

***Lactobacillus gasseri*: effects on mouse intestinal flora enzyme activity and isoflavonoids in the caecum and plasma**

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(Received 27 April 2004 – Revised 23 July 2004 – Accepted 3 August 2004)

The effects of *Lactobacillus gasseri* JCM 1131^T on isoflavonoid levels within the caecum and plasma were assessed in adult mice. Male 5-week-old mice were fed an AIN 93M diet for 30 d. Two groups of mice were administered either *L. gasseri* JCM 1131^T (the LGI group) or physiological saline solution (the control (CI) group) daily for 5 d before dissection. The plasma daidzein concentration was significantly higher in the LGI group, however, their plasma equol concentration was significantly less than in the CI group. The total amount of equol present as aglycone in the caecum was significantly greater in the CI group, but there was no significant difference in the total daidzein present as caecal aglycone. In an *in vitro* incubation of daidzin with the faecal flora of mice, the equol concentration was significantly higher in the CI group. The numbers of lactobacilli present were significantly higher in the LGI group. The present data suggest that the administration of *L. gasseri* is likely to influence the effect of isoflavonoids on the host via changes in the gastrointestinal environment.

Daidzein: Equol: *Lactobacillus gasseri*: Caecal enzyme activity

Phyto-oestrogens are defined as compounds that exert oestrogenic effects on the central nervous system, induce oestrus, and stimulate growth of the genital tract in female animals (Lieberman, 1996). Plant phyto-oestrogens include the soya isoflavones, which in addition to their oestrogenic effects may be partially responsible for the protective effects of soya against CVD (Anthony *et al.* 1998). Daidzin is a major component of these isoflavones. Daidzin is the 7-glucoside form of daidzein. Daidzein is metabolised to equol by the intestinal bacterial flora (Bowey *et al.* 2003). Equol is considerably more oestrogenic than daidzein (Shutt & Cox, 1972).

The intestinal flora plays a key role in the metabolism and bioavailability of isoflavones (Setchell *et al.* 1984). As such, infants fed soya-containing infant formula in the first 4 months of life (when gut microflora are underdeveloped) cannot produce large quantities of equol (Cruz *et al.* 1994; Setchell *et al.* 1997). Furthermore, only about 30–40% of adult subjects excrete significant quantities of equol after isoflavone consumption (Setchell *et al.* 1984; Lampe *et al.* 1998). Probiotics have been defined as live microbial food supplements which benefit the recipient host by improving the intestinal microbial balance (Fuller, 1989). Lactic acid bacteria have been used as probiotics with the aim of managing intestinal disorders such as lactose intolerance, acute rotavirus gastroenteritis (and other enteric pathogens), the adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease and food

allergy (Salminen *et al.* 1988, 1996; Gilliland, 1990; Iso-lauri *et al.* 1991; Hammes & Tichaczek, 1994).

Lactobacillus gasseri, in the *L. acidophilus* group, is a major species of the human faecal flora. It has been reported that the *L. gasseri* strain, isolated from infant faeces, shows wide inhibitory activity against some pathogenic and food-spoilage species, including *Clostridium*, *Listeria* and *Enterococcus* (Zhu *et al.* 2000). *L. gasseri* may affect the intestinal flora and hence the metabolism of isoflavonoids within the digestive tract. However, there are few reports of the effects of *L. gasseri* on caecal and plasma isoflavonoids, or on caecal enzyme activity. Therefore, the aim of the present study was to investigate these effects in mice.

Materials and methods

Materials

A soyabean extract with a high isoflavone–glucoside content was kindly provided by Fuji Oil Co., Ltd. (Osaka, Japan). It contained 31.1% (w/w) daidzin, 9.6% (w/w) genistin and 38.5% (w/w) gycitin. Daidzein used as a standard for HPLC analysis was purchased from Fujicco (Kobe, Japan), as was daidzin. Equol was purchased from Extrasynthese (Genay, France). β -Glucuronidase type H-5, *p*-nitrophenyl- β -D-glucopyranoside and *p*-nitrophenyl- β -D-glucuronide were obtained from Sigma,

Abbreviations: CI group, control group; LGI group, *Lactobacillus gasseri* group.

