Effect of Administration of Milk Fermented with *Lactobacillus acidophilus* LA-2 on Fecal Mutagenicity and Microflora in the Human Intestine

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ABSTRACT

To demonstrate the antimutagenic effect of fermented milk in the human intestine, fecal mutagenicity and bacterial composition of six healthy subjects consuming their regular diet were investigated before and during the administration of milk fermented with *Lactobacillus acidophilus* LA-2. The administration of the fermented milk caused a remarkable decrease (71.9% on the average; range of 19.4 to 90.6%) in fecal mutagenicity compared with that before the administration; *Lactobacillus* spp. and *Bifidobacterium* spp. population increased in the feces of all subjects. The suppression of fecal mutagenicity appeared to be due to the change in fecal microflora caused by the presence of strain LA-2 in the human intestine.
(**Key words**: *Lactobacillus acidophilus* LA-2, antimutagenicity, fecal mutagenicity, fecal microflora)

INTRODUCTION

Epidemiological data have indicated that the incidence of colon cancer is increasing in Japan and that a strong linkage exists between such incidence and changes in Japanese lifestyle, especially diet (18). The increased mutagenicity in the human intestine arising from dietary change (1) has been considered to be a major factor responsible for colon cancer (21).

In contrast, fermented milk products prepared with lactic acid bacteria are known to exhibit antimutagenicity toward amino acid pyrolysates, such as 3-amino-1,4-dimethyl-5*H*-pyrido-[4,3-*b*]indole (Trp-P1) and 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole (Trp-P2) (12, 13, 14). This antimutagenic effect indicated that intake of fermented milk products might be potentially beneficial to lower cancer incidence.

We have investigated the antimutagenicity of milk cultured with lactic acid bacteria against N-methyl-N′-nitro-N-nitrosoguanidine and tryptophane pyrolysates by an in vitro test using *Salmonella typhimurium* TA 100 and TA 98. Each cultured milk sample displayed antimutagenic activities against each chemical. Among the cultured milk samples, those cultured with *Lactobacillus acidophilus* LA-2 exhibited the highest inhibitory effect against these mutagens (9, 10).

Hence, the present study was conducted to provide information regarding the antimutagenic properties in the human intestine of milk cultured with lactic acid bacteria. Mutagenicity of fecal samples from six subjects before and during the administration of milk cultured with *L. acidophilus* LA-2 was investigated by the Ames test (17). In addition, the bacterial composition of these samples was also examined.

MATERIALS AND METHODS

Preparation of Fermented Milk and Selection of Human Subjects

Pasteurized milk base (8.0% SNF, 3.0% milk fat, and 5.0% sucrose) was inoculated with *L. acidophilus* LA-2 strain (a strain very similar to *Lactobacillus casei* ssp. *rhamnosus* by determination of molar percentage of G + C of the DNA) (22) and then incubated at 38°C for 18 h. The pH, acidity, and bacterial count of this LA-2 fermented milk were 4.60, 0.80%, and 2.5×10^8 cfu/g, respectively.

Of 20 volunteers, six subjects were chosen on the basis of the following criteria: 1) general health, 2) no prescription drugs for at least a month, 3) absence of constipation, and 4) higher and more stabilized fecal mutagenicity than other volunteers in pretrials. The experiments began after the informed consent of the subjects had been obtained. The 6 volunteers selected were male; mean age was 32.6 yr (range of 26 to 38 years).

Administration of Fermented Milk and Collection of Feces

One hundred grams of LA-2 fermented milk were given three times a day after the meal for 7 d. Before and after consumption of LA-2 fermented milk, the

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subjects continued their usual diet without prescription drugs or fermented food.

Fecal samples were collected daily for 3 d prior to intake of LA-2 fermented milk and during 5 to 7 d of fermented milk intake.

Determination of Fecal Mutagenicity

The procedure to determine fecal mutagenicity was essentially the method described by Hayatsu et al. (6). Fresh feces (100 to 200 g) were mixed with water (350 ml) and homogenized for 5 min in a blender. The homogenate was centrifuged at 10,000 × *g* and 20°C for 30 min.

The slurry collected by decantation was treated twice with blue rayon (7) (Funakoshi Chemical Co., Ltd., Tokyo, Japan). One gram and then 0.5 g of the blue rayon were used with 30 min of shaking for each treatment. The combined blue rayon samples were washed with water and extracted twice with 130 ml of methanol and ammonia (50:1, vol/vol). The pooled extracts were evaporated at 37°C to dryness. The residue was dissolved in 0.6 ml of methanol, and the solution was mixed with 50 ml of water. The aqueous solution was again treated with blue rayon (0.1 g) twice, and the rayon was twice extracted with 30 ml of methanol and ammonia. After evaporation at 37°C to dryness, the sample was dissolved in 1.2 ml of dimethylsulfoxide and frozen at –80°C until assay by the Ames test (17). The mutagenicity test was carried out according to methods of Maron and Ames (17); the test strain (*Salmonella typhimurium* TA 98) was kindly supplied by Bruce N. Ames (University of California, Berkeley). The S-9 mix was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Each sample was tested in three dose levels: 50, 100, and 200 μl. Colonies were counted in an automated colony counter (System Science Co. Ltd., Tokyo, Japan).

