

論 文
Articles

Effect of Fermented Milk on the Activities of Fecal Bacterial Enzymes of Mice

By

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Summary : The fecal enzymes originating from the injurious bacteria in the intestinal tract, azoreductase and nitroreductase, convert pro-carcinogens to proximal carcinogens. The effect of fermented milk on the activity of fecal enzymes in mice was studied. The fermented milk was prepared using 1% low-fat milk with the starter of *Lactobacillus gasseri* JCM 1130 and *Lactobacillus amylovorus* JCM 5811. Mice were fed with diets containing high contents of protein and fat. When administered fermented milk as *Lactobacillus* strains supplement, the mice exhibited significantly lower levels of azoreductase and nitroreductase activities in the feces as compared with the levels of their activities in the feces of nontreated mice. The number of viable lactic acid bacteria increased in the mice feces for 5 days after the administration of fermented milk, and the same species of lactic acid bacteria from fermented milk was detected in their feces by using random amplified polymorphic DNA (RAPD) method.

Key Words : fecal azoreductase, fecal nitroreductase, *Lactobacillus*, PCR

The incidence of colon cancer shows an increasing trend in Japan. The role of diets with high contents of protein and fat in the etiology of colon cancer has not been well established. Changes in the metabolic activity of intestinal microflora could occur without appreciable changes in the numbers or types of viable microorganisms in the gut. Bacteria possess many inducible or repressible enzymes for colon cancer. Nitroreductase and azoreductase from the injurious bacteria in the intestinal tract produce nitroso- and N-hydroxy compounds, which are well-known carcinogens. GOLDIN *et al.*¹⁾ reported that the supplementation of the normal diet of mice with *Lactobacillus acidophilus* suppressed the activities of fecal nitroreductase, azoreductase and β -glucuronidase.

The recent development of PCR method using genomic DNA for obtaining random amplified polymorphic DNA (RAPD) fingerprints allows easy identification of lactic acid bacteria from foods or environmental and intestinal samples.

In this experiment, the effect of oral administration

of fermented milk on the activities of azoreductase and nitroreductase, and the number of viable lactobacilli and the detection of orally administered lactobacilli in the mice feces were studied.

Materials and Methods

Animals and diets

Specific pathogen-free male C3H/Hen mice (5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were divided into three groups and five mice were housed in each plastic cage. Group 1 mice (experimental group) and group 2 mice (control group) were fed with a diet of high protein and fat contents (MR-A1, Nihon Nosan Kogyo K.K., Kanagawa, Japan). Group 3 mice (normal group) were fed with a regular diet (F-2, Funabashi Farms Co., Ltd., Chiba, Japan). All mice were provided with tap water *ad libitum*.

Preparation of fermented milk

Lactobacillus gasseri JCM 1130 and *Lactobacillus amylovorus* JCM 5811, were used for the fermented milk

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preparation. Two strains isolated from human feces were kindly provided by Japan Collection of Microorganisms (The Institute of Physical and Chemical Research, Saitama, Japan). The strains were stored in 10% solid-reconstituted skim milk at -80°C . They were grown in 10% solid-reconstituted skim milk for 18–24 hours at 37°C under aerobic conditions. This operation was continuously repeated three times prior to use in the preparation of fermented milk. Two actively grown *Lactobacillus* strains were added into 5 ml of 1% low-fat milk. The milk was incubated for 16–18 hours at 37°C under aerobic condition. The resulting fermented milk (10^8 cfu/ml) was used after being diluted with equal an volume of ultrapure water to decrease the viscosity.

Schedules of oral administration

The fermented milk as lactobacilli supplement was orally administered to group 1 mice in a dose of 10 ml/kg/day using a blunt tipped feeding needle for the first 10 days (term I) and the last 10 days (term III) of the experimental period (74 days), however the mice were not fed with the fermented milk for the middle 54 days (term II) between term I and term III. Group 2 and 3 mice were not supplied with the fermented milk throughout the experiment period.