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Aldrich Corp. (St Louis, MO, USA). *L. gasseri* JCM 1131^T (type strain) was obtained from the Institute of Physical and Chemical Research (RIKEN, Wako-shi, Saitama, Japan).

Treatment of animals with Lactobacillus gasseri

Male Crj: CD-1 (ICR) mice (5 weeks old) 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 $24 \pm 0.5^\circ\text{C}$ and a relative humidity of 65%, with alternating 12 h periods of light and dark. They were fed an AIN-93M purified diet (Reeves *et al.* 1993) for 30 d. The *L. gasseri* JCM 1131^T type strain was incubated on EG agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) for 2 d at 37°C in an anaerobic atmosphere generated using an AnaeroPack[®] (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). An inoculating loop of the bacteria was then suspended in 2 ml physiological saline solution.

The mice were randomly divided into two groups of seven animals each. The mice had been fed the AIN 93M diet for 30 d. One group received *L. gasseri* (the *L. gasseri* group; LGI group) and one did not (control group; CI group). A 0.2 ml dose of *L. gasseri* (10^9 colony-forming units/0.2 ml) was administered via a stomach tube to the LGI group of mice for 5 d. The mice in the CI group were administered 0.2 ml physiological saline solution via a stomach tube, under the same dietary and living conditions.

At 5 d before the administration of *L. gasseri* (or physiological saline), mouse faeces were collected and immediately processed for bacteriology. On the fifth day after the administration of *L. gasseri* (or physiological saline) mouse faeces were again collected and a faecal incubation test with daidzin was performed. During the night before dissection, a soyabean extract with a high isoflavone–glucoside content (136 mg; 31.1% (w/w) daidzin, 9.6% (w/w) genistin and 38.5% (w/w) glycitin) was freshly prepared as a 3 ml suspension in water. A 0.2 ml dose was administered to the mouse via a stomach tube. At 12 h later, all mice were killed and dissected. The mice had unrestricted access to food throughout the experiment. The mice were then anaesthetised with diethyl ether and blood samples were taken from the abdominal aorta and placed in heparinised tubes. The plasma was separated from whole blood by centrifugation and stored at -80°C before HPLC analysis for isoflavonoids. Caecal contents were also collected and stored at -80°C until caecal enzyme activity and caecal isoflavonoids content were measured. The present study was carried out in accordance with the 'Guidelines for Animal Care and Experimentation' of the National Food Research Institute.

Analysis of plasma and caecal isoflavonoids

The analysis of plasma isoflavonoids was performed as follows. Plasma (200 μl) was added to 200 μl β -glucuronidase type H-5 solution (35 mg/ml; Sigma-Aldrich Corp., St Louis, 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 methanol–acetic acid (100:5, v/v), vortexing for 30 s, sonication for 30 s, vortexing again for 30 s and centrifugation at 5000 g for 10 min at 4°C . The supernatant fractions were transferred to an eggplant-type flask and evaporated completely using a rotary evaporator. The sample was then dissolved within the mobile phase of the HPLC system in 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 C18-5 μm column (Shiseido, Tokyo, Japan). Isoflavonoids were detected using an ECD with a guard cell (model 5020), and an analytical cell (model 5010) (Coulchem II; ESA Inc., Bedford, MA, USA). The mobile phase consisted of methanol–acetic acid–water (28:5:67, by vol.). The running conditions for HPLC were as follows: column temperature, 40°C ; flow rate, 1 ml/min; guard cell, +850 mV; analytical cell, +300 mV for electrode 1 and +800 mV for electrode 2. Electrochemical data were collected from electrode 2. To perform the analysis of caecal isoflavonoid aglycones, caecal contents were treated with 2 ml methanol–acetic acid (100:5, v/v), vortexed for 30 s, sonicated for 30 s, vortexed again for 30 s, and centrifuged at 5000 g for 10 min. The supernatant fraction was then transferred to the eggplant-type flask. The same volume as the first extraction of methanol–acetic acid (100:5, v/v) was added to the sediment, and the procedure was repeated. The supernatant fractions from both extractions were pooled and evaporated completely using a rotary evaporator. Then, the sample was dissolved within 3 ml of the mobile phase of the HPLC system and filtrated through a 0.2 μm filter. We used the same HPLC analytical method as described earlier.

