Chapter 3

Survivability of *Lactobacillus paracasei paracasei* 06TCa19 strain in the intestine of healthy women and its effects on the bowel habits and intestine environment

3.1 Introduction

The effects of lactic acid bacteria (LAB) and bifidobacteria on human health have been demonstrated in many studies, *in vitro* and *in vivo* (Xiao *et al*. 2003; Ishida *et al*. 2005; Lin *et al*. 2009; Hong *et al*. 2010; Takeda *et al*. 2011a). Some LAB and bifidobacteria are described as probiotics, living microorganisms that confer a health benefit to the host when they were administered in adequate amounts (World Health Organization 2001). Many commercial yogurts contain probiotics (Stanton *et al*. 2001) and the effects of probiotics on the human intestine environment have been studied. *Lactobacillus* (*L*.) *casei* strain Shirota is a strain that is popular as a probiotic because it has been proven not only to reach and survive in human intestine, but also to affect human immune function (Takeda & Okumura 2007; Ivory *et al*. 2008). One function of probiotics is to regulate the host's intestinal environment (Parves *et al*. 2006). Previous reports have proven these effects on the intestine by investigating the improvement in defecation, frequency, fecal characteristics, and intestine microbiota (Ogata *et al*. 1997; Matsumoto *et al*. 2006; Olivares *et al*. 2006; Yamano *et al*. 2006; Verdenelli *et al*. 2011). In those reports, LAB and bifidobacteria in the feces were suggested to be involved in the intestine regulation.

In my previous report, I isolated many strains of LAB from traditional Mongolian dairy products, and demonstrated *in vitro* that several strains were candidate probiotics because they

could tolerate low pH and bile acid and could adhere to Caco-2 cells (Takeda *et al*. 2011b). *L. paracasei paracasei* 06TCa19 strain was deemed to be the most promising of the candidate strains for use in developing dairy products, especially yogurt. However, it was not demonstrated whether this candidate strain could reach and survive in human intestine.

Therefore, the aims of this study were to demonstrate that the 06TCa19 strain reaches and survives in the intestine, and to evaluate the effects of the 06TCa19 strain on human defecation, frequency, fecal characteristics, and intestine microbiota, including *Bifidobacterium* species. They were investigated through administration of the 06TCa19 strain in a placebo-controlled, double-blind, crossover study.

3.2 Materials and methods

3.2.1 Lactic acid bacteria strains, test fermented milks

In this study, I used 06TCa19 strain in a test fermented milk, which had a pH of 4.38 and contained 1.3×10^9 CFU/g of the only 06TCa19 strain. As a control, a placebo fermented milk with a pH of 4.43 that contained 4.1×10^8 CFU/g of the only non-probiotic *Streptococcus thermophilus* 510 strain was used. Both fermented milks were made with milk, defatted milk, milk whey protein, glucose, gelatin, and agar. The ingredient composition was identical for both fermented milks except for the difference in bacterial content. The tastes of fermented milks were almost same.

3.2.2 Study schedule and fecal sampling

A double-blind, crossover trial designed to investigate the effects of administering the 06TCa19 strain fermented milk was carried out. Throughout the experimental period, the subjects regulated their diet to avoid eating other fermented products and products containing

probiotics and oligosaccharides. Forty-six female students (with ages from 18 to 39 years old) were assigned as subjects into two groups at random. Observations were taken for a week before the intake periods, to set the baseline for the test. During the first intake period, the subjects consumed 100 g of fermented milk, twice a day (in the morning and the evening), for 3 weeks. This was followed by a one-week washout interval, in which no fermented milk was consumed. Then the subjects consumed the other fermented milk for another 3 weeks. At the end of each week of the experiment, fecal samples were collected in sterile packs under anaerobic conditions with AneroPack (Mitsubishi Gas Chemical, Tokyo, Japan) from the subjects who agreed to offer their excrement as samples. In this study, it was 8 of 46 subjects that agreed to offer their excrement. The fecal samples from 8 subjects on each week were carried out the following analysis within the day of collection. These examinations were performed following the guidelines of the Helsinki Declaration and those of the Ethical committee of Minami Kyushu University, Japan, which approved this study (Permission No. 35).

