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Intake of a fermented soymilk beverage containing moderate levels of isoflavone aglycones enhances bioavailability of isoflavones in healthy premenopausal Japanese women: a double-blind, placebo-controlled, single-dose, crossover trial

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This study aimed to investigate the bioavailability of serum isoflavones after the intake of soymilk fermented by *Lactobacillus casei* strain Shirota containing 32.5% isoflavone aglycones (FSM) or placebo soymilk containing no isoflavone aglycones (SM). In a double-blind, placebo-controlled, single-dose, crossover trial, 7 healthy premenopausal Japanese women (mean age: 35.3 ± 11.0) consumed FSM or SM on day 1 and crossed over to the other soymilk after a 6-day washout period. Serum isoflavones in blood samples collected at 0, 1, 2, 3, 4, and 5 hr after intake were analyzed by liquid chromatography coupled with tandem mass spectrometry. The area under the curve (AUC) values for the serum concentrations of genistein and total isoflavones were significantly higher, by about 1.4-fold, up to 5 hr after FSM intake compared with SM intake (each $p<0.05$), and that of daidzein tended to be higher after FSM intake. In addition, AUC analysis of total isoflavones for individual subjects revealed that 5 out of 7 subjects had higher AUC values after FSM intake compared with SM intake and that the 2 remaining subjects had similar AUC values. These 2 subjects had higher AUC values after SM intake (mean, 2,502 ± 348) than those of the other subjects (mean, 1,158 ± 269). These results indicate that the bioavailability of isoflavones, especially genistein, is enhanced after the intake of FSM containing 32.5% isoflavone aglycones compared with intake of SM containing no isoflavone aglycones and that the enhancement is observed in healthy premenopausal Japanese women whose isoflavone absorption capacity is low after SM intake.

Key words: isoflavone aglycone, bioavailability, pharmacokinetics, area under the curve, fermented soymilk, premenopausal women

INTRODUCTION

In Japan, the soybean has long been consumed as a traditional foodstuff. Several epidemiological studies have suggested that populations with the highest consumption of soybean products have the lowest risk of breast and prostate cancer, and furthermore, women in this group experience less severe menopausal symptoms [1–4]. Isoflavones, which are contained in large quantities in soybeans, are thought to be involved in many of these benefits, as there are similar chemical structures in isoflavones and estrogen, the main female sex hormone, and studies have demonstrated that isoflavones not only have estrogen-like effects but also can suppress the effects of excessive estrogen [5].

The glycoside form of isoflavones, which is normally present in soybeans, does not demonstrate physiological effects; these kinds of effects are instead observed when the aglycone form is consumed [6]. When glycosides are ingested, they are hydrolyzed by β-glucosidase derived from gut microbiota and the gastrointestinal mucosa, and the resulting aglycones are absorbed through the

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intestine and circulated throughout the body, where their physiological actions take place [7, 8]. However, there are great interindividual differences in the absorption and metabolism of isoflavones (due to ethnicity, dietary habits, the form that the isoflavones are consumed in, etc.), which are mainly attributed to differences in gut microbiota. It is possibly due to these interindividual differences in isoflavone absorption and metabolism that there are inconsistencies in studies examining their activities; in contrast to the abovementioned studies [1–5], several other epidemiological studies have denied that soybean products and isoflavone consumption are correlated with lowered risk of breast cancer [9–12]. Therefore, blood levels of isoflavone are considered good markers of physiological effects. In fact, other epidemiological studies have demonstrated that there is a low risk of breast cancer and prostate cancer in subjects with higher plasma isoflavone levels compared with those with lower levels [13, 14].

