Lactobacillus fermentum ATCC9338: Effects on Mouse Intestinal Flora and Plasma Concentration of Isoflavonoids

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The effects of Lactobacillus fermentum ATCC9338 on the metabolism of daidzein and on the composition of intestinal flora were assessed for adult mice. Lactobacillus fermentum ATCC9338 altered the equol production status in the in vitro incubation of daidzein with fecal flora of mice. For in vivo investigation, mice were fed an AIN-93M purified diet for 19 days followed by a 0.05% daidzein diet for 4 days in the in vivo experiment. The LD group received L. fermentum for 10 days. The control (CD) group did not receive lactic acid bacteria. In analysis of plasma isoflavonoids, plasma equol concentration tended to be high in the LD groups. However, no significant differences in plasma daidzein and equol concentrations were observed between the LD and CD groups. In analysis of the composition of intestinal flora, the occupation ratio of Lactobacillales was significantly higher in the LD group. We demonstrated that Lactobacillus fermentum ATCC9338 could affect the intestinal flora and in vitro fecal equol production in mice.

Keywords: Lactobacillus fermentum, intestinal flora, daidzein, equol, mouse

Introduction

The health benefits of soy-based foods, which have attracted much attention, have been largely attributed to isoflavones. Human metabolism and excretion of isoflavones following the consumption of soy products show considerable variation (Kelly et al., 1995; Xu et al. 1995). Equol is a bacterial metabolite of the widespread isoflavone daidzein (Bowey et al., 2003). These isoflavonoids are estrogenic compounds (phytoestrogens). In animals, phytoestrogens exert estrogenic effects on the central nervous system, induce estrus, and stimulate the growth of the female genital tract (Lieberman, 1996). Investigation of the absorption and metabolism of isoflavonoids is essential for understanding their biological activity.

Intestinal flora play key roles in the metabolism and bioavailability of isoflavones (Setchell et al., 1984). Infants fed soy-containing infant formula in the first four months of life (when gut microflora are underdeveloped) cannot produce large quantities of equol (Cruz et al., 1994; Setchell et al., 1997). Furthermore, in some studies, only 30% to 40% of adult subjects excreted significant quantities of equol after isoflavone consumption (Lampe et al., 1998; Setchell et al., 1984). Probiotics have been defined as live microbial food supplements that benefit the recipient host by improving the intestinal microbial balance (Fuller, 1989). It has also been reported that probiotics can potentially enhance the calcium bioavailability of calcium-fortified soymilk due to increased calcium solubility and bioactive isoflavone aglycone enrichment (Tang et al., 2007). Oteino et al. (2007) suggested that beta-glucosidase from some probiotics may have much potential in the development of various aglycone-rich functional soy beverages. Probiotics containing dietary isoflavones seem to be important food supplements. Since lactic acid bacteria have also been used in probiotics, it is important to estimate the effects of lactic acid bacteria on the metabolism of isoflavonoids. However, few reports have focused on the effects of lactic acid bacteria on mouse intestinal flora and isoflavonoids in the plasma. Therefore, the aim of the present study was to investigate these effects in mice.

Materials and methods

Materials The equol used as a standard for HPLC analysis was purchased from LC Laboratories (Woburn, MA, USA). The daidzein used as a diet supplement or a standard for HPLC analysis was also purchased from LC Laboratories.

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The purity of daidzein was more than 99%. *Lactobacillus fermentum* ATCC9338 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