3.2.3 Random amplified polymorphic DNA PCR analysis

Lactobacilli from fecal samples of 8 subjects on each week were isolated as single colonies from streak cultures on modified-LBS (mLBS) agar plates (Mitsuoka & Hirayama 2006). A pure culture of the 06TCa19 strain on Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany) was used as the positive control, and pure cultures of the 510 strain on M17 agar (Oxoid, Hampshire, UK) and *L. acidophillus* ATCC 43121 on MRS agar were used as negative controls. 16S ribosormal DNA was extracted from the isolated cultures and control cultures by using Dr. GenTLE for yeast (TAKARA Bio, Siga, Japan) and was subjected to the Random amplified polymorphic DNA (RAPD) PCR analysis. The primers for RAPD PCR were 1254 (5′-CCGCAGCCAA-3′) (Akopyanz *et al*. 1992) and AT41

(5′-CGGATGTTGT-3′) (Takeda *et al*. 2011b). The PCR reaction was performed in a DNA thermal cycler, GeneAmp PCR System 2700 (Applied Biosystems Japan, Tokyo, Japan) and the PCR reaction mix consisted of a total volume of 25 μL, containing 1.0 μL bacterial DNA, 2.0 μL dNTP mixture, 2.5 μL 10× buffer, 0.2 μL 5 units/μL *Taq* polymerase (Gene *Taq* FP, Nippon Gene, Tokyo, Japan), 0.5 μL of each primer (100 μmol/L), and 18.8 μL pure water. The cycling program for primer 1254 consisted of 4 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min; followed by 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; with a final extension at 72°C for 10 min. The cycling program for primer AT41 consisted of an initial denaturation step at 94°C for 4 min; followed by 40 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min; with a final extension at 72°C for 5 min. RAPD products were electrophoresed at 100 V on a 2.0% (W/V) agarose gel including 0.3 μg/mL ethidium bromide. The amplified DNA bands from 06TCa19 and other colonies were visualized by ultraviolet irradiation (254 nm) and photographed using an AE-6911FXN print graph (ATTO, Tokyo, Japan).

3.2.4 Fecal characteristics and defecation frequency

Throughout the examination period, all the subjects recorded their defecation frequency and quantity and assessed their fecal characteristics using a score sheet. The defecation quantity was expressed as a number of table tennis balls, one of which was given to each subject as a reference. The fecal shape was described as one of six conditions, ranging from “Watery” to “Very hard”. The fecal color was described as one of six colors, ranging from “Yellow” to “Black”. The fecal smell was described as being of one of three levels, from “Weak” to “Strong”.

3.2.5 Fecal property analysis

At several points, all the subjects were asked to directly measure fecal pH after defecation using bromothymol blue pH test paper (Advantec, Tokyo, Japan). For measurements of ammonia and lactic acid concentration, 1 g feces was suspended in 9 mL sterilized phosphate buffered saline (pH 7.3) at 4°C. Then, the sample suspension was centrifuged at 3000 rpm at 4°C and the supernatant was collected and stored at –80°C until analysis. For ammonia measurement, the supernatant was diluted with 2% perchloric acid and the concentration was determined using an ammonia test kit (Wako Pure Chemical Industries, Tokyo, Japan). Lactic acid concentration was determined using the F-kit L-lactic acid (J. K. International, Tokyo, Japan).

3.2.6 Enumeration of intestinal bacteria by culture method

The fecal samples of 8 subjects agreed to offer their excrement as samples were prepared for the enumeration of intestinal bacteria by culture method. Enumeration of anaerobes (BL agar), aerobes (TS agar), Bifidobacteria (BS agar), Clostridia (NN agar), Bacteroidaceae (NBGT agar), and Lactobacilli (mLBS) was done according to the culture methods of Mitsuoka & Hirayama (2006).

3.2.7 Enumeration of intestinal bacteria by real time PCR method

For quantification by real time PCR, DNA was extracted from fecal samples of 8 subjects on each week and standard strains using ISOFECAL for Beads Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Standard strains were used for each group, including *Bifidobacteria longum* JCM 1217 for the *Bifidobacterium* subspecies, the 06TCa19 strain for the *Lactobacillus* group, *Clostridium* (*C.*) *coccides* JCM 1395 for the *C. coccides* group, *C. perfringens* JCM 1290 for the *C. perfringens* group, and *Bacteroides fragilis* JCM