Soybean products fermented with microbes having β-glucosidase activity, such as miso and soy sauce, have a moderate level of isoflavone aglycones (from 10 to 60%), but unfermented soybean products have almost no isoflavone aglycones [15, 16]. Epidemiological studies among the Japanese population, whose consumption of soybean products is high, have reported that higher miso consumption is associated with lower risk of breast cancer and coronary heart disease [17, 18]. These results suggest that fermented soybean products have better isoflavone effects than unfermented soy products, presumably because of a higher bioavailability of isoflavone aglycones. Additionally, Toi et al. reported in an epidemiological study among Japanese women that dietary habits involving the intake of both soy foods and dairy products containing *Lactobacillus casei* strain Shirota (LcS) may be useful for preventing breast cancer [19]. Our previous studies have also demonstrated that the development and growth of mammary tumors are suppressed by soymilk and LcS, respectively, and that soymilk fermented by LcS containing isoflavone aglycones has more potent suppression of this development compared with unfermented soymilk in an animal carcinogenic model [20, 21]. However, it remains unclear how the fermentation of LcS enhances the suppressive potential of soymilk and whether it enhances isoflavone bioavailability.

To investigate the effect of the fermentation of LcS on the pharmacokinetics of isoflavones after the intake of soymilk fermented by LcS containing moderate levels of isoflavone aglycones (“fermented soymilk”; FSM hereafter), a double-blind, placebo-controlled, single-dose, crossover trial was conducted in a group of healthy premenopausal Japanese women.

MATERIALS AND METHODS

Subjects

Ten healthy premenopausal Japanese women participated in this trial based on the inclusion criteria of age over 18 and below 55, no medication, no diseases, and maintenance of their habitual lifestyle during the trial. Exclusion criteria were history of severe disorders, history of abnormal cardiopulmonary function, history of abnormal results in clinical laboratory tests, habitual consumption of Chinese herbal medicine, food allergy, drug allergy, soymilk (soy) allergy, milk allergy, unable to consume soymilk, pregnant or lactating, planning to become pregnant, enrollment in another clinical trial, and unsuitability for the trial as judged by the director.

Three subjects fulfilling the discontinuation criteria (1 case due to a cold and 2 cases of withdrawal requests by subjects due to pain in the arm upon blood sample collection after the first intake of test beverage) were withdrawn from the study. The remaining 7 subjects who did not fulfil the criteria for discontinuation or exclusion from the analysis and whose adherence to isoflavone intake restriction (20 mg isoflavone aglycones/day) could be shown by their dietary logs were included in the following analyses (Table 1).

Test beverages

To produce FSM, soymilk (Fuji Oil Co., Ltd., Osaka, Japan) was fermented with *Lactobacillus casei* strain Shirota YIT 9029 (LcS) obtained from Yakult Central Institute at 37°C for 20 hr to convert a portion of the isoflavone glycosides into aglycones. Placebo soymilk (SM) was prepared from the unfermented soymilk by adding flavoring, and the final lactic acid level, taste, color, flavor, and nutritional composition of the SM were almost the same as the FSM. The compositions of the FSM and SM are shown in Table 2.

The isoflavone compositions of the FSM and SM were analyzed by high-performance liquid chromatography. As shown in Table 3, the FSM contained a moderate amount of isoflavone aglycones (32.5%; daidzein, 7.1 μmol/100 g; genistein, 16.3 μmol/100 g), and the SM contained no isoflavone aglycones (0%).

Study design

A double-blind, placebo-controlled, single-dose, crossover trial (UMIN No. 000017190) was conducted at the Nutrition Clinic of Kagawa Nutrition University

Table 1. Background data of the subjects

Number	Age	Height (cm)	Weight (kg)	BMI	Body fat percentage
7	35.3 ± 11.0	157.1 ± 5.5	49.3 ± 6.7	19.9 ± 1.5	26.2 ± 5.2

Values are shown as the mean ± SD. All subjects were healthy premenopausal Japanese women.