Fecal Microflora Assay

Each fecal sample was homogenized, and 10-fold serial dilution was made to 10^{-9} within 6 h using the method of Mitsuoka et al. (20). The 0.05 ml of the appropriate dilutions were streaked on numerous agar plates, and then fecal bacteria were analyzed as reported by Hosoda et al. (11).

Eggerth Gagnon agar (Nissui Seiyaku Co. Ltd., Tokyo, Japan), blood liver agar (Nissui Seiyaku Co. Ltd.), and trypticase soy blood agar (BBL, Cockeysville, MD) were used as a nonselective medium. *Bifidobacterium* selection agar, neomycin Nagler agar, modified *Lactobacillus* selection agar (BBL), and desoxycholate hydrogen sulfide lactose agar (Eiken Chemical Co., Ltd., Tokyo, Japan) were used as selective media. The anaerobic incubation was carried out by Aeropack Kenki System (Mitsubishi-Gas Chemical Co. Ltd., Tokyo, Japan). *Bifidobacterium* spp., *Bacteroides* spp., *Eubacterium* spp., *Peptostreptococcus* sp., and lecithinase-negative *Clostridium* spp. were enumerated on the Eggerth Gagnon and blood liver agar. Enterobacteriaceae, *Enterococcus* spp. from trypticase soy blood agar, *Bifidobacterium* spp. from *Bifidobacterium* agar, lecithinase-positive *Clostridium* (mainly *Clostridium perfringens*) from neomycin Nagler agar, *Lactobacillus* from *Lactobacillus* agar, and Enterobacteriaceae from desoxycholate hydrogen sulfide lactose agar were counted, respectively. The isolated bacteria were identified mainly based on the aerobic test, Gram stain, morphology of colony and cell, spore formation, and carbohydrate fermentation (19).

Determination of Fecal Moisture, pH, Ammonia Concentration, and Defecation

Fecal moisture was determined by forced-air convection oven (PC-410; Advantec Co., Tokyo, Japan), pH by pH meter (F-22; Horiba Co., Tokyo, Japan), and ammonia by test kit (F-kit; Boehringer Yamanouchi Mannheim Co., Tokyo, Japan). During the study (14 d), excretion time, frequency, fecal quality (hardness, color, and smell), and health condition (subjective feelings) were recorded by subjects with questionnaires.

Statistical Analysis

Student's *t* test was conducted with Microsoft Excel, Version 5.0 to determine the significance of the changes of fecal mutagenicity and microflora before and during administration of LA-2 fermented milk.

RESULTS

During the administration of LA-2 fermented milk, all subjects defecated once a day, and the health condition of subjects remained similar to that before intake. The fecal mutagenicity of all subjects is presented in Table 1. Before intake, mutagenicity varied from 345 to 59 in the feces of all subjects (mean revertant count: 151/100 g of feces). During the LA-2 fermented milk intake, fecal mutagenicity decreased ($P < 0.01$). The mean revertant count decreased 71.9%. The reduction was especially significant for subject 3, for whom the mean revertant count was as low as 10% of the initial count.

The bacterial composition of the feces collected from the subjects was analyzed in detail. The total

TABLE 1. Effect of administration of milk fermented with *Lactobacillus acidophilus* LA-2 on fecal mutagenicity.

Subject	His⁺-Revertant colonies in feces[1]				Inhibition[3]
	Before intake		During intake[2]		
	(no./100 g)				(%)
	$\bar{X}$	SD	$\bar{X}$	SD	
1	140	13	36	3	74.3
2	345	12	50	4	85.5
3	203	12	19	2	90.6
4	99	11	55	3	44.4
5	59	7	45	3	23.7
6	62	7	50	3	19.4
$\bar{X}$	151.3		42.5**		71.9
SEM	100.2		12.5		

[1]The net increase from the solvent control values (30 ± 6); His⁺ = histidine-dependent.

[2]The LA-2 fermented milk (100 g) was administered three times a day for 5 to 7 d.

[3]Inhibition percentage = [1 − (b)/(a)] × 100, where a = before intake, and b = during intake.