11019 for the *Bacteroides–Prevotera* group. These strains, except for the 06TCa19 strain, were cultured at 37°C for 24–48 h in GAM broth and on GAM agar (Nissui, Tokyo, Japan) containing 0.5% glucose, in jars with anaerobic packs, for enumeration of their colony forming units (CFU). The 06TCa19 strain was cultured at 37°C for 24 h in MRS broth and on MRS agar for enumeration of CFU. *Bifidobacterium* subspecies, *C. coccides* group, *C. perfringens* group, *Bacteroides–Prevotera* group, and *Lactobacillus* group were detected by specific SYBR-Green real time PCR. Specific primers and their annealing temperatures are shown in Table 3-1. Gene quantification was performed on an Applied Biosystems 7300 real time PCR system (Applied Biosystems Japan, Tokyo, Japan). The reaction mixture (20 μL) contained 10 μL of 2× SYBR Green Master Mix (Applied Biosystems Japan), 0.1 μL of each primer (100 μmol/L), 4 μL of 10^1–10^2-fold diluted template DNA, and 5.8 μL pure water. The amplification reaction consisted of 50°C for 2 min and 95°C for 10 min; then 40 cycles of 95°C for 15 s, primer specific annealing temperature (Table 3-1) for 15 s, and extension at 72°C for 30 s. Following the amplification reaction, melting curve analysis was conducted to determine the specificity of PCR reactions by heating to 95°C for 15 s, cooling to 60°C for 1 min, heating to 95°C for 15 s, then 60°C for 15 s. For each cycle, the fluorescence signal was detected at the end of the extension step. The bacterial CFU was calculated using a standard regression curve of threshold cycle (Ct) values generated from 10-fold serial dilution of DNA samples from standard strains having known CFU. For determining total bacteria from feces, the fecal sample was diluted 10^3-fold and stained with 4',6-diamidino-2-phenylindole (DAPI) solution (Dojindo Laboratories, Kumamoto, Japan), and bacteria were counted with a hemocytometer for bacteria (Asone Corporation, Osaka, Japan) with a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Table 3-1 Real time PCR primers

Target bacteria	Amplicon size (bp)	Annealing temperature (℃)	Sequence (5'-3')	Reference
Bifidobacterium subspecies	243	58	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	Rinttilä and others (2004)
Lactobacillus group	341	58	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Rinttilä and others (2004)
Clostridium coccoides group	440	50	F: AAATGACGGTACCTGACTAA R: TTTGAGTTTCATTCTTGCGAA	Matsuki and others (2004)
Clostridium perfringens group	120	55	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	Rinttilä and others (2004)
Bacteroides-Prevotella group	418	59	F: GAAGGTCCCCCACATTG R: CAATCGGAGTTCTTCGTG	Xu and others (2011)

3.2.8 Statistical analysis

The results of defecation frequency and intestinal bacterial enumeration were analyzed statistically with one-way analysis of variance (ANOVA), followed by the Tukey test and a 2-tailed paired Student's *t* test. The *Chi*-square test was used to analyze the association between fecal characteristics and consumption of fermented milks. The Mann-Whitney U-test was used to analyze the fecal constituents. A *P*-value of less than 0.05 was defined as statistically significant.

3.3 Results

3.3.1 Random amplified polymorphic DNA PCR analysis for detecting 06TCa19 strain from fecal sample

To demonstrate that the 06TCa19 strain in the fermented milk reached and was alive in the intestines of the subjects, I collected fecal samples from subjects following intake weeks and used mLBS plates to isolate 72 colonies from these samples (Fig. 3-1). Twelve of these 72 colonies were randomly chosen to be subjected to genotyping by RAPD PCR analysis (Lanes 4–15), along with pure cultures of the 06TCa19 strain (Lane 1), 510 strain (Lane 2), and *L. acidophilus* ATCC 43121 strain (Lane 3). All genotypes observed while using primer 1254, and 9 genotypes observed while using primer AT41 (Lane 4, 6, 7, 8, 9, 11, 12, 14, and 15), were consistent with that of the pure culture of the 06TCa19 strain. Therefore, 9 genotypes (Lanes 4, 6, 7, 8, 9, 11, 12, 14, and 15) of the randomly chosen 12 Lactobacilli colonies from the subject's fecal samples were suggested to be a homologue of the 06TCa19 strain.