Collection of fecal specimens and preparation of fecal extract

The bedding of the mice was cleaned, and at 7 : 00–7 : 30 am of the next day soft and fresh feces of the mice were collected. The fecal specimens were assayed immediately after collection.

The fresh fecal samples (150 mg/ml) were suspended in cold prerduced 0.2M Tris-HCl buffer (pH 7.8). Glass beads (0.2mm diameter) were added to the fecal suspension for complete homogenization. The suspension was agitated in a tightly stoppered tube for 5 minutes on a vortex mixer. After centrifugation at $200\times g$ for 10 minutes, the supernatant was used for the enzyme assay.

Azoreductase assay

The specific activity of fecal azoreductase was determined by the modified method of GOLDIN *et al*²⁾. All solutions were prerduced. The enzyme reaction was run anaerobically at 37°C in a total volume of 0.5 ml consisting of 0.2 ml of 1 mM sunset yellow [1-(4 sulfo-1-phenylazo)-2-naphthol-6-sulfonic acid, 191-11072, Wako, Japan] solution and 0.3 ml of fecal extract. After 2 hours, the reaction was terminated by the addition of 0.75 ml of 1.2N HCl. The reaction mixture was

centrifuged at $20,000\times g$ for 30 minutes, and the amounts of reduced products of sunset yellow in the supernatant were measured by the BRATTON and MARSHALL method³⁾. The results were standardized by calculating the amounts of reduced sunset yellow ($\mu\text{g/hr/mg-fecal protein}$).

Nitroreductase assay

The specific activity of the fecal nitroreductase was determined by the modified method of GOLDIN *et al*²⁾. All solutions were prerduced. The enzyme reaction was run anaerobically at 37°C in a total volume of 1 ml consisting of 0.2 ml of 1.75 mM *m*-nitrobenzoic acid solution, 0.2 ml of 2.5 mM NADPH (N-6505, Sigma, St. Louis, MO, USA) solution, 0.2 ml of 5 mM NADH (N-8129, Sigma) solution and 0.4 ml of fecal extract. The amount of *m*-aminobenzoic acid formed was then measured by the BRATTON and MARSHALL method³⁾. The results were standardized by calculating the amount of *m*-aminobenzoic acid ($\mu\text{g/hr/mg-fecal protein}$).

Discrimination of the supplemented lactobacilli in feces by RAPD method

The colonies of *Lactobacillus* were isolated from the collected feces using BCP plate count agar (E-MB31, Eiken Chemical Co., Ltd., Tokyo, Japan). Ten individual colonies were picked up from the colonies formed on BCP plate count agar and cultured in MRS broth (Oxoid, Unipath Ltd, Hampshire, UK) for 16–20 hours at 37°C . The extraction of DNA from lactobacilli was performed by the benzyl chloride method of HENG *et al*⁴⁾. PCR amplification was performed in a Thermal Cycler TP-3000 (Takara Biomedicals, Tokyo, Japan) using Takara EX TaqTM (RR001A, Takara Biomedicals) and three primers of arbitrary nucleotide sequences (primer A : 5' CCGCAGCCAA 3', primer B : 5' AACGCGCAAC 3', primer C : 5' GCGGAAATAG 3')⁵⁾. The products of PCR were electrophoresed on 1.5% agarose gel. After staining with 1% ethidium bromide solution, the gels were photographed under UV light. As control, the purified *L. gasseri* JCM 1130 and *L. gasseri* JCM 5811 were used.

Number of viable lactic acid bacteria in the feces of mice

The mice and fermented milk were used in this experiment in the same manner as described above. The mice were fed with a regular diet throughout the experiment period. The fermented milk was administered to the mice consecutively for 3 days using a blunt tipped feeding needle. Fresh feces of mice were collected 1, 3, 5 and 10 days after the termination of the

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administration of fermented milk to mice. The number of viable lactic acid bacteria in feces was determined from the colonies formed on BCP plate count agar.

Statistical analysis

The results were expressed as the mean and standard deviation of triple identical assays. A statistical difference in mean was determined by Dunnett's or Scheffe's multiple comparison test after evaluating the data using Bartlett's test and Kruskal-Wallis's test.

