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ScienceDirect
Food Chemistry 109 (2008) 278–284
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

Enrichment of isoflavone aglycones in soymilk by fermentation with single and mixed cultures of *Streptococcus infantarius* 12 and *Weissella* sp. 4

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Received 5 October 2007; received in revised form 29 November 2007; accepted 13 December 2007

Abstract

Soymilk was fermented with either *Streptococcus infantarius* 12 (*Si* 12), *Weissella* sp. 4 (*Ws* 4), or their mixed cultures with different mixing ratios (*Si* 12:*Ws* 4 = 1:1, 1:3, 1:5, and 1:10, v/v) for 12 h at 37 °C. All cultures in soymilk readily proliferated and reached about 10^{8-9} CFU/mL. After 12 h, pH and titratable acidity of soymilk ranged 4.19–4.47 and 0.57%–0.64%, respectively. The pH of soymilk fermented with *Si* 12 was the lowest while that obtained with *Ws* 4 the highest. A sharp increase in β-glucosidase (β-glu) activity corresponded well with a rapid decrease in isoflavone glucosides and an increase in aglycone contents. The rate of hydrolysis of isoflavone glucosides was the least with *Si* 12 while the highest with *Ws* 4, resulting in about 23%–33% and 98%–99% hydrolysis of the glucosides with *Si* 12 and *Ws* 4, respectively, after 12 h. Mixed cultures with 1:3, 1:5, and 1:10 ratios seem to be more effective starters for bioactive fermented soymilk with more aglycones and appropriate acidity in a short time than single cultures.

Keywords: Isoflavones; Lactic acid bacteria; Lactic fermentation; Soymilk

1. Introduction

Isoflavones are rich in leguminous plants, especially in soybeans. They are called phytoestrogens due to structural similarity to estrogen hormone and have been reported to have the potential to reduce the risk of age-related and hormone-related diseases including cancer, menopausal symptoms, cardiovascular diseases, and osteoporosis (Adlercreutz, 2002; Jacobsen, Knutsen, & Fraser, 1998; Omoni & Aluko 2005). Soy isoflavones consist of 12 chemical forms including three aglycones (daidzein, genistein and glycitein) and their glucosides, acetyl-, malonyl-, and β-glucosides. Their different chemical structures may be responsible for the different bioavailabilities of isoflavones in human. In general, isoflavones in soybeans exist mainly as glucoside forms and rarely as aglycone forms and the concentrations of isoflavones in soy foods depend on how the food is prepared (Wang & Murphy, 1994; Wang & Murphy, 1996). Biotransformation and the production of metabolites of isoflavones from food intake in human are highly dependent on the nature of microflora in human intestines (Chang & Nair 1995; Omoni & Aluko, 2005; Xu, Keecha, Wang, Murphy, & Hendrich, 1995). It suggests that any change in intestinal microflora could affect the bioavailability of isoflavones.

The extent of bioavailability and the mechanism of intestinal absorption of isoflavones in human are unclear. Izumi et al. (2000) stated that soy isoflavone aglycones were absorbed faster and in larger amounts than their glucoside forms in humans. Setchell et al. (2002) reported that isoflavone glucosides were not efficiently absorbed across the intestinal epithelium in human adults, and

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doi:10.1016/j.foodchem.2007.12.024

hydrolysis of the sugar moiety by intestinal β-glu was required for efficient absorption. However, a study by Richelle, Pridmore-Merten, Bodenstab, Enslen, and Offord (2002) showed that hydrolysis of isoflavone glycosides to aglycones did not alter plasma and urine isoflavone pharmacokinetics in women. Despite of these contradictory findings, several studies have been conducted attempting the enrichment of isoflavone aglycones in soymilk by fermentation with β-glu-producing lactic acid bacteria (LAB) (Chien, Huang, & Chou, 2006; Otieno, Ashton, & Shah, 2006; Pyo, Lee, & Lee, 2005). These studies proceeded on the premise that both the enhancement of isoflavone aglycones before consumption of soy foods and the modulation of intestinal microflora through the ingestion of viable LAB could improve the bioavailability of isoflavones from soy foods.