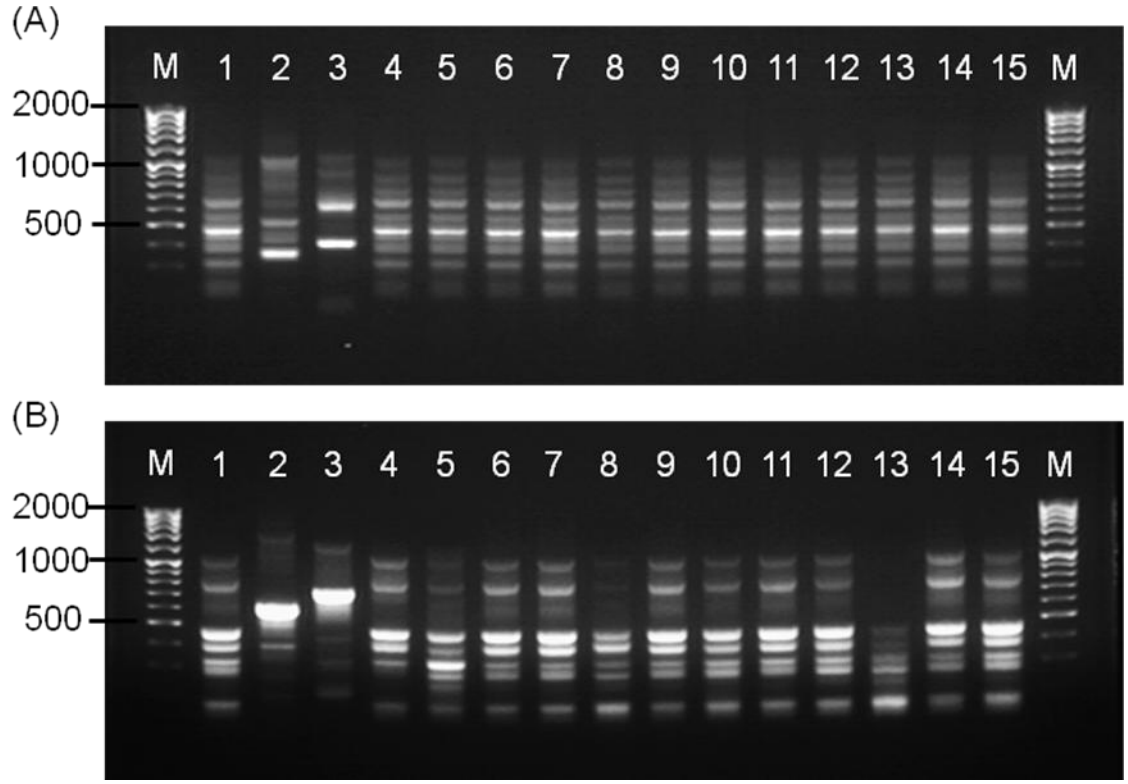

Figure 3-1 RAPD PCR profiles obtained from randomized Lactobacilli isolates from the fecals.

(A) used primer 1254. (B) used primer AT41. Lane M, DNA size marker; Lane 1, *L. paracasei paracasei* 06TCa19 strain; Lane 2, *S. thermophilus* 510 strain; Lane 3, *L. acidophillus* ATCC 43121 strain; Lanes 4-15, the Lactobacilli isolates from the fecals of 8 subjects who agreed to offer their excrement as samples. Of all the 72 isolates from the 8 subject's samples on intake weeks, 12 isolates were randomly chosen to be subjected to RAPD PCR analysis.

3.3.2 Effects of 06TCa19 strain on human defecation frequency and fecal characteristics

In this study, no subjects dropped out due to ill health. The defecation frequencies and quantities of the 06TCa19 and control groups are shown in Tables 3-2 and 3-3. In both the 06TCa19 and control groups, defecation frequencies and quantities during intake week 1 were significantly increased when compared with the values from the previous week by a one-way ANOVA, followed by the Tukey test ($P < 0.05$). However, there were no significant differences in the defecation frequencies and quantities between the 06TCa19 and control groups during any week, based on a 2-tailed paired Student's *t* test (Table 3-2). Also, the numbers of each fecal condition (regarding shape, color, and smell) self-reported by all subjects on their score sheets were counted (Table 3-3). The relationships between the distribution of each of the fecal characteristics and the consumption of 06TCa19 or control fermented milks, during the week before intake and during intake week 1, were analyzed by *Chi*-square test. Results showed significant differences in fecal shape and color, but not smell, in the 06TCa19 group between the baseline week and intake week 1 ($P < 0.05$). Regarding fecal shape, the number of reports of "Hard + very hard" was decreased and the number of reports of "Watery + muddy" was increased during intake week 1 compared to during the baseline week. In addition, regarding fecal color, the number of reports of "Yellow + browny yellow" was increased from the baseline week to intake week 1. On the other hand, in the control group, these significant differences in fecal characteristics were not observed by *Chi*-square test.

Table 3-2 Effects of 06TCa19 strain on human defecation frequency and quantity

	06TCa19 group (n=46)[a]				**Control group (n=44)[a]**			
	Before intake week	Intake week 1	Intake week 2	Intake week 3	Before intake week	Intake week 1	Intake week 2	Intake week 3
Frequency (times)	5.6 ± 2.3	6.6 ± 2.9*	5.9 ± 2.6	5.9 ± 2.2	5.4 ± 2.2	6.7 ± 2.5*	6.1 ± 2.7	5.9 ± 2.8
Quantity[b]	18.7 ± 9.6	22.5 ± 13.2*	20.8 ± 11.3	20.6 ± 10.7	18.4 ± 10.6	23.1 ± 11.9*	21.9 ± 10.9	20.8 ± 11.9

The numbers were shown the mean ± SD.

a: The number of subjects answered fully about the defecation frequency and quantity in the score sheet.

b: Fecal quantity was estimated by using a table tennis boll as 1 unit.