Measurement of enzyme activity

We measured enzyme activities as previously described (Rowland *et al.* 1983). A 1:100 caecal suspension was prepared in 0.1 M-phosphate buffer (pH 7.0) and non-bacterial debris was removed by centrifugation at 700 g for 2 min. The supernatant fraction was used immediately in the β -glucosidase and β -glucuronidase assays. β -Glucosidase activity was measured with *p*-nitrophenyl- β -D-glucopyranoside as the substrate. β -Glucuronidase activity was measured with *p*-nitrophenyl- β -D-glucuronide as the substrate. β -Glucosidase and β -glucuronidase activities were expressed as μmol *p*-nitrophenol liberated/60 min per g wet weight caecal contents.

Bacteriology

Faeces were weighed, serial 10-fold dilutions were prepared and then homogenised using a sterile glass homogeniser. The diluted samples were spread on the surface of LBS agar media (Becton Dickinson and Company, Sparks, MD, USA) and incubated at 37°C in an anaerobic atmosphere generated using an AnaeroPack[®] (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). Bacteria were identified to the level of genus on the basis of colony form, Gram stain and cell morphology. Bacterial numbers were expressed as \log_{10} colony-forming units/g wet weight faeces.

In vitro incubation of daidzin with faecal flora of mice

Freshly voided mouse faeces were collected in sterile glass homogenizers. Fifty volumes of the anaerobic broth were added to the faeces, and the mixture was homogenized. Daidzin (20 mg) was dissolved in 1 ml dimethyl sulfoxide. Daidzin solution (3 μ l) was transferred into 0.3 ml homogenate then incubated anaerobically at 37°C for 24 h. After incubation, the reaction mixture was treated with nine volumes of methanol–acetic acid (100:5, v/v). The mixture was vortexed for 30 s, sonicated for 30 s, vortexed again for 30 s and centrifuged at 5000 *g* for 10 min. The supernatant fraction was transferred to a test-tube and filtered through a 0.2 μ m filter. HPLC analysis was performed by injecting 20 μ l of each preparation into a 250 \times 4.6 mm Capcell Pak C18-5 μ m column (Shiseido, Tokyo, Japan). The anaerobic broth used in this experiment was prepared as follows. Brain–heart infusion (37 g), agar (1 g), L-cysteine HCl (0.5 g), and Na₂CO₃ (4 g) were dissolved in 1000 ml distilled water. Samples of broth (9 ml) were distributed into test-tubes, gassed with O₂-free CO₂ gas, sealed with a butyl rubber stopper, and sterilised by autoclaving. HPLC analysis was performed as described earlier.

Statistics

The data are expressed as mean values with their standard errors. Data were analysed using SigmaStat for windows (Jandel Corporation, San Rafael, CA, USA) and *t* test analysis.

Results

Body weight, food consumption, and caecal contents

No significant differences in final body weight (g) were observed between the LGI (38.4 (SE 0.9)) and the CI (37.3 (SE 1.0)) groups. No significant differences in food consumption (g/d) were observed between the LGI (4.8 (SE 0.1)) and the CI (4.8 (SE 0.1)) groups. There were no significant differences in the caecal contents between the LGI and CI groups.

Plasma isoflavonoids and caecal isoflavonoid aglycones

The plasma daidzein concentration was significantly higher in the LGI group compared with the CI group (Fig. 1). Conversely, the plasma equol concentration was significantly lower in the LGI group (Fig. 1). There was a significant difference in the plasma equol:plasma daidzein ratio between the LGI and CI groups. The plasma equol:plasma daidzein ratio in the LGI group (4.3 (SE 2.6)) was significantly lower compared with the CI group (19.7 (SE 4.9)) ($P < 0.05$). The total amount of equol present as aglycone in the caecum was significantly higher in the CI group compared with the LGI group (Fig. 2). However, there was no significant difference in the total amount of daidzein present as aglycone in the caecum (Fig. 2). The ratio of the total amount of daidzein present as aglycone in the caecum to the total amounts of equol present as aglycone in the caecum tended to be high in the CI