**Effects of *L. fermentum* ATCC9338 on the in vitro incubation of daidzein and fecal flora of mice** Five-week-old male Crj: CD-1 (ICR) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in suspended stainless-steel cages with wire mesh bottoms in a room kept at a temperature of 24 ± 0.5°C and a relative humidity of 65% with alternating 12-h periods of light and dark. They were fed an MF pelleted diet (Oriental Yeast Co., Ltd., Tokyo, Japan) for 3 weeks. The diet was then replaced with an AIN-93M diet, which the mice received for 10 weeks (Table 1). Freshly voided feces of three mice were collected in sterile glass homogenizers and homogenized with 30 volumes of sterile, anaerobic medium (37 g brain heart infusion, 1 g agar, 0.5 g L-cysteine HCl, and 4 g Na₂CO₃ in 1L distilled water). Aliquots of medium (9 mL) were distributed into test tubes, gassed with O₂-free CO₂ gas, sealed with a butyl rubber stopper, and sterilised by autoclaving. Thirty volumes of the anaerobic medium were added to the feces, and the mixture was homogenised. 1μL of the daidzein solution (20 mg of daidzein was dissolved in 1 mL DMSO) was transferred into 0.2 mL of homogenate. In the control incubation, 10μL of the anaerobic medium was added to the reaction mixture. In the incubation with *L. fermentum* ATCC9338, the bacteria were pre-incubated for 24 h on MRS (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) agar plates, suspended in the anaerobic medium, adjusted to a concentration of 10¹⁰/mL, and used in 10μL aliquots for inoculation. All samples were incubated anaerobically at 37°C for 24 h. Then, the reaction mixture was treated with four volumes of methanol/acetic acid (100/5, v/v), vortexed for 120 s, and centrifuged at 5000 g for 10 min. The supernatant was transferred to a test tube and filtered through a 0.2-μm filter. HPLC analysis was performed by injecting 20μL of each preparation onto a 250×4.6 mm Capcell Pak C18-5 micron column type MG (Shiseido, Tokyo, Japan). To detect isoflavonoids, a JASCO MD-1515 photodiode array detector (JASCO Co., Ltd., Tokyo, Japan) was used to monitor the spectral data from 200 to 400 nm for each peak. To measure the isoflavonoids, we used daidzein and equol as standard samples. We used the spectral data of 254 nm of daidzein and 280 nm of equol. The mobile phase consisted of methanol/acetic acid/water (50:0.5:49.5, v/v/v). The HPLC running conditions were a column temperature of 40°C and a flow rate of 1 mL/min.

**Treatment of animals with *L. fermentum*** Five-week-old male Crj: CD-1 (ICR) mice were purchased from Charles River Japan, Inc. The animals were housed in suspended stainless-steel cages with wire mesh bottoms in a room kept at a temperature of 24 ± 0.5°C and a relative humidity of 65% with alternating 12 h periods of light and dark. They were fed an AIN-93M purified diet (Reeves, 1993) for 13 weeks. The purity of daidzein was more than 99%. *Lactobacillus fermentum* ATCC9338 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>AIN-93M (g/kg diet)</th>
<th>0.05% daidzein (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>465.692</td>
<td>465.692</td>
</tr>
<tr>
<td>Casein</td>
<td>140.0</td>
<td>140.0</td>
</tr>
<tr>
<td>α-Cornstarch</td>
<td>155.0</td>
<td>154.5</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>100.0</td>
</tr>
<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Cellulose</td>
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<td>50.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>(AIN-93M-Mix)</td>
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<td></td>
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<tr>
<td>Vitamin mix</td>
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<td>10.0</td>
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<tr>
<td>(AIN-93-Mix)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Cystine</td>
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<td>1.8</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Daidzeina</td>
<td>-</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Daidzein was purchased from LC laboratories (Woburn, MA, USA).*
days, and then randomly divided into two groups of seven animals each. All mice were fed an AIN-93M diet for six days; then the diet was replaced with an 0.05% daidzein diet (Table 1), which the mice received for four days. All mice were pair-fed. *L. fermentum* ATCC9338 was pre-incubated on MRS agar plates for 24 h at 37°C in an anaerobic atmosphere generated with the use of AnaeroPack® (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) and transferred by inoculating loop to 2 mL of physiological saline solution. The LD group received *L. fermentum*. The CD group (control) did not receive lactic acid bacteria. The LD group received 0.2 mL of *L. fermentum* (10⁶ CFU/0.2 mL) via a stomach tube for 10 days; dissection was performed on the following day. The CD group received 0.2 mL of physiological saline solution via a stomach tube, under the same dietary and living conditions. After the experimental diet feeding period, the mice were sacrificed, and blood and cecal contents were collected. The blood samples were then centrifuged, the plasma was stored at -80°C until HPLC analysis for isoflavonoids. Cecal contents were stored at -80°C until T-RFLP analysis.

This study was carried out in accordance with the “Guidelines for Animal Care and Experimentation” of the National Food Research Institute.

**Analysis of plasma isoflavonoids** Plasma isoflavones were analyzed as follows. A total of 200 μL plasma was added to 200 μL of beta-glucuronidase type H-5 solution (35 mg/mL, Sigma, MO, USA) in 0.2 M sodium acetate buffer (pH 5.0). Next, the mixture was incubated at 37°C in a shaking water bath for 2 h, followed by treatment with 3600 μL of methanol/acetic acid (100/5, v/v), vortexed for 120 s, and centrifugation at 5000 × g for 10 min at 4°C. The supernatants were transferred to an eggplant-type flask and evaporated to dryness by rotary evaporation. The sample was then dissolved with 400 μL of 80% methanol, which is a volume equivalent to the original plasma, and filtrated through a 0.2-μm filter. During HPLC analysis, 20 μL of each preparation was injected into a 250×4.6 mm Capcell Pak C18-5μ column-type MG (Shiseido). HPLC analysis was performed as described above.