Results and Discussion

Figure 1 shows that the activity of fecal azoreductase is affected by the oral administration of fermented milk to mice. The fecal azoreductase activity of mice was suppressed during the consumption of fermented milk. Even 5 days after the interruption of administration, a significantly low level of fecal azoreductase activity was observed. This activity progressively increased again 10 days after the interruption of administration. However, the resumption of oral administration of fermented milk repressed the markedly increasing activity of fecal azoreductase.

Figure 2 shows the changes in the activity of fecal nitroreductase during the experiment period. By the consumption, interruption and resumption of administration of fermented milk to mice, changes in the activ-

ity of fecal nitroreductase similar to those in the azoreductase activity described above were observed.

Figure 3 shows the effect of consumption of fermented milk on the number of viable lactic acid bacteria in feces of mice. It was observed that the number of viable lactic acid bacteria increased after the administration of fermented milk, but the increase was not significant. Although the increase continued for 5 days after the termination of administration of fermented milk, the number of bacteria decreased after that and returned to the initial level prior to the oral administration of fermented milk.

Plate 1 shows RAPD patterns of lactic acid bacteria in feces of mice 5 days after the oral administration of fermented milk. *L. gasseri* JCM 1130 and *L. amylovorus* JCM 5811, used for the preparation of fermented milk, were detected in the feces of mice 5 days after the termination of administration.

Azoreductase results in the hydrolysis of azocompounds such as food dyes. An intermediate free radical is believed to be formed during the course of this reductive hydrolysis⁶⁾. The final products of this reaction are aromatic amines, which can be converted to N-hydroxyl compounds in the intestinal tract⁷⁾. Nitroreductase can convert aromatic nitro-compounds to aromatic amines. Nitroso- and N-hydroxy compounds are formed during the course of this reduction^{8,9)}, and they are well-known