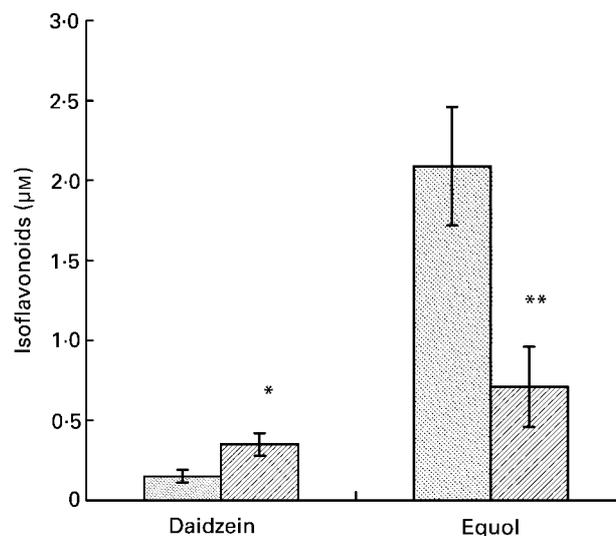


Fig. 1. Plasma isoflavonoids (aglycones + metabolites) of the mice in the control group (□) and the *Lactobacillus gasseri* (LGI) group (▨). Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that for the CI group ($P < 0.05$). **Mean value was significantly different from that for the CI group ($P < 0.01$).

group (6.3 (SE 3.5)) compared with the LGI group (1.1 (SE 0.5)). However, no significant difference was observed in these ratios between the LGI and CI groups ($P = 0.158$).

Caecal enzyme activity

No significant differences in caecal β -glucosidase and β -glucuronidase activities were detected between the LGI and CI groups (Fig. 3).

Measurement of numbers of lactobacilli

Significant differences were observed in the numbers of lactobacilli between the two groups. There were significantly higher numbers in the LGI group (Fig. 4).

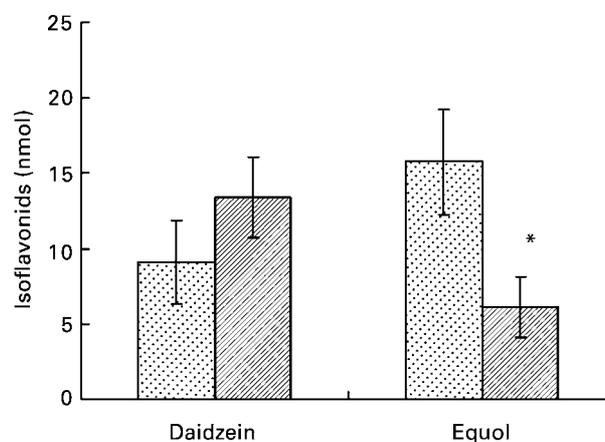


Fig. 2. Total amounts of isoflavonoids present as aglycone in the caecum of the mice in the control group (□) and the *Lactobacillus gasseri* group (▨). Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that for the CI group ($P < 0.05$).