* : Significant differences from the before intake by one-way ANOVA followed by the Tukey test ($P < 0.05$).

Table 3-3 Effects of 06TCa19 strain on human fecal characteristics

Characteristics	06TCa19 group (n=46)				Control group (n=44)			
	Before intake week	Intake week 1	Intake week 2	Intake week 3	Before intake week	Intake week 1	Intake week 2	Intake week 3
Shape								
Watery + muddy	13[a] (5.5%)[b]	27 (10.7%)	10 (4.3%)	25 (10.6%)	18[a] (8.2%)[b]	15 (5.5%)	16 (6.6%)	19 (7.9%)
Soft + banana shape	154 (65.8%)	175 (69.4%)	168 (72.1%)	168 (71.5%)	142 (64.8%)	193 (70.2%)	174 (71.6%)	174 (72.5%)
Hard + very hard	67 (28.6%)	50 (19.8%)	55 (23.6%)	24 (10.2%)	59 (26.9%)	67 (24.4%)	53 (21.8%)	47 (19.6%)
Total	234 (100%)	252 (100%)	233 (100%)	235 (100%)	219[c] (100%)	275 (100%)	243 (100%)	240 (100%)
Color								
Yellow + browny yellow	12 (5.2%)	32 (12.8%)	18 (7.9%)	23 (9.8%)	21 (9.6%)	22 (8.4%)	25 (10.0%)	15 (6.2%)
Brown + tea brown	139 (60.7%)	145 (58%)	135 (59.5%)	135 (57.4%)	125 (57.1%)	169 (64.7%)	164 (65.9%)	158 (65.6%)
Dark brown + Black	78 (23.1%)	73 (29.2%)	74 (32.6%)	74 (31.5%)	73 (33.3%)	70 (26.8%)	60 (24.1%)	68 (28.2%)
Total	229 (100%)	250 (100%)	227 (100%)	235 (100%)	219 (100%)	261 (100%)	249 (100%)	241 (100%)
Smell								
Weak	33 (15.2)	56 (23.0%)	55 (25%)	50 (22.4%)	46 (20.8%)	49 (19.1%)	50 (21.2%)	46 (19.8%)
Normal	140 (64.5%)	144 (59.0%)	132 (60%)	129 (57.8%)	131 (59.3%)	158 (61.5%)	146 (61.9%)	128 (55.2%)
Strong	44 (20.3%)	44 (18.0%)	33 (15%)	44 (19.7%)	44 (19.9%)	50 (19.5%)	40 (16.9%)	58 (25.0%)
Total	217 (100%)	244 (100%)	220 (100%)	223 (100%)	221 (100%)	257 (100%)	236 (100%)	232 (100%)

a: The number of subjects answered from each fecal characteristics in the score sheet.

b: The rate of subjects answered from each fecal characteristics in the score sheet.

The significant differences were observed about shape and color in 06TCa19 group between before intake week and intake week 1, but not observed in control group by the *Chi*-square tests. ($P < 0.05$)

3.3.3 The analysis of fecal properties

The results of the fecal pH, ammonia and L-lactic acid concentration are shown in Table 3-4. No notable changes were observed in the fecal pH and ammonia concentrations in the 06TCa19 and control groups, however, during the fermented milk intake period, the L-lactic acid concentration tended to be higher in the 06TCa19 group than in the control group. In particular, during intake week 2, the concentration of L-lactic acid was significantly higher in the 06TCa19 group than in the control group ($P < 0.05$).

Table 3-4 Effects of 06TCa19 strain on human fecal properties

	06TCa19 group				**Control group**			
	Before intake week	Intake week 1	Intake week 2	Intake week 3	Before intake week	Intake week 1	Intake week 2	Intake week 3
pH	7.0 ± 0	7.0 ± 0	7.0 ± 0	7.0 ± 0	6.9 ± 0.2	6.9 ± 0.2	7.0 ± 0	7.0 ± 0.1
	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)
NH_3 (mg/g)	3.9 ± 1.6	2.8 ± 1.4	4.0 ± 2.0	2.2 ± 1.0	3.3 ± 2.2	2.7 ± 2.0	6.7 ± 5.0	4.1 ± 2.1
	(n = 8)	(n = 7)	(n = 6)	(n = 7)	(n = 8)	(n = 7)	(n = 6)	(n = 7)
L-lactic acid (μg/g)	33.1 ± 15.2	65.7 ± 39.2	65.2 ± 34.2*	106.0 ± 78.3	34.7 ± 10.2	41.0 ± 26.3	29.7 ± 18.7	67.4 ± 28.7
	(n = 8)	(n = 8)	(n = 8)	(n = 7)	(n = 8)	(n = 8)	(n = 8)	(n = 7)

The numbers were shown mean ± SD. The numbers in the parentheses was detected in the experiment.