**Difference before and during intake ($P < 0.01$).

bacterial count of anaerobes, *Bacteroides* spp., *Eubacterium* spp., *Peptostreptococcus* spp., and *Bifidobacterium* spp. and the percentage of *Bifidobacterium* spp., lecithinase-positive *Clostridium* spp., lecithinse-negative *Clostridium* spp., *Lactobacillus* spp., aerobes, Enterobacteriaceae, and *Enterococcus* spp. are shown in Table 2. Moisture, pH, and ammonia concentration of these feces were also recorded (Table 2). Lactobacilli and bifidobacteria in the feces of most of the subjects increased during the intake of LA-2 fermented milk ($P < 0.05$; Table 2) and Figure 1. The total bacterial count, anaerobes, and aerobes of feces were not greatly changed, but the percentage of *Bifidobacteria* sp. in fecal bacteria rose from 11.0% to 33.3%. Lecithinase-positive *Clostridia* were present in the feces of one subject before LA-2 fermented milk but not in the feces of any of the subjects during the administration of fermented milk. Moisture, pH, and ammonia concentration in feces of all subjects remained unchanged during administration of the LA-2 fermented milk (Table 2).

DISCUSSION

Previous in vitro studies of fermented milk samples using *Lactobacillus delbrueckii* sp. *bulgaricus* or *Streptococcus thermophilus* against fecal extracts of cat, monkey, and dog indicated that these types of fermented milk display antimutagenic effects (12). In the present study, the fecal mutagenicity of subjects during the administration of the LA-2 fermented milk was reduced ($P < 0.01$; Table 1), demonstrating the antimutagenic effects of LA-2 fermented milk in humans. Although the literature deals very little with the effect of lactic acid bacteria on fecal mutagenicity

TABLE 2. Effect of administration of milk fermented with *Lactobacillus acidophilus* LA-2 on fecal bacterial composition and properties in six healthy volunteers.

Microorganism	Before intake		Frequency	During intake[1]		Frequency
	(log cfu/g)		(%)	(log cfu/g)		(%)
	$\bar{X}$	SD		$\bar{X}$	SD	
Total counts	10.4	0.2		10.3	0.2	
Anaerobes	10.3	0.2		10.2	0.2	
Bacteroides	10.1	0.3	100[2]	9.8	0.4	100
Eubacteria	9.2	0.4	100	9.3	0.3	100
Peptostreptococci	8.5	0.1	50	8.9	0.9	50
Bifidobacteria	9.2	0.1	100	9.9*	0.4	100
Bifidobacteria, %[3]	11.0	4.8		33.3	21.9	
Clostridia, lecithinase-positive	3.3		17	0.0		0
Clostridia, lecithinase-negative	8.8	0.2	100	9.3	0.3	100
Lactobacilli	6.4	1.1	100	7.6*	0.8	100
Aerobes	7.7	0.8		7.8	0.8	
Enterobacteriaceae	7.3	0.2	100	7.2	0.3	100
Enterococci	7.7	0.8	100	7.8	0.8	100
Moisture, %	77.9	7.2		78.2	10.6	
pH	6.6	0.3		6.6	0.3	
Fecal ammonia, μg/g of feces	472.8	168.8		476.2	143.7	

[1]The LA-2 fermented milk (100 g) was administered three times a day for 5 to 7 d.

[2]Bifidobacteria population in relation to fecal bacteria.

*Difference before and during intake ($P < 0.05$).

of humans, our findings are in good agreement with the results obtained by Lidbeck et al. (16), who reported decreased excretion of mutagenic substances in feces and urine by 11 healthy human subjects who consumed fried beef while taking *L. acidophilus*. The results of the present study confirmed that LA-2 fermented milk inhibits fecal mutagenicity similar to the *L. acidophilus* milk examined by Lidbeck et al. (16). Because the present study was conducted without any dietary condition, such as the inclusion of fried meat containing various heterocyclic amines, the results obtained are thought to reflect the activities of cultured milk with lactic acid bacteria in human intestine more accurately than results of other studies (5, 6, 7, 16).

The present study was conducted without any dietary condition, so the fecal mutagenicity varied greatly from 345 to 59 (Histidine-dependent revertant colonies/100 g of feces) before administration of LA-2 fermented milk. Further studies about relative change of individual fecal mutagenicity and intestinal metabolisms must be carried out.

The LA-2 strain exhibited a strong antimutagenic effect against not only N-methyl-N'-nitro-N-nitrosoguanidine, 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole, aflatoxin B_1 in vitro (8, 9, 10) but also the mutagenicity of human feces; moreover, LA-2 was resistant to artificial gastric juices and bile acid (data not shown). Therefore, LA-2 strain conceivably contributed directly to the reduction of fecal mutagenicity.