Table 2. Compositions of the FSM and SM

Component	FSM	SM
Moisture (g/100 g)	86.2	85.4
Protein (g/100 g)	2.5	2.6
Lipids (g/100 g)	0.4	0.3
Ash (g/100 g)	0.4	0.4
Carbohydrates (g/100 g)	10.5	11.3
Energy (kcal/100 g)	56	58
Sodium (mg/100 g)	8.8	8.4
Lactic acid (g/100 g)	0.5	0.4
LcS (CFU/ml)	2.1×10^9	N.D.
pH	4.1	4.2

N.D.: not detected

Table 3. Isoflavone contents of the FSM and SM

Isoflavone	FSM	SM
	μmol/100 g	
Daidzein	7.1	N.D.
Genistein	16.3	N.D.
Glycitein	N.D.	N.D.
Malonyl daidzin	13.1	13.3
Malonyl genistin	21.2	21.2
Malonyl glycitin	N.D.	N.D.
Daidzin	8.4	15.6
Genistin	5.8	22.9
Glycitin	N.D.	N.D.
Total	71.9	73.1
Aglycone/total (%)	32.5	0.0

Determination limit of isoflavones: 0.5 mg/100 g. Aglycone/Total (%) = (daidzein + genistein + glycitein)/total isoflavones × 100. N.D.: not detected

(Tokyo, Japan). The trial schedule is shown in Fig. 1. After a 6-day washout period, 10 subjects were randomly assigned to Group A or Group B, and as mentioned above, 7 subjects were analyzed. Subjects in Group A (n=4) and Group B (n=3) consumed 100 ml of FSM or SM, respectively. About 10 ml of venous blood was collected from a brachial vein before and at 1, 2, 3, 4, and 5 hr after intake. In consideration of their physical conditions, subjects were given an isoflavone-free rice ball snack after the 2-hr blood collection. After a 6-day washout period, the crossover test was carried out under the same conditions.

During the trial, subjects complied with dietary restrictions to avoid the intake of other fermented milks, yogurt, lactic acid bacteria beverages, and probiotic and prebiotic products and heavy intake of soy products such as tofu and miso; these foods were recorded in a diary if consumed. Daily intake of soy products was restricted to less than 20 mg isoflavone aglycones and was also recorded in a diary. On the day before the test beverages were consumed, subjects were served an isoflavone-free breakfast, lunch, and dinner, and they fasted from 9:00 PM until the test (subjects were allowed to freely drink mineral water).

Ethics statement

This trial was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of Kagawa Nutrition University (Tokyo, Japan). Written informed consent was obtained from all subjects prior to enrollment.

Serum isoflavones

Serum was prepared according to general methods, and isoflavones (daidzein, genistein, glycitein, dihydrodaidzein (DHD), *O*-desmethylangolensin (*O*-DMA), and equol) in serum were analyzed by tandem mass spectrometry [22] at Sumika Chemical

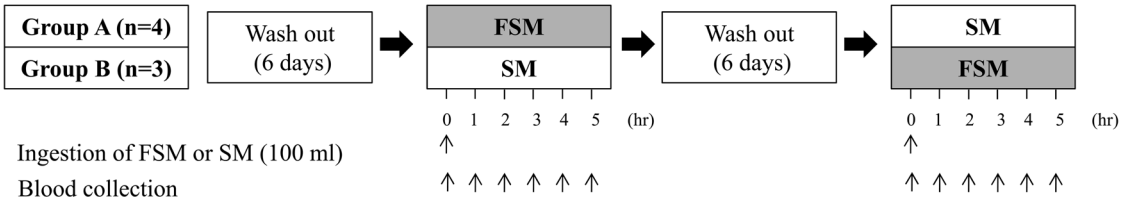

Fig. 1. Design of crossover trial

Analysis Service, Ltd. (Osaka, Japan). Pharmacokinetic parameters including maximum concentration (C_{max}), time to maximum concentration (T_{max}), and area under the curve (AUC) were calculated from serum concentration curves. The AUC values of total isoflavones were evaluated as the primary outcome, and the other metrics were evaluated as secondary outcomes.