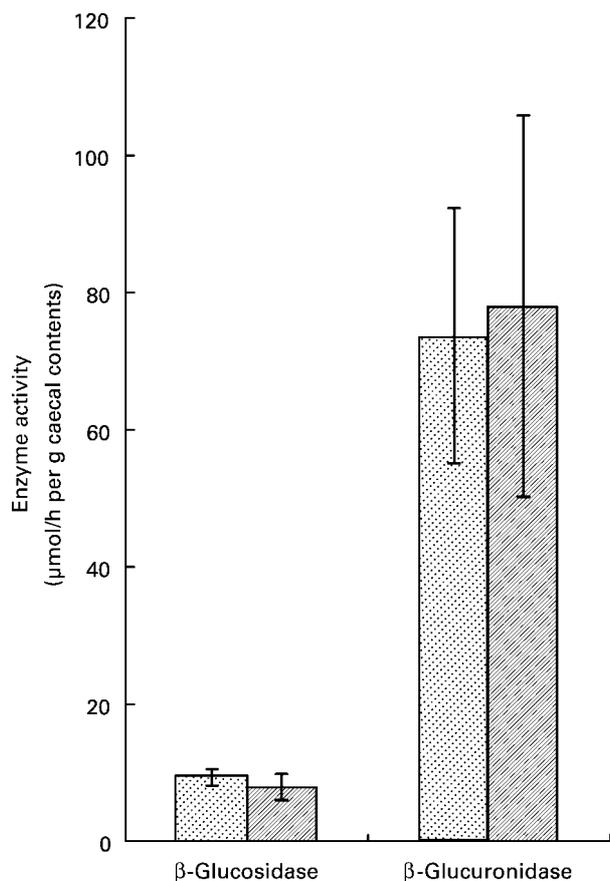


Fig. 3. Caecal β -glucosidase and β -glucuronidase activities of the mice in the control group (▨) and the *Lactobacillus gasseri* group (▩). Values are means, with their standard errors represented by vertical bars.

In vitro incubation of daidzin with the faecal flora of mice

There was a significant difference in equol concentration between the two groups, with the CI group having a

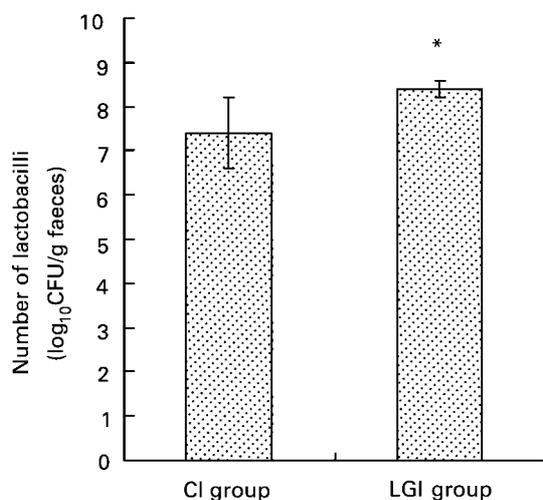


Fig. 4. Number of lactobacilli (\log_{10} colony-forming units (CFU)/g wet weight faeces) of the mice in the *Lactobacillus gasseri* (LGI) group and the control (CI) group. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that for the CI group ($P < 0.05$).

higher concentration. The average equol concentration in the CI group was ten times higher compared with the LGI group (Fig. 5).

Discussion

Daidzein is a major component of the isoflavones, and it is metabolised to equol by the intestinal bacterial flora (Bowey *et al.* 2003). Both compounds have an oestrogenic effect and may contribute to protection against CVD. When used as a probiotic, *L. gasseri* can affect the intestinal flora and thus influence isoflavonoid metabolism in the digestive tract. In the present study we investigated the effects of *L. gasseri* on caecal and plasma isoflavonoids and caecal enzyme activity in mice.

Among mice that were administered *L. gasseri* (LGI group), the equol concentration in the plasma was significantly lower than in untreated mice (CI group). However, the plasma daidzein concentrations were significantly higher in the LGI group. This may be because high concentrations of daidzin (the glucoside of daidzein) were present in the soyabean extract included in the diet of the mice. It has been reported that absorption of daidzin is lower than daidzein in human subjects (Izumi *et al.* 2000). Setchell *et al.* (2002b) reported that daidzein was absorbed as the aglycone form after hydrolysis of the sugar moieties. There was a significant difference in the plasma equol:plasma daidzein ratio between the LGI and CI groups. The plasma equol:plasma daidzein ratio in the CI group was significantly higher compared with that in the LGI group. However, no significant difference in the ratio of the total amount of daidzein present as aglycone in the caecum to the total amounts of equol present as aglycone

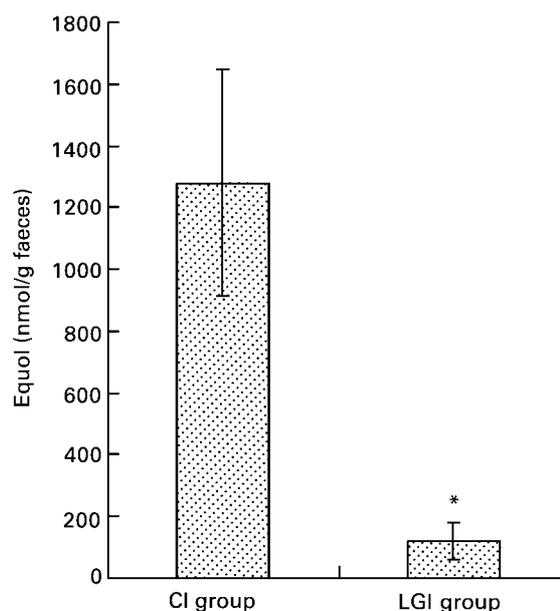


Fig. 5. Amounts of equol (nmol/g wet weight faeces) of the mice in the *Lactobacillus gasseri* (LGI) group and the control (CI) group in an *in vitro* incubation of daidzin with the faecal flora of mice. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that for the CI group ($P < 0.05$).