**DNA extraction from cecal contents** DNA was extracted from cecal contents according to the Matsuki’s methods (2006). Cecal samples (20 mg) were washed three times by suspending them in 1.0 mL of phosphate-buffered saline and centrifuging each preparation at 14,000 × g in order to remove possible PCR inhibitors. The cecal pellets were resuspended in a solution containing 250 μL of extraction buffer (200 mM Tris-HCl, 80 mM EDTA; pH 9.0) and 50 μL of 10% sodium dodecyl sulphate. Glass beads (300 mg; diameter, 0.1 mm) and 500 μL of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 60 s using a Mini Bead-Beater (BioSpec Products Inc., Bartlesville, OK, USA) at a power level of 4800 rpm. After centrifugation at 14,000 × g for 5 min, 400 μL of the supernatant was collected. Subsequently, phenol-chloroform-isoamyl alcohol extractions were performed, and 250 μL of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1 mL of Tris-EDTA buffer. The DNA preparation was adjusted to a final concentration of 10 μg/mL in TE and checked by 1.5% agarose gel electrophoresis.

**PCR conditions and restriction enzyme digestion** The PCR mixture (25 μL) was composed of EX Taq buffer, 2 mM Mg²⁺, 200 μM each deoxynucleoside triphosphate, 10 ng cecal DNA. Primers 5’ HEX-labeled 516f (5’-TGCCACG-CAGCCGCGGTA-3’) and 1510r (5’-GGTTACCTTGT-TACGACTT-3’) at a concentration of 0.10 μM, and 0.625 U of TaKaRa EX Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). Amplification was carried out using the Dice PCR System (Takara Bio Inc.) with one cycle of 95°C for 15 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and finally one cycle at 72°C for 10 min. The amplification products were subjected to gel electrophoresis in 1.5% agarose followed by ethidium bromide staining. The PCR products were purified using QIAquick spin columns (Qiagen KK, Tokyo, Japan) according to the manufacturer’s instructions and treated with 2 U of Bsl I (New England Biolabs) for 3 h, at 55°C (Nagashima et al., 2003).

**T-RFLP analysis** Fluorescently labeled T-RFs (terminal restriction fragments) were analyzed by electrophoresis on an ABI PRISM 310 Genetic Analyzer automated sequence analyzer (Applied Biosystems) in GeneScan mode. Restriction enzyme digestion mixture (2 μL) was mixed with 0.5 μL of MapMarker 1000 size standard (BioVentures, Inc.) and 12 μL of deionized formamide, followed by denaturation at 96°C for 2 min and immediate chilling on ice. The injection time was 30 s for analysis of T-RFs from the digestion with Bsl I. The run time was 40 min. The lengths and peak areas of T-RFs were determined with the GeneMapper software. From the predominant operational taxonomic units (OTUs, which correspond to either T-RFs or T-RF clusters) that were detected in the T-RFLP profiles from the cecal contents were applied to the phylogenetic groups of intestinal flora (Nagashima et al., 2003).

**Statistics** The data in Figure 1 are expressed as means. The data in Figures 2 and 3 are expressed as means ± SE. Data in Figures 2 and 3 were analysed using SigmaStat for Windows (Jandel Corporation, San Rafael, CA, USA) and t-test analysis.
**Results**

**Effects of L. fermentum ATCC9338 on in vitro incubation of daidzein with fecal flora of mice**  
The in vitro incubation of daidzein with fecal flora of mice showed higher equol concentrations for feces supplemented with *L. fermentum* ATCC9338 than in feces without any addition of bacteria (Fig. 1). However, daidzein concentrations were lower in the feces supplemented with *L. fermentum* ATCC9338 (Fig. 1). Equol concentration in the fecal suspension supplemented with *L. fermentum* ATCC9338 was 2.0 times higher than that in the control fecal suspension.

**Effects of L. fermentum ATCC9338 on in vivo test**  
No significant differences in final body weight (g), food consumption (g/day), or cecal contents were observed between the CD and LD groups. No significant differences in plasma daidzein concentrations were observed between the CD and LD groups (Fig. 2). Plasma equol concentrations tended to be high in the LD groups; however, no significant differences in plasma equol concentrations were observed between the two groups (Fig. 2).