Statistics

Data are shown as the mean ± standard deviation (SD). All data were analyzed using SAS preclinical package ver. 5.0 (SAS Institute Japan, Tokyo, Japan). The period effect and order effect on the AUC were analyzed by variance with the general linear model, and other data were analyzed by paired t-test. Two-sided p-values less than 0.05 were considered statistically significant.

RESULTS

Crossover trial

We opted for a crossover trial due to large interindividual differences in the amount of isoflavone absorbed. To more precisely determine the amount of isoflavones absorbed from the test beverages, subjects reduced their daily consumption of soy products to less than 20 mg of isoflavone aglycones during the 6-day washout period, and on the day before the intake of test beverages, they were served 3 isoflavone-free meals and were allowed to freely drink mineral water. However, a possible difficulty in the complete washout of isoflavones was anticipated, because the Japanese diet is generally based on soybean products. Indeed, isoflavones were detected in the sera of all subjects before the first and second intake of test beverages. Therefore, this study assessed the changes in values before the intake of test beverages.

The AUC values of daidzein, genistein, and total isoflavones were analyzed for period and order effects, but no such effects were found (Table 4).

Table 4. Analysis of crossover trial

	p-value		
	Daidzein	Genistein	Total isoflavones
Period effect	0.384	0.143	0.212
Order effect	0.386	0.261	0.194

Period and order effects were examined for the AUC values of daidzein, genistein, and total isoflavones for each subject. Data were analyzed by variance with the general linear model.

Serum isoflavones

Figures 2, 3, and 4 show the serum concentration-time curves of daidzein, genistein, and total isoflavones, respectively, which were analyzed after the intake of FSM and SM. Higher values were found for all three serum concentrations after the intake of FSM compared with SM intake. Significantly higher concentrations were found for daidzein at 1, 2, and 5 hr after the intake of FSM compared with SM intake, for genistein at 1 and 2 hr, and for total isoflavones at 1, 2, and 5 hr ($p<0.05$ in all cases). The serum concentrations of glycitein and daidzein metabolites (DHD, *O*-DMA, and equol) were barely detectable (data not shown).

The pharmacokinetic parameters derived from the serum concentrations of daidzein, genistein, and total isoflavones are shown in Table 5. Higher C_{max} values were found for daidzein, genistein, and total isoflavones with FSM intake compared with SM intake, but these differences were not significant. The AUC values of genistein and total isoflavones were significantly higher after FSM intake compared with SM intake ($p<0.05$ in both cases), while the AUC value for FSM daidzein was higher than that for SM, but not significantly so. No differences in T_{max} were found between FSM and SM.

Fig. 5 shows the serum concentration-time curves of total isoflavones for individual subjects. Two peaks were observed in all subjects after the intake of FSM and SM. All subjects had higher concentrations of total isoflavones from 0 to 2 hr (the first peak) after the intake of FSM compared with SM intake. However, from 2 to 5 hr (the second peak), higher concentrations of total isoflavones were observed in 5 out of 7 subjects (Subjects 1, 4, 5, 6, and 7) after the intake of FSM and in the 2 remaining subjects (Subjects 2 and 3) after the intake of SM. Analysis of the AUC values of total isoflavones for individual subjects revealed that 5 out of 7 subjects (Subjects 1, 4, 5, 6, and 7) had higher AUC values after the intake of FSM compared with SM intake and that the 2 remaining subjects (Subjects 2 and 3) had similar AUC values (Fig. 6). These 2 subjects had higher AUC values after SM intake (mean, 2,502 ± 348) than those of the other subjects (mean, 1,158 ± 269).

DISCUSSION

In the present study, a placebo-controlled, double-blind, crossover trial was conducted in a group of healthy premenopausal Japanese women to investigate the effect of fermentation of LcS on the pharmacokinetics of isoflavones after the intake of FSM compared with SM intake. This study demonstrated that the AUC value of total isoflavones was significantly higher up to 5 hr after the intake of FSM compared with SM intake.