in the caecum was observed between the LGI and CI groups. It has been reported that the slower plasma clearance of equol is striking compared with its precursor, daidzein (Setchell *et al.* 2002a). A significant difference of plasma clearance between equol and daidzein might contribute to the difference of the equol:daidzein ratio between the plasma and caecal contents.

The lactobacilli can survive long periods in mucin (Sims, 1964). Most lactobacilli have been shown to adhere via binding to collagen type I (Aleljung *et al.* 1991). We showed that the *L. gasseri* strain used in the present experiment possesses β -glucosidase activity *in vitro* (data not shown). β -Glucosidase from the *L. gasseri* in the upper gastrointestinal tract could contribute to the higher plasma daidzein concentrations found in the LGI group of mice by hydrolysing the daidzin to daidzein. No significant differences were observed in the present experiments in the caecal β -glucosidase and β -glucuronidase activities of the two groups. However, it has been reported that *L. gasseri* administration actually reduces faecal β -glucuronidase activities (Pedrosa *et al.* 1995) in human subjects. Tamura *et al.* (1996) reported that some strains of *Bacteroides* and *Clostridium* have β -glucosidase and β -glucuronidase activities *in vitro*. The inconsistent reports of *L. gasseri* effects on β -glucuronidase activity in the gastrointestinal tract might be due in part to differences in the compositions of human and mouse intestinal flora.

The numbers of lactobacilli were significantly greater in the LGI group than in the CI group. Saxelin *et al.* (1991) demonstrated that all human volunteers were colonized following the administration of *L. casei* strain GG after only 4 d of treatment. Lactobacilli shedding also increased after only 4 d of treatment in the present study (although we did not identify the lactobacilli in the mouse faeces to the species level).

The present study is the first to demonstrate the effects of *L. gasseri* on faecal floral equol production. Equol is a bacterial metabolite derived from daidzein (Bowey *et al.* 2003) and it has been reported that equol excretion in human subjects varies among individuals (Rowland *et al.* 2000). We found that in the CI group, which was not fed lactobacilli, both the plasma equol concentration and the total amount of equol present as aglycone in the caecal contents were significantly higher compared with those which were administered lactobacilli. When daidzein was incubated *in vitro* with mouse faecal flora, the concentrations of equol produced were again significantly higher in the CI group. This result suggests that equol production of the faecal flora was inherently greater in the CI group and that *L. gasseri* may suppress its production.

Several metabolic compounds produced by lactic acid bacteria such as organic acids, fatty acids, and H₂O₂ have antimicrobial effects (Ouweland, 1998). Bacteriocins produced by some strains of lactic acid bacteria also have antimicrobial effects (Ouweland, 1998). Increased numbers of lactobacilli would therefore affect the composition and/or metabolic activity of intestinal flora in the LGI mice. It has been reported that antibiotic treatments result in a marked reduction in plasma equol concentrations and altered plasma isoflavonoid patterns in cynomolgus monkeys (*Macaca fascicularis*; Blair *et al.* 2003). The alterations in

the plasma isoflavonoid profile are unique to the antibiotic used. We also observed different plasma isoflavonoid profiles in the LGI and CI groups. Administration of *L. gasseri* could have caused the change of equol production by changing the gastrointestinal environment including the intestinal microflora. On the other hand Morotomi & Mutai (1986) have investigated the *in vitro* binding to intestinal bacteria of amino acid pyrolysates. The administration of *L. gasseri* might also affect the binding capacity of intestinal flora for isoflavonoids. In summary, the present data suggest that the administration of *L. gasseri* influences the effect of isoflavonoids on the host, probably through changes in the gastrointestinal environment.

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