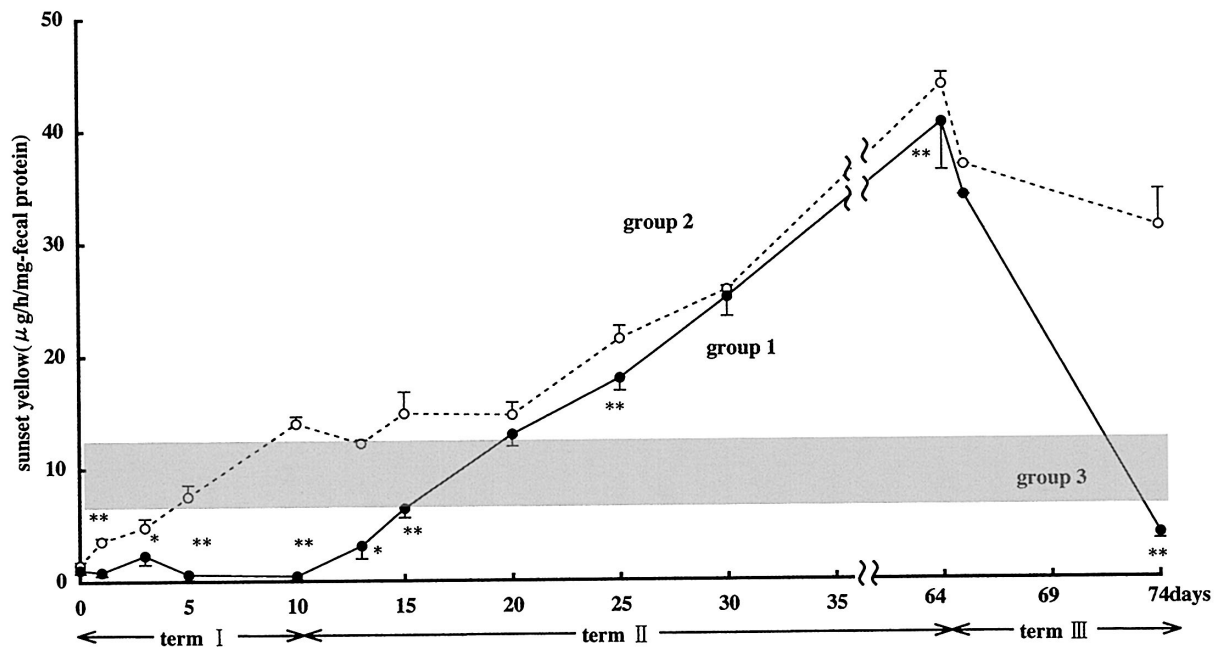


Fig. 1 Effect of oral administration of fermented milk on azoreductase activity in feces of mice fed with a high protein and fat diet

The results were expressed as means \pm standard deviations of triple identical assays.

The vertical bar represented the standard deviation.

**P < 0.01 as compared with group 2. *P < 0.05 as compared with group 2.

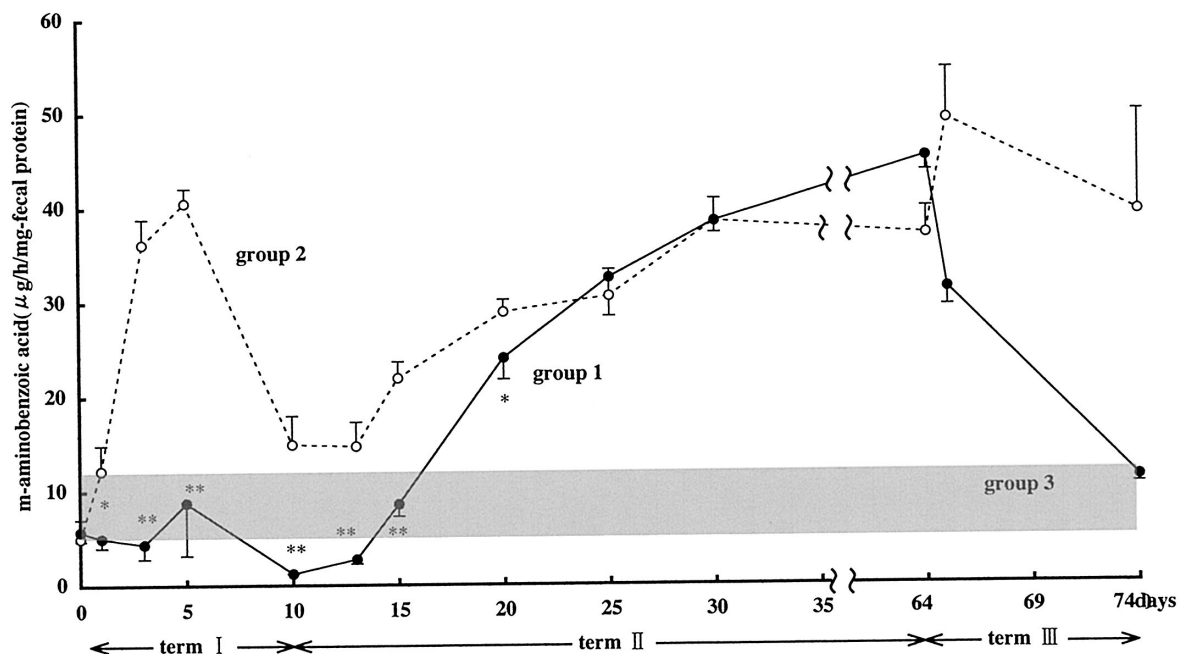


Fig. 2 Effect of oral administration of fermented milk on nitroreductase activity in feces of mice fed with a high protein and fat diet

The results were expressed as means \pm standard deviations of triple identical assays.

The vertical bar represented the standard deviation.