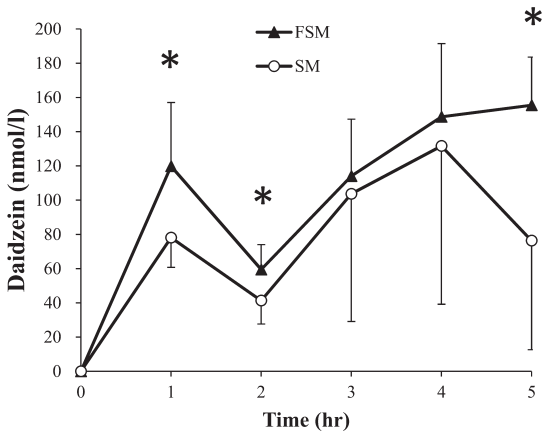

Fig. 2. Serum concentration-time curve of daidzein after intake of FSM or SM. Determination limit: 0.5 ng/ml. Values are shown as the mean ± SD (n=7). Data were analyzed by paired t-test. *p<0.05 vs. SM

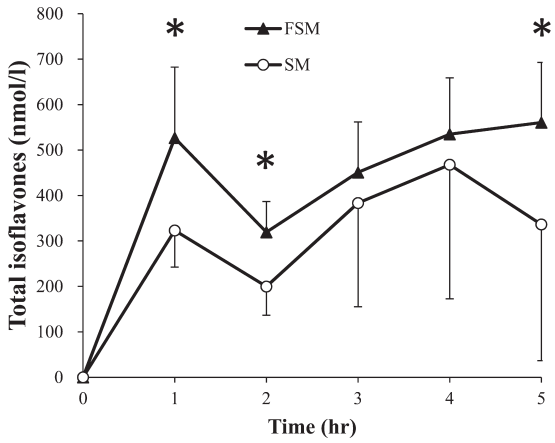

Fig. 4. Serum concentration-time curve of total isoflavones after intake of FSM or SM. Values are shown as the mean ± SD (n=7). Data were analyzed by paired t-test. *p<0.05 vs. SM

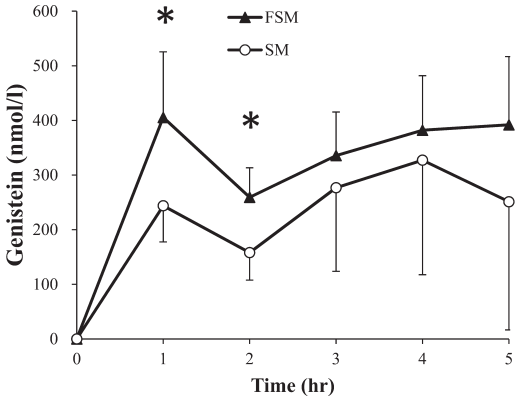

Fig. 3. Serum concentration-time curve of genistein after intake of FSM or SM. Determination limit: 2.0 ng/ml. Values are shown as the mean ± SD (n=7). Data were analyzed by paired t-test. *p<0.05 vs. SM

Table 5. Pharmacokinetic parameters derived from the serum concentrations of daidzein, genistein, and total isoflavones after intake of FSM and SM

	Pharmacokinetic parameter		
	C_{max}	T_{max}	AUC
Isoflavone/beverage	nmol/l	hr	5 hr*nmol/l
Daidzein			
FSM	178.3 ± 14.2	4.0 ± 1.4	519.9 ± 64.7
SM	149.1 ± 73.0	3.0 ± 1.4	392.9 ± 91.8
Genistein			
FSM	470.7 ± 106.3	2.9 ± 1.8	1,578.4 ± 330.4 *
SM	376.9 ± 181.5	2.9 ± 1.8	1,131.5 ± 519.4
Total isoflavones			
FSM	650.8 ± 98.1	3.4 ± 1.7	2,112.1 ± 376.8 *
SM	533.0 ± 243.7	3.1 ± 1.6	1,542.1 ± 706.2