* : Significant differences on each weeks between 06TCa19 and control group by Mann-Whitney U test ($P < 0.05$).

3.3.4 Enumeration of human intestinal bacteria

The results of enumeration of intestine bacteria by culture and real time PCR methods are shown in Figures 3-2 and 3-3, respectively. The anaerobe and aerobe counts measured by the culture method and the total bacteria counts measured by DAPI staining did not differ between the 06TCa19 and control groups, and remained almost unchanged throughout the weeks in both groups. When the 06TCa19 fermented milk was eaten, the counts of Lactobacilli and *Lactobacillus* were significantly increased during the intake period compared with the baseline, as measured by culture and real time PCR methods ($P < 0.05$). Using the culture method, no significant differences were observed in the counts of the other kinds of bacteria between the baseline week and the intake period or between the two groups during any of the weeks. However, using the real time PCR method, some significant differences were observed in the counts in the other kinds of bacteria. In the 06TCa19 group, *Bifidobacteria* count was gradually increased in a week-dependent manner, and significantly increased at intake week 3 as compared with the baseline week ($P < 0.05$). The count of the *C. coccides* group in the 06TCa19 group was significantly decreased at intake week 1 as compared with in the baseline week ($P < 0.05$), but it recovered at intake week 2. *Bacteroides* class count in the 06TCa19 group was decreased in a week-dependent manner, and it was significantly lower than that of the control group at intake week 3 ($P < 0.05$). On the other hand, no significant effects of administration of the control fermented milk on the bacterial counts were observed by the real time PCR method.

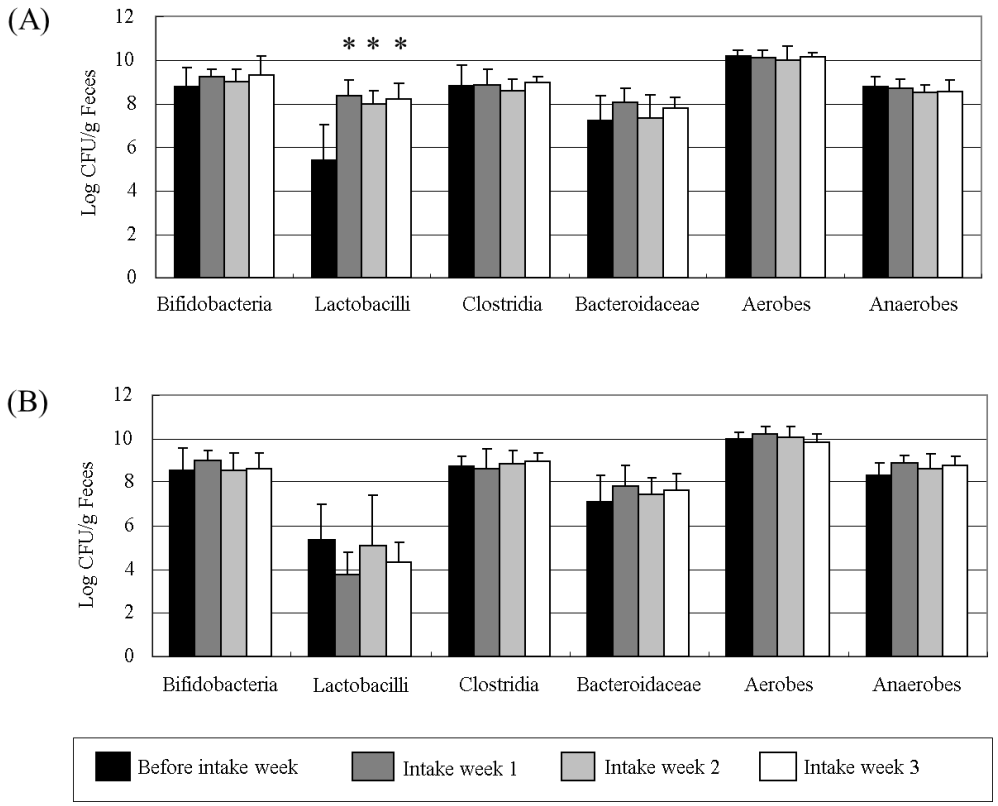

Figure 3-2 Enumeration of intestinal bacterium from fecal samples by culture method.