More than 100 species of bacteria reside in the human intestine. These intestinal bacteria produce various kinds of enzymes, which greatly affect the activity of mutagens in human intestine (2). Recently, the intake of fermented milk has been demonstrated to have a beneficial impact on intestinal microflora, changing their composition by substantially increasing *Bifidobacterium* spp. and decreasing *Clostridium* spp. or *Escherichia coli* (4, 15). In the present study, *Bifidobacterium* spp. and *Lactobacillus* spp. were also increased in the feces of subjects given LA-2 fermented milk. These changes in intestinal microflora caused by the intake of fermented milk are in good

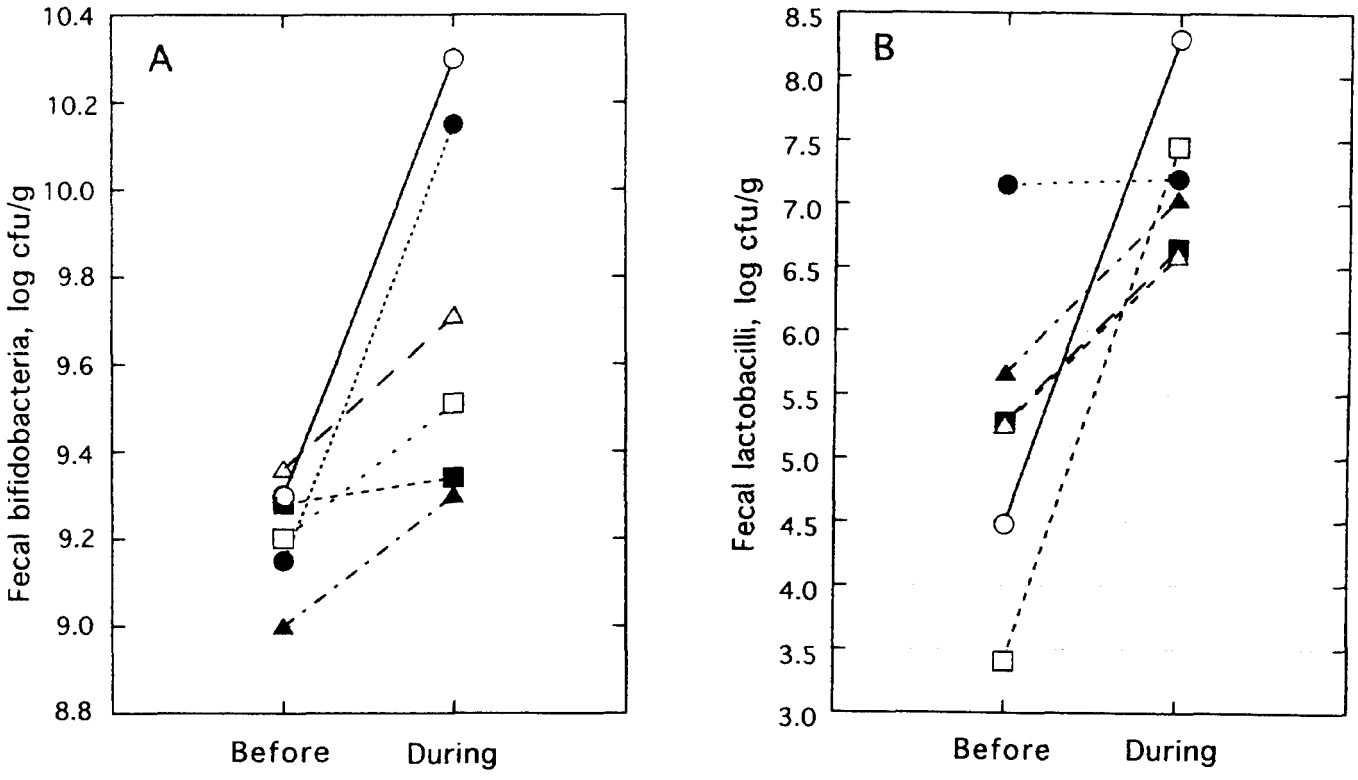

Figure 1. Effect of administration of *Lactobacillus acidophilus* LA-2 fermented milk on fecal bifidobacteria (A) and lactobacilli (B) for human subject number 1 (□), 2 (■), 3 (○), 4 (●), 5 (△), and 6 (▲).

agreement with the findings of our previous study of the fecal microflora of eight subjects administered milk that had been fermented with *L. acidophilus* GG (11). For subject 3 especially, *Bifidobacterium* spp. increased, and fecal mutagenicity decreased. This result indicated a positive correlation between fecal mutagenicity and microflora.

Friend and Shahani (3) reported that specific strains of lactobacilli possess anticarcinogenic properties. Because the blue rayon used in this study also binds carcinogens, LA-2 fermented milk may possess anticarcinogenic properties. Further studies are necessary to generalize this observation with more lactic cultures to evaluate this correlation further.

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