Values are shown as the mean ± SD (n=7). Data were analyzed by paired t-test. *p<0.05 vs. SM

Daidzein and genistein were detected in serum after the intake of FSM and SM, but glycitein was barely detected. This is likely because both the FSM and SM contained glycitein and the glycoside of glycitein at levels below the detection limit. The daidzein metabolites, DHD, *O*-DMA, and equol, were also barely detectable in serum. These metabolites are produced from daidzein by gut microbiota, and they have been reported to be detectable in the serum from 5 hr to 48 hr after intake [23, 24]. Due to ethical considerations, we were able to collect blood samples only up to 5 hr after intake in the present trial, and it is possible that such metabolites had yet to become detectable.

The concentrations of daidzein, genistein, and total isoflavones changed over the course of the trial, with higher concentrations seen after the intake of FSM compared with SM intake. Specifically, significantly higher concentrations were detected at 1, 2, and 5 hr after intake for daidzein, 1 and 2 hr after intake for genistein, and 1, 2, and 5 hr after intake for total isoflavones (Figs. 2, 3, and 4). Although there were no significant differences in C_{max} and T_{max} between FSM and SM, the AUC value of daidzein was higher and the AUC values of genistein and total isoflavones were significantly higher after the intake of FSM compared with SM intake (Table 5). These findings support the previously reported results indicating that genistein has a higher absorption