Soymilk provides high quality proteins while containing no cholesterol, gluten or lactose. However, soymilk has often limited human use in Western diets due to undesirable flavour and flatulence caused by high levels of oligosaccharides. Fermentation of soymilk with some organisms, mainly LAB, has been attempted to overcome these limitations (Granata & Morr 1996; Wang, Yu, Yang, & Chou, 2003). *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* are the most commonly used organisms for fermentation of dairy products. There is a continuing need to improve existing cultures or to screen new organisms for development of new products. Recently, authors isolated LAB from human feces including *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Weissella* with β-glu activities. There are many studies on fermentation of soymilk with different species of LAB, but no information about *Weissella* species related with bioconversion of isoflavone glucosides and growth characteristics in soymilk is available. It was expected that especially *Weissella* species could effectively convert isoflavone glucosides to aglycones in soymilk due to their relatively high β-glucosidase activities (data not shown).

Therefore, the objective of this study was to investigate changes in the contents of isoflavone glucosides (daidzin and genistin) and aglycones (daidzein and genistein) and other characteristics (viable cell growth, acid production, and β-glucosidase activity) in soymilk fermented with *Weissella* sp. 4. Furthermore, another LAB, *Streptococcus infantarius* 12, was tested as a single and mixed culture with *Weissella* sp. 4.

2. Materials and methods

2.1. Organisms

S. infantarius 12 (*Si* 12) and *Weissella* sp. 4 (*Ws* 4) were selected from our LAB collections isolated from human feces according to Chun et al. (2007) because of their high β-glucosidase activities and ability to grow rapidly in MRS lactobacilli broth (data not shown). They were identified based on the carbon source utilization patterns (API 50 CHL kit, BioMerieux, France) and 16S rDNA sequence analyses (GenBank of National Center for Biotechnology Information, Bethesda, MD, USA).

2.2. Preparation of soymilk

Tae-Kwang variety soybean grown in Kyungpook province of Korea (2004 crop year) was used for soymilk preparation. Whole beans were washed and soaked in distilled water overnight. The swollen soybeans were dehulled and then ground with warm water using a blender (Waring Commercial, Torrington, CT, USA) for 3 min at low speed and 2 min at high speed. The ratio of dry soybean (500 g) to water (5 L) used for grinding was 1:10 (w/v). The slurry was filtered through a double-layered cheese cloth and the filtrate was centrifuged at $7000\times g$ for 20 min. The supernatant was transferred into glass bottles and autoclaved at 121 °C for 15 min. Cooled soymilk was stored in a refrigerator (4 °C) before use.

2.3. Fermentation of soymilk

Each strain of *Si* 12 and *Ws* 4 (100 μL) was propagated twice in 10 mL of Difco™ lactobacilli MRS broth (Difco, Sparks, MD, USA) at 37 °C for 12–15 h. Incubation time was adjusted for each culture to obtain $O.D_{600}$ of 1.5. Exactly 150 mL of soymilk was transferred into a sterile flask and inoculated with either single (*Si* 12 and *Ws* 4) or mixed cultures (*Si* 12:*Ws* 4 = 1:1, 1:3, 1:5, and 1:10, v/v). Total volume of the single or the mixed cultures inoculated into soymilk was exactly 2% of soymilk (3 mL, v/v), which gives about 3.4×10^7 CFU/mL of soymilk. Inoculated soymilk was aliquoted into sterile 15 mL centrifuge tubes after thoroughly mixed. The soymilk tubes tightly screw-capped were placed in an incubator at 37 °C. Tubes were taken out at 3 h intervals up to 12 h. Total viable cell numbers, pH, titratable acidity (TA), and β-glucosidase activity of soymilk samples were immediately measured after sampling. For isoflavone analysis, samples were freeze-dried at −70 °C.

2.4. Growth and acid production

LAB in soymilk fermented with either single or mixed cultures were enumerated using the pour plate method (Case & Johnson, 1984). Soymilk (1 mL) was serially diluted with 0.1% peptone water and then 100 μL of diluted samples were used. Plates of lactobacilli MRS agar seeded with the samples were incubated at 37 °C for 24 h. Total viable cells counted in triplicates were expressed as $\log_{10}$ colony forming units (CFU)/mL of soymilk. pH was measured using a pH meter (DMS, Seoul, Korea). TA was determined by titration with 0.1 N NaOH solution and expressed as % lactic acid (AOAC, 1995).

