through stimulating the growth and activity of bifidobacteria and lactobacilli as was reported in the studies with other strains of lactic acid bacteria (Tuohy et al., 2007; Yamano et al., 2006).

It is very interesting that *L. paracasei* KW3110 showed a high adhesive activity to the human epithelial cell line Caco-2, and HT29 as a strain of *L. paracasei*. There are many in vitro studies of bacterial adhesion to the intestinal tract. In these reports, several cell surface proteins such as MapA protein of *L. reuteri* (Miyoshi et al., 2006), CbsA protein of *L. crispatus* (Sillanpaa et al., 2000) and EF-Tu of *L. jonsonii* (Granato et al., 2004) were found as important factors that were involved in adhesion to the intestinal tract. The genomic sequences of *L. casei* ATCC334 strain, which have been found recently, contain the sequence of the EF-Tu factor. However, another report has demonstrated

that cell surface-associated lipoteichoic acid (LTA) is the determinant of the adhesive property of *L. jonsonii* to Caco-2 cells (Granato et al., 1999). Further studies using microarray and/or deletion mutant of those genes would elucidate whether the adhesive property of *L. paracasei* KW3110 to the intestinal tract is mediated by protein factors such as EF-Tu or by LTA.

In the study using human subjects, we showed that *L. paracasei* KW3110 stayed in the intestinal tract even 1 week after the termination of intake (Fig. 4). Furthermore, in our preliminary study of oral administration of *L. paracasei* KW3110 to rats, we found that this strain stayed in the intestinal tract for a longer period than did other strains of *L. paracasei* (data not shown) after the termination of the administration. Schultz has reported that, following oral administration of *L. rhamnosus* GG strain to pregnant women, the strain was eliminated from the maternal feces within 1 week whereas a stable inhabitation was observed in the babies over several months (Schultz et al., 2004). Taken together, it is likely that the *L. paracasei* KW3110 strain can stay in the intestine for a similar or longer period than *L. rhamnosus* GG. Though more studies are necessary to address the anti-allergic mechanism, it is likely that this good-colonizing property of *L. paracasei* KW3110 in the human gut may positively affect the anti-allergic effect of this strain in vivo. It has been reported that the administration of the *L. rhamnosus* GG strain to pregnant women resulted in reduction of the incidence of allergy development in the newborn babies (Kalliomaki et al., 2001, 2007). In the future, administration tests of *L. paracasei* KW3110 to pregnant woman might be interesting to determine whether this strain is useful to alleviate the development of allergic diseases such as dermatoid in newborn babies.

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