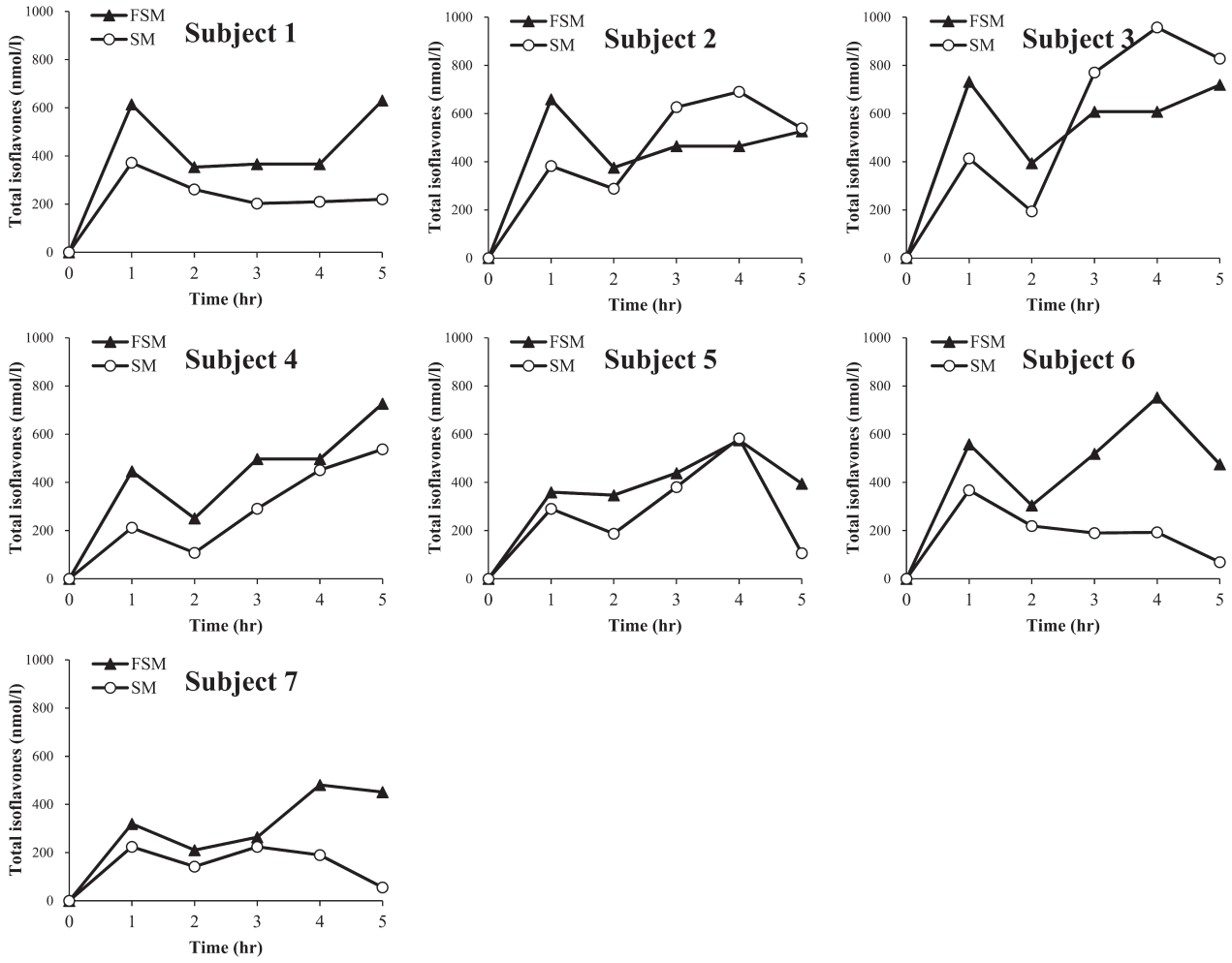

Fig. 5. Serum concentration-time curves of total isoflavones after intake of FSM or SM in individual subjects

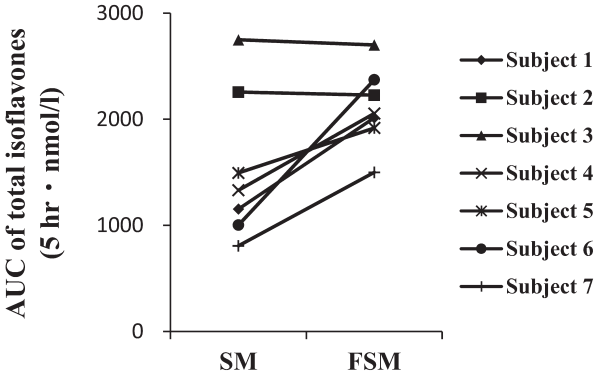

Fig. 6. AUC values of total isoflavones after intake of FSM or SM in individual subjects

efficiency than daidzein [25].

Our previous study demonstrated that the absorption efficiency of total isoflavones for the 24 hr after intake is 35.8% for normal soymilk as opposed to 65.9% for soymilk fermented by *Bifidobacterium breve* Yakult and *Lactobacillus mali* with 90% or more isoflavone aglycones [22], indicating that fermentation enhanced bioavailability by approximately 1.8-fold. The present study revealed absorption efficiencies of total isoflavones of 11.2% up to 5 hr after the intake of FSM with 32.5% isoflavone aglycones and 8.0% up to 5 hr after the intake of SM, suggesting that fermentation enhanced bioavailability by approximately 1.4-fold. Our previous study also demonstrated that 35.8% of the total isoflavones in SM are absorbed during the 24 hr after intake and that the concentration returns to the baseline level at 24 hr after intake. There was a similar pattern in the changes

in the serum concentration of total isoflavones up to 5 hr after intake in our previous study [22] and the present study. These findings suggest that about 22% of the amount of total isoflavones absorbed over the course of 24 hr would be absorbed in the first 5 hr, and that the bioavailability of isoflavones can be adequately assessed from analysis of the serum isoflavone concentration up to 5 hr after intake with fewer blood collections.