2.5. β-Glucosidase activity

β-Glu activity in soymilk during fermentation was determined by a modified McCue and Shetty (2005) method. Two grams of fermented soymilk samples were homogenized with 20 mL of 0.2 M sodium acetate buffer (pH 5.5) at 4 °C. Aliquots of the homogenate were placed into a 1.8 mL eppendorf tube followed by centrifugation (14,000×*g*, 15 min, 4 °C). Sediment was suspended in 1 mL of 0.2 M acetate buffer (pH 5.5) and used for enzyme assay. Exactly 100 μL of suspended samples was mixed with 350 μL of 0.2 M sodium acetate (pH 5.5) and 50 μL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma, St. Louis, MO, USA) and incubated at 50 °C for 30 min. The reaction was stopped by addition of 500 μL of 1 M sodium carbonate, and the reaction mixture was centrifuged at 14,000×*g* for 15 min at 4 °C. The amount of *p*-nitrophenol released was determined by measuring the absorbance of the clarified reaction mixture at 400 nm. One unit of β-glu activity (U) was defined as the amount of enzyme which released 1 mM of *p*-nitrophenol from the substrate (*p*-NPG) per minute under the assay conditions. Enzyme activity was expressed as U/g of soymilk sample.

2.6. Isoflavone analysis by HPLC

Isoflavones were extracted from freeze-dried soymilk samples (−70 °C) with 80% methanol (Barnes, Kirk, & Coward, 1994). Exactly 0.5 g of dried soymilk was weighed into a 15 mL centrifuge tube and mixed with 5 mL of 80% methanol. The tubes were tightly screw-capped and shaken in a water bath at 60 °C for 2 h. The samples were centrifuged at 12,000×*g* for 10 min at 4 °C. Supernatant was filtered through a 0.45 μm membrane filter (Waters, Milford, MA, USA) and used for HPLC analysis.

Isoflavone separation was performed by a reverse-phase HPLC system consisting of a HPLC pump (Dionex P680, Germany), an auto-sampler (Dionex, Germany), a Nova-Pak C_{18} column (150 × 3.9 mm I.D., 4 μm, Waters, Milford, MA, USA) and a photodiode array detector (PDA-100, Dionex, Germany). The mobile phase A and B were 0.1% glacial acetic acid in water and 0.1% glacial acetic acid in methanol, respectively. Solvent A and B were run at a flow rate of 1.0 mL/min, using a gradient of 85% A (15% B) at 0 min, decreasing to 50% A for 42 min, steady at 50% A for 3 min, and then increasing to 85% A for 5 min. A column was equilibrated with 85% A for 10 min before the next injection. The mobile phases were filtered through a 0.45 μm nylon membrane filter (Waters, Milford, MA, USA) prior to use. Isoflavones were identified by comparing retention times and PDA spectra (200–350 nm) of sample peaks with those of standards. Quantification of each isoflavone was carried out by electronic integration of the chromatographic peak. Standards of isoflavone glucosides and aglycones were dissolved in dimethyl sulphoxide (DMSO) and diluted with 80% methanol. Daidzin, glycitin, and genistin were obtained from Indofine (Somerville, NJ, USA) and daidzein, glycitein, and genistein were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and spectral analyzed-grade DMSO were obtained from Fisher Scientific (Pittsburg, PA, USA).

2.7. Statistical analysis

All assays were done in triplicates and the results were given as mean ± standard deviation (SD). The analysis of variances was performed by ANOVA and differences among the means of samples were analyzed by Duncan's test at a significant level of 0.05 (SAS, 1990).