**P < 0.01 as compared with group 2. *P < 0.05 as compared with group 2.

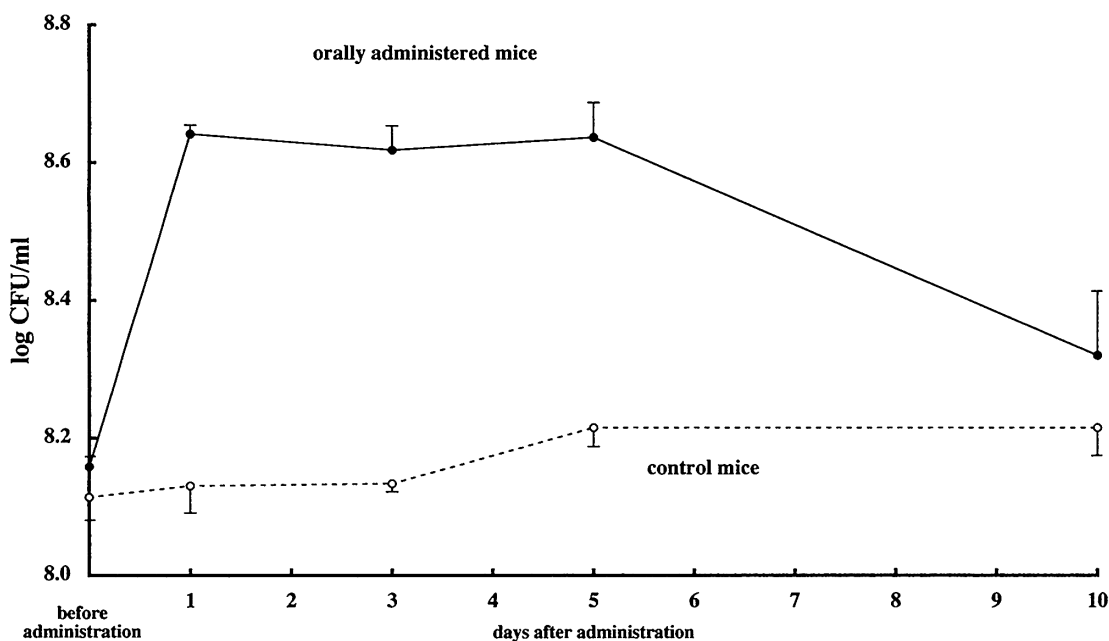


Fig. 3 Viable counts of lactic acid bacteria in feces of mice

carcinogens^{10,11}). Bacterial β -glucuronidase and β -glucosidase are also important in the generation of toxicants and carcinogens^{12,13}). β -Glucuronidase influences the enterohepatic circulation of carcinogenic conjugates¹⁴). Azoreductase, nitroreductase and β -glucuronidase are produced in large amounts by anaerobic bacteria such as *Clostridium bifermentans*

and aerobic bacteria such as *Escherichia coli*, whereas *Lactobacillus* strains produce small amounts of these enzymes¹⁵).

In this study, mice (control mice) in group 2 fed with diets of high protein and fat contents exhibited a significant increase in the fecal azoreductase and nitroreductase activities. Administration of fer-

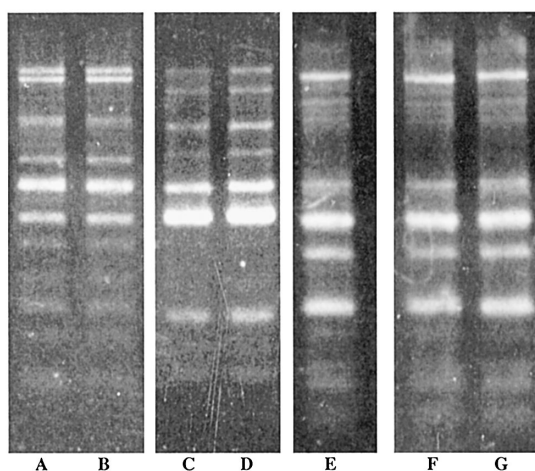


Plate 1 RAPD patterns of different strains of lactic acid bacteria in feces of mice

A, B : *Lactobacillus gasseri* JCM1130.

C, D : Lactobacilli 5 days after the termination of oral administration.

E : *Lactobacillus amylovorus* JCM 5811.