The pharmacokinetic analysis in the present study showed that there were 2 peaks in the changes in the serum concentrations of daidzein, genistein, and total isoflavones after intake of FSM and SM (Figs. 2, 3, and 4), which is consistent with other previously reported results [22, 24, 26]. The second peak (at 2–5 hr after intake) may be due to enterohepatic circulation, in which the absorbed daidzein and genistein are released as glucuronide and sulfate conjugates in the gut together with bile and then subsequently reabsorbed after being deconjugated by gut microbiota. However, the concentrations of total isoflavones were almost the same between the first peak (0–2 hr after intake) and the second peak (Figs. 2, 3, and 4). This suggests that the 2-peak phenomenon is based on not only enterohepatic circulation but also other factors.

All subjects showed 2 peaks after the intake of FSM and SM in the pharmacokinetic analysis of total isoflavones for individual subjects (Fig. 5). Interestingly, in all subjects, the serum concentrations of total isoflavones after the intake of FSM were higher at the first peak (0–2 hr after intake), and the AUC value of total isoflavones up to 2 hr after intake was significantly higher after intake of FSM compared with SM intake (data not shown). Therefore, it seems that other fermented soy products with a moderate proportion of isoflavone aglycones may have enhanced bioavailability at least up to 2 h after intake. However, there were two patterns regarding the second peak (2–5 hr after intake); in 5 of the 7 subjects (Subjects 1, 4, 5, 6, and 7), there was a higher concentration after the intake of FSM, and in the 2 remaining subjects (Subjects 2 and 3), there was a higher concentration after the intake of SM. Isoflavone aglycones (daidzein and genistein), but not their glucosides, have been reported to be absorbed in the stomach in animal models [27]. It has been also reported that isoflavone glycosides are converted to aglycones by β-glucosidase located in the mucosa of the upper gastrointestinal tract and that the aglycones are then absorbed in the upper gastrointestinal tract into the blood circulation in GF mice [8, 28]. It is well known that gut microbiota are very diverse and show great interindividual differences, although it can be assumed that the activity of β-glucosidase, located in the mucosa of the upper gastrointestinal tract, will show fewer interindividual differences than gut microbiota. Our findings indicate that the first peak may be derived from isoflavone aglycones ingested from FSM directly as well as those converted from the glycosides in FSM and SM by mucosal β-glucosidase in the upper gastrointestinal tract; furthermore, the second peak may be derived from isoflavone aglycones via not only enterohepatic circulation but also conversion from the glycosides contained in FSM and SM by β-glucosidase in gut microbiota in the lower gastrointestinal tract.

A group of 5 subjects (Subjects 1, 4, 5, 6, and 7) had higher AUC values after the intake of FSM compared with SM intake. However, the remaining 2 subjects (Subjects 2 and 3) had similar AUC values after the intake of FSM and SM. These 2 subjects had higher AUC values after SM intake (mean, 2,502 ± 348) than those of the other subjects (mean, 1158 ± 269) (Fig. 6). These findings suggest that the 2 subjects may have had strong gut microbiota β-glucosidase activity that facilitated the production of isoflavone aglycones from the glycosides, leading to high AUC values after the intake of SM. We also speculate that bioavailability was enhanced in the 5 subjects after the intake of FSM compared with intake of SM, who had lower AUC values after the intake of SM, due to high absorption in the upper gastrointestinal tract (shown as the first peak) as well as activation of β-glucosides in the gut microbiota in association with LcS and/or fermentation, which was observed as a the second peak (2–5 hr after intake).

In conclusion, our placebo-controlled, double-blind, crossover trial in premenopausal healthy Japanese women revealed that the bioavailability of isoflavones, especially genistein, is enhanced after the intake of FSM containing 32.5% isoflavone aglycones compared with intake of SM containing no isoflavone aglycones and that the enhancement is observed in healthy premenopausal Japanese women whose isoflavone absorption capacity is low after intake of SM. Furthermore, the results of pharmacokinetic analysis of serum isoflavones for each subject suggest that the enhancement is associated with beneficial effects of LcS and/or soy fermentation on gut microbiota having β-glucosidase activity as well as the increase of isoflavone aglycones due to fermentation by LcS. Further studies are necessary to clarify these points in greater detail.

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