3. Results and discussion

3.1. Growth of LAB in soymilk

Growth of LAB in soymilk inoculated with either single (*Si* 12 and *Ws* 4) or mixed cultures (*Si* 12:*Ws* 4 = 1:1, 1:3, 1:5, and 1:10, v/v) during fermentation at 37 °C was investigated. LAB in soymilk fermented with mixed cultures was enumerated as a whole using MRS plates. Fig. 1 shows changes in total viable cell numbers of soymilk during 12 h of fermentation. Cell numbers, initially 3.4×10^7 CFU/mL of sample, rapidly increased and reached about 10^{8-9} CFU/mL of sample during the first 6 h of fermentation. Rates of cell growth in soymilk varied depending on the types of cultures and the respective fermentation stages. Comparing single cultures, cell number of soymilk fermented with *Si* 12 was higher than that of *Ws* 4 after 6 h of fermentation although growth rate of *Ws* 4 was much higher than that of *Si* 12 until 3 h. At 3 h, cell number of the mixed culture with a 1:10 ratio was outstandingly higher than those of other mixed cultures. However, there was no significant difference in cell numbers among mixed

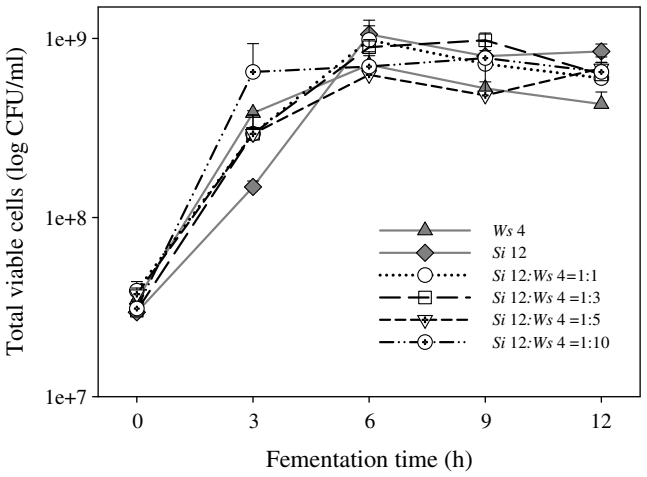

Fig. 1. Changes in viable cell numbers of soymilk fermented with LAB at 37 °C. *Si* 12 and *Ws* 4 indicate *S. infantarius* 12 and *Weissella* sp. 4, respectively.

cultures at 12 h. The cell numbers of the mixed cultures after 12 h were lower than that of *Si* 12 but higher than that of *Ws* 4 ($p < 0.05$).

3.2. Acid production by LAB in soymilk

Appropriate acid concentration is one of the most important factors ensuring good quality of fermented soymilk (Granata & Morr, 1996). It has been reported that the amount of acid produced in soymilk depends on the types of organisms involved (Angeles & Marth, 1971). In this study, acid production by single and mixed cultures of *Si* 12 and *Ws* 4 was investigated by measuring pH and TA of soymilk during fermentation (Figs. 2 and 3). A sharp decrease in pH and a rapid increase in TA were observed during the first 6 h of fermentation and then the change became slowed during the rest period of fermentation. These time points corresponded well to the exponential and stationary growth phases of cultures (Fig. 1). After 12 h of fermentation, pH and TA of soymilk ranged from 4.19 ± 0.08 to 4.47 ± 0.05 and from $0.57 \pm 0.04\%$ to $0.64 \pm 0.01\%$, respectively. At this stage, the lowest pH and the highest TA were observed in soymilk fermented with single culture of *Si* 12, indicating that *Si* 12 produced more acid than *Ws* 4. During the overall fermentation period, pHs of soymilk fermented with mixed cultures were lower than that of *Si* 12 but higher than that of *Ws* 4. It suggested that acid production in soymilk fermented with *Ws* 4 could be improved by using mixed cultures of *Ws* 4 and *Si* 12. pH of soymilk was significantly affected by the ratios of mixed cultures ($p < 0.05$), but the differences in the pH values among mixed cultures were very small to be ignorable. On the other hand, the TA values among soymilks fermented with different cultures were not significantly different during all the fermentation stages except at 12 h.