**Effects of *L. fermentum* on cecal flora of mice**  
It has been shown that human intestinal microbiota are predominantly comprised of the members of approximately ten phylogenetic bacterial groups and that these bacterial groups can be distinguished by T-RFLP system developed by Nagashima *et al.* (2003, 2006). Figure 3 depicts the compositions of the cecal flora, which differed between the two dietary groups. The occupation ratio of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTUs 919) tended to be high in the LD group ($P=0.08$). The occupation ratio of *Bacteroides, Clostridium* cluster IV (OTUs 370) tended to be low in the CD group ($P=0.132$). The occupation ratio of *Lactobacillales* was significantly higher in the LD group. No significant differences in the occupation ratio of *Bifidobacterium* and *Prevotella* were observed between the two groups.

**Discussion**

Daidzein, a major component of the isoflavones, is metabolized to equol by intestinal bacterial flora (Bowey *et al.*, 2003). Both compounds have an estrogenic effect and may help protect against cardiovascular disease. In the present study, we investigated the effects of *L. fermentum* ATCC9338 on mouse intestinal flora and isoflavonoids in the plasma. The in vitro incubation of daidzein with fecal flora of mice showed higher equol concentrations in feces supplemented with *L. fermentum* ATCC9338 than in the feces with no addition of bacteria. It has been reported that administration of equol-producing bacteria alters the equol production status in the Simulator of the Gastrointestinal Microbial Ecosystem (SHIME) (Decroos *et al.*, 2006). In this report, bacteria-related equol production changed the intestinal microflora from equol non-producing status to equol-producing status by changing the total metabolic activity of isoflavonoids. We produced higher equol production by adding *L. fermentum* to fecal flora of the mice in the in vitro study. *L. fermentum* could not produce equol from daidzein in vitro. However, Decroos *et al.* (2006) reported that the equol production status of the SHIME could be changed with the addition of equol-producing bacteria. *L. fermentum* might affect the in-

![Fig. 1. Effects of Lactobacillus fermentum ATCC9338 on in vitro incubation of daidzein with fecal flora of mice. LD: The fecal suspension supplemented with L. fermentum ATCC9338 CD: Control; fecal suspension supplemented with anaerobic medium.](image1)

![Fig. 2. Plasma isoflavonoids (aglycones+metabolites) of the mice in the LD and CD groups. Values are means±SE. (n=7)](image2)
Effects of *L. fermentum* on microflora and isoflavonoids

The numbers of lactobacilli might be too small to increase equol production in vivo. To achieve increased equol production in vivo, a large increase in lactobacilli within the gut microflora would be required. The occupation ratio of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTUs 919) tended to be high in the LD group (P<0.01). The *Bacteroides, Clostridium* cluster IV (OTUs 370) tended to be low in the CD group. Many kinds of intestinal bacteria live in the microecology of the gut and affect each other by competing for nutrition. *L. fermentum* might affect the intestinal flora by changing the environment of equol production in the intestinal flora.

It has been reported that administration of *L. gasseri* to mice reduces plasma equol concentration and fecal equol production in vitro (Tamura et al., 2004). It has also been reported that 2-month intervention with probiotic capsules did not significantly alter equol excretion, plasma hormones, or leptin concentrations in subjects (McMullen et al., 2006). In our examination, lactic acid bacteria did not reduce the plasma equol concentrations. Inter-individual variation of the effects of lactic acid bacteria on equol production by intestinal microflora has been reported. Intestinal bacterial composition may influence bone mineral density (BMD) in postmenopausal women who regularly consume soy (Frankenfeld et al., 2006). It seems important to provide lactic acid bacteria that can produce an increase in equol production in vivo. To achieve increased equol production in vivo a large increase in lactobacilli within the gut microflora would be required. The occupation ratio of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTUs 919) tended to be high in the LD group (P<0.01). The *Bacteroides, Clostridium* cluster IV (OTUs 370) (P=0.132) tended to be low in the CD group. Many kinds of intestinal bacteria live in the microecology of the gut and affect each other by competing for nutrition. *L. fermentum* might affect the intestinal flora by changing the environment of the gut.

In summary, we demonstrated that *Lactobacillus fermentum* ATCC9338 could affect the intestinal flora and in vitro fecal equol production in mice.

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**References**