F, G : Lactobacilli 5 days after the termination of oral administration.

mented milk suppressed the high levels of azoreductase and nitroreductase activities caused by feeding of the high protein and fat diet. This result is in good agreement with the finding of GOLDIN *et al.*^{1,2)} that the rats fed with meat diets exhibited significantly higher levels of fecal azoreductase, nitroreductase and β -glucuronidase activities as compared with the rats fed with grain and that the supplementation with *L. acidophilus* significantly decreased the levels of fecal azoreductase and nitroreductase activities in meat-feeding rats. However, ROMOND *et al.*¹⁶⁾ showed that the consumption of cell-free concentrated whey from milk was not related to the decrease in the number of viable *Bacteroides fragilis* and Clostridia. These results revealed that the suppression of fecal azoreductase and nitroreductase activities was associated with a partial replacement of the flora with *Lactobacillus* strains given by the supplement of fermented milk.

In this study, the number of viable lactic acid bacteria in feces increased during the administration of fermented milk and the increase continued for 5 days after the termination of administration of fermented milk. The detection of *L. gasseri* JCM 1130 and *L. amylovorus* JCM 5811 demonstrated that the increase in the number of viable lactic acid bacteria in feces was associated with the administration of fermented milk. However, there was no statistically significant increase in the number of fecal lactic acid bacteria, and the number of the bacteria decreased 10 days after the

termination of administration of fermented milk. The result revealed that *L. gasseri* JCM 1130 and *L. amylovorus* JCM 5811, which exhibited high adhesional property to Caco-2 cells (human adenocarcinoma, ATCC HTB 37), did not colonize by adhesion to the intestinal tract of mice. It was considered that a slight increase in the number of fecal lactic acid bacteria was due to the high competition between the administered lactic acid bacteria and the pre-existing microflora or the replacement of the pre-existing lactic acid bacteria with the administered lactic acid bacteria.

TANNCOK¹⁷⁾ reported that transient microbes in the intestinal tract might exert beneficial effects without adhesion to the intestinal cells. The consumption of *L. acidophilus*¹⁸⁾ and yoghurt bacteria¹⁹⁾ has been reported to change the composition of microflora in the feces of humans and rats, respectively.

On the basis of these results, it is reasonable to conclude that viable lactic acid bacteria in fermented milk transfer to the intestinal tract and that if they do not colonize therein, transient lactic acid bacteria in the intestinal tract repress either the increase of the number of fecal microflora producing large amounts of azoreductase and nitroreductase or the production of these enzymes by fecal microflora. Our finding in mice cannot be directly applied to humans, but *L. gasseri* JCM 1130 and *L. amylovorus* JCM 5811 used for the preparation of fermented milk could exert beneficial effects on the intestinal environment because of their high adhesional property to Caco-2 cells.

References

- 1) GOLDIN, B. and GORBACH, S.L., 1977. Alternations in microflora enzymes related to diet, age, *Lactobacillus* supplements and dimethylhydrazine. *Cancer*, **40**, 2421-2426.
- 2) GOLDIN, B. and GORBACH, S.L., 1976. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. *J. Natl. Cancer Inst.*, **57**, 371-375.
- 3) BRATTON, C.A. and MARSHALL, E.K., 1939. A new coupling component for sulfanilamide determination. *J. Biol. Chem.*, **128**, 537-555.
- 4) HENG, Z., FENG, Q. and LI-HUANG, Z., 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res.*, **21**, 5279-5280.
- 5) NATALIA, A., NICKOLAI, O.B., WESTBLOM, T.U., STEPHEN, K. and DOUGLAS, E.B., 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res.*, **20**, 5137-5142.
- 6) MASON, R.P. and HOLTZMAN, J.L., 1975. The mechanisms of microsomal and mitochondrial nitroreductase electron resonance evidence for nitroaromatic free radical intermediates. *Biochemistry*, **14**, 1626-1636.
- 7) UEHLEKE, H., 1968. Extrahepatic microsomal drug metabolism. *Proc. Eur. Soc. Study Drug Toxicity*, **10**, 94-100.
- 8) GILLETTE, J.R., KAMM, J.J. and SUSAME, H.A., 1968. Mechanism of *p*-nitrobenzoate reduction in liver-The possible