(A) shows administrating fermented milk of *L. paracasei paracasei* 06TCa19 strain.

(B) shows administrating fermented milk of *S. thermophilus* 510 strain (Control).

The fecals from 8 subjects who agreed to offer their excrement as samples on each week were investigated for the examination. The asterisks show the significant differences compared with the before intake week by one-way ANOVA, followed by the Tukey test ($P < 0.05$).

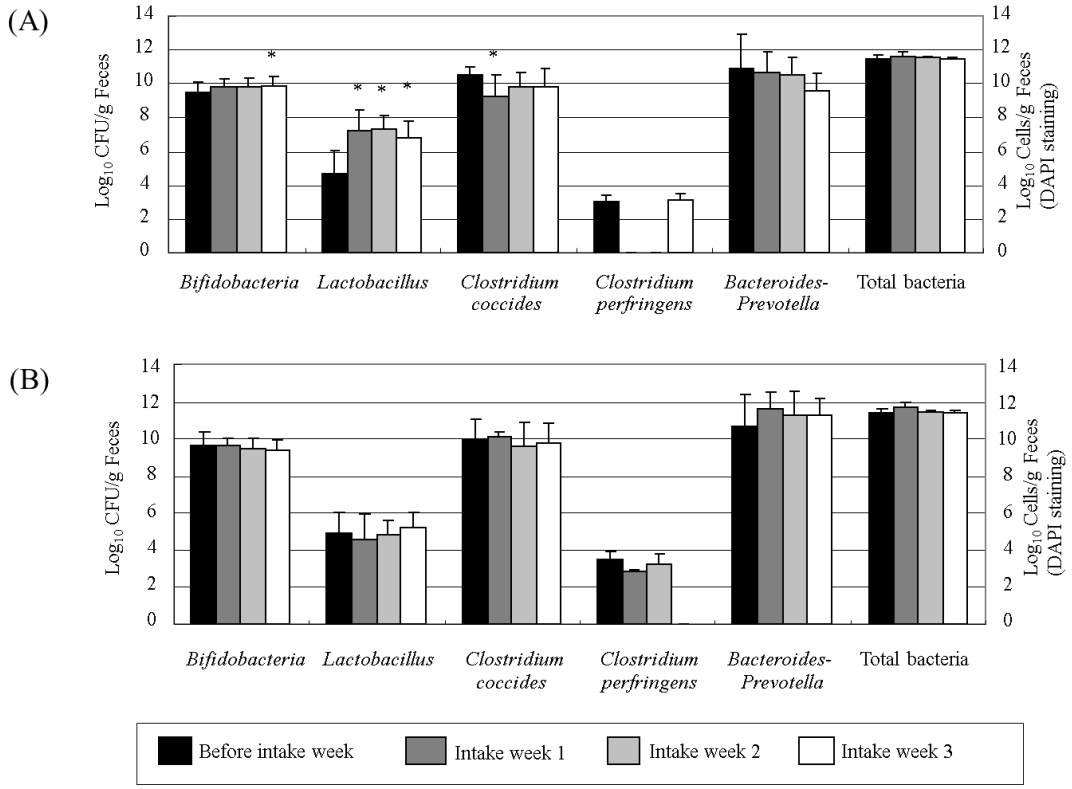

Figure 3-3 Enumeration of intestinal microorganisms from fecal samples by real time PCR and DAPI staining.

(A) shows administrating fermented milk of *L. paracasei paracasei* 06TCa19 strain.

(B) shows administrating fermented milk of *S. thermophilus* 510 strain (Control).

The fecals from 8 subjects who agreed to offer their excrement as samples on each week were investigated for the examination. The asterisks show the significant differences compared with the before intake week by one-way ANOVA, followed by the Tukey test ($P < 0.05$).

3.4 Discussion

In this study, the 06TCa19 and control fermented milks were prepared and they were given in the placebo-controlled double blind crossover test to healthy women subjects. Lactobacilli were cultured from feces of the subject administered 06TCa19 fermented milk and the DNAs extracted from the isolated Lactobacilli were subjected to RAPD analysis. Genotypes of the isolates consisted with that of pure cultured 06TCa19 strain by RAPD analysis (Fig. 3-1). This result suggested that 06TCa19 strain could reach and survive in human intestine. Furthermore, the effects of the 06TCa19 strain on defecation frequency, quantity and fecal characteristics comparing to the control group were also investigated. A significant increase in the defecation frequency and quantity was not only observed in the 06TCa19 group but also the control group at intake week 1 comparing with the before intake week (Table 3-2). Generally, fermented milk is known to have a potent effect on intestine regulation, with or without the inclusion of probiotics. This fact is consistent with the increases observed in defecation frequency and quantity during intake week 1, in both 06TCa19 and control groups. However, the data suggest that 06TCa19 fermented milk improved bowel habits better than the control, because the group that consumed 06TCa19 fermented milk showed significant changes in fecal shape and color, which were not observed in the group that consumed the control fermented milk (Table 3-3). In particular, it seemed to be notable for the reduction of the proportion of ‘Hard + very hard’ stool from intake week to intake week 1 in 06TCa19 fermented milk group.