- role of cytochrome P-450 in liver microsomes. *Mol. Pharmacol.*, **4**, 541-548.
- 9) KATO, R., OSHIMA, T. and TAKANAKA, A.L., 1968. Studies on the mechanism of nitro reduction by rat liver. *Mol. Pharmacol.*, **4**, 541-548.
- 10) MAGEE, P.N., 1971. Toxicity of nitrosoamines-Their possible human health hazards. *Food Cosmet. Toxicol.*, **9**, 207-219.
- 11) MILLER, J.A. and MILLER, E.C., 1969. The metabolic activation of carcinogenic aromatic amines and amides. *Prog. Exp. Tumor Res.*, **11**, 273-301.
- 12) CHIPMAN, J.K., 1982. Bile as a source of potential reactive metabolites. *Toxicology*, **25**, 99-111.
- 13) WILLIAMS, R.T., 1972. Toxicologic implications of biotransformation by intestinal microflora. *Toxicol. Appl. Pharmacol.*, **23**, 769-781.
- 14) TAKADA, H., HIROOKA, T., HIRAMATSU, Y. and YAMAMOTO, M., 1982. Effect of beta-glucuronidase inhibitor on azoxymethane-induced colonic carcinogenesis in rats. *Cancer Res.*, **42**, 331-334.
- 15) MCBAIN, A.J. and MACFARLANE, G.T., 1998. Ecological and physiological studies on large intestinal bacteria in relation to production of hydrolytic and reductive enzymes involved in formation of genotoxic metabolites. *J. Med. Microbiol.*, **47**, 407-416.
- 16) ROMOND, M.B., AIS, A., GUILLEMOT, F., BOUNOUADER, R., CORTOT, A. and ROMOND, C., 1998. Cell-free whey from milk fermented with *Bifidobacterium breve* C50 used to modify the colonic microflora of healthy subjects. *J. Dairy Sci.*, **81**, 1229-1235.
- 17) TANNCOK, G.W., 1997. Normal microbiota of the gastrointestinal tract of rodents. In *Gastrointestinal Microbiology*. Ed, I.R. WHITE, and A.E. ISAACSON, Chapman and Hall, New York, 187-215.
- 18) AYEBO, A.D., ANGELE, I.A. and SHAHANI, K.M., 1980. Effect of ingesting *Lactobacillus acidophilus* milk upon fecal flora and enzyme activity in humans. *Milch-wissenschaft*, **35**, 730-733.
- 19) DJOUZI, Z., ANDRIEUX, C., DEGIVRY, M.C., BOULEY, V. and SYLIT, O., 1997. The association of yogurt starters with *Lactobacillus casei* DN 114.001 in fermented milk alters the composition and metabolism of intestinal microflora in germ-free rats and in human flora-associated rats. *J. Nutr.*, **127**, 2260-2266.

発酵乳がマウス糞便中の発癌関連酵素活性に及ぼす影響

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要約：腸管内の有害微生物によって生成される糞便中のアゾレダクターゼ及びニトロレダクターゼは、発癌前駆物質を発癌物質に変換する作用がある。発酵乳の投与がマウス糞便中のこれらの酵素活性に及ぼす影響について調査した。発酵乳は、*Lactobacillus gasseri* JCM 1130 と *Lactobacillus amylovorus* JCM 5811 をスターター菌株として 1% 低脂肪乳に加えて製造した。これら 2 つの酵素活性はマウスに高蛋白・高脂肪食を与えることにより高くなったが、発酵乳を投与することで非投与群と比較し、有意に低下した。さらに、マウス糞便中の乳酸菌数は、発酵乳の経口投与により増加し、経口投与終了後でも数日間維持された。これら糞便中に供試乳酸菌株が含まれることを Random amplified polymorphic DNA fingerprinting (RAPD) 法によって確認した。

キーワード：乳酸菌，糞便，発ガン関連酵素，アゾンレダクターゼ，ニトロレダクターゼ

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