Some previous reports have suggested that the improvement in the frequency of defecation and fecal quality in humans following ingestion of probiotics was due to changes in the intestinal microbiota, in particular, the increase of the *Lactobacillus* or *Bifidobacterium* (Ogata *et al*. 1997; Matsumoto *et al*. 2006; Olivares *et al*. 2006; Yamano *et al*. 2006;

Verdenelli *et al*. 2011). In this study, the *Lactobacillus* level in the 06TCa19 group significantly increased after consumption of the 06TCa19 fermented milk as compared with the baseline level (Figs. 3-2 and 3-3). Moreover, almost all of the randomly selected Lactobacilli isolates from fecal samples collected after the intake period in the 06TCa19 group were suggested to be the 06TCa19 strain according to the results of RAPD analysis (Fig. 3-1). Therefore, the enhancement of Lactobacilli level during the intake period might reflect the portion of the 06TCa19 strain that reached and survived in the intestine.

In studies of probiotics, it is important to develop a method for selective enumeration of the strain. Previous reports demonstrated that *L. casei* strain Shirota could survive in human gastrointestinal tract and some molecular biological methods have been developed for selective enumeration of this strain in feces (Yuki *et al*. 1999; Fujimoto *et al*. 2008). Moreover, previous reports showed that *Lactobacillus* and *L. casei* strain Shirota were detected at the same levels in the feces from the subjects who drank the fermented milk beverage containing the strain Shirota (Matsumoto *et al*. 2006; Tuohy *et al*. 2007; Matsumoto *et al*. 2010). In the present study, I did not establish methods for the selective enumeration of the 06TCa19 strain or for accurately assaying the number of 06TCa19 strain in fecal samples. To investigate the more detailed effects of 06TCa19 strain in the human intestine, further study is needed to develop a selective enumeration method for 06TCa19 strain, as has been established for other strains in previous reports.

On the other hand, I confirmed that the 06TCa19 strain mainly produces L–lactic acid (data not shown). Thus, the increase of L-lactic acid in feces of the 06TCa19 group was likely produced by the 06TCa19 strain in the subject's intestine (Table 3-4). Organic acids, including lactic acid, are thought to promote gastrointestinal motility (Yokokura *et al*. 1977). Therefore, the increased L-lactic acid produced by the 06TCa19 strain in the intestine might cause the 06TCa19 fermented milk to have a greater effect on fecal characteristics than the control.

Moreover, the level of *Bifidobacteria* in the 06TCa19 group tended to increase in a week-dependent manner during the intake period. The real time PCR method revealed that the level of *Bifidobacteria* in the 06TCa19 group at intake week 3 was significantly higher than that during the baseline week (Fig. 3-3). Therefore, the data suggested that the 06TCa19 strain reached the intestine and increased the frequency of defecation and fecal quantity in humans, as well as improved the fecal characteristics, as a result of the increase in the number of *Lactobacillus* and *Bifidobacteria* following the consumption of the 06TCa19 fermented milk. It was also suggested that the 06TCa19 strain regulated the host's intestine through the enhancement of the number of *Bifidobacteria*.

3.5 Conclusions

A randomized, double-blind, crossover study with 46 young, healthy women who consumed either the 06TCa19 strain or control fermented milks was conducted. The 06TCa19 strain was observed from the Lactobacilli colonies from cultures of feces of subjects who consumed the 06TCa19 fermented milk, suggesting that this strain was able to reach and survive in the human intestine. After consumption of either fermented milk, the subjects' defecation frequencies were significantly increased. However, subjects who consumed the 06TCa19 fermented milk also exhibited significantly improved defecation characteristics, particularly regarding fecal shape and color, compared to those of the control group, indicating that the 06TCa19 strain is superior for use as a probiotic to regulate the human intestine. It is presumed that the mechanism of action is through the increased level of L-lactic acid produced by the 06TCa19 strain and/or the increased number of *Lactobacillus* and *Bifidobacteria* in the human intestine after the ingestion of the 06TCa19 fermented milk.