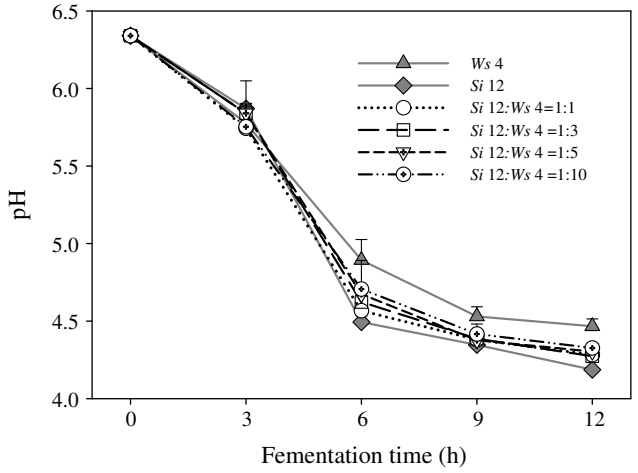

Fig. 2. Changes in pH and TA of soymilk fermented with LAB at 37 °C. *Si* 12 and *Ws* 4 indicates *S. infantarius* 12 and *Weissella* sp 4, respectively.

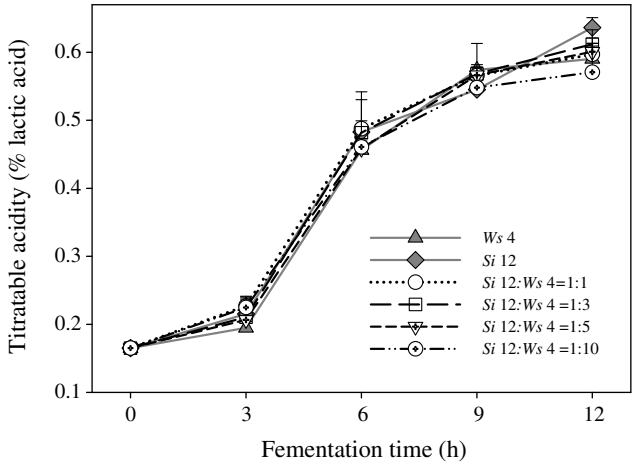

Fig. 3. Changes in titratable acidity of soymilk fermented with LAB at 37 °C. *Si* 12 and *Ws* 4 indicates *S. infantarius* 12 and *Weissella* sp 4, respectively.

Commercial yogurts were reported to have a pH range of 4.2–4.4 (Pinthong, Macrae, & Dick, 1980). In the present study, pH of soymilk fermented with *Ws* 4 was slightly higher than the pH ranges found in commercial yoghurts at the end of fermentation. It indicates that 12 h of fermentation may be not enough for *Ws* 4 to obtain the pH range for commercial yoghurts. Otherwise, pH of the other soymilk reached the pH range of 4.2–4.4 within 9 h. It indicated that mixed cultures of *Ws* 4 and *Si* 12 could be more desirable starters for producing fermented soymilk with high content of isoflavone aglycones and the appropriated amount of acids than a single culture of *Ws* 4. Compared with other organisms previously studied by several researchers (Angeles & Marth, 1971; Matsuyama et al., 1992; Pyo et al., 2005; Wang et al., 2003), acid production by *Ws* 4 as well as *Si* 12 was relatively good at the equivalent fermentation stages under the conditions of the present study. Pyo et al. (2005) reported that the values of 4.4–5.8 for pH were obtained by fermentation of soymilk with *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Bifidobacterium breve*, or *Bifidobacterium thermophilum* for 24 h. On the other hand, Wang et al. (2003) reported that a lower pH (5.59–6.07) in soymilk was obtained by fermentation with a mixed culture of *Lactobacillus acidophilus* and bifidobacteria than with *L. acidophilus* (pH 6.44) alone after 32 h of fermentation.

3.3. β-Glucosidase activity of LAB

It has been proposed that intestinal microorganisms with β-glu activities play a key role in the metabolism and bioavailability of isoflavones in human (Setchell, 2000). β-Glu catalyzes the hydrolysis of β-glucosidic bond found in isoflavone glucosides, releasing the bioactive aglycone forms (Esaki, Watanabe, Hishikawa, Osawa, & Kawakishi, 2004). Previous studies have demonstrated that β-glu activity in fermented soymilk varies depending on the starter organism and the fermentation period (Chien et al., 2006; Otieno et al., 2006; Pyo et al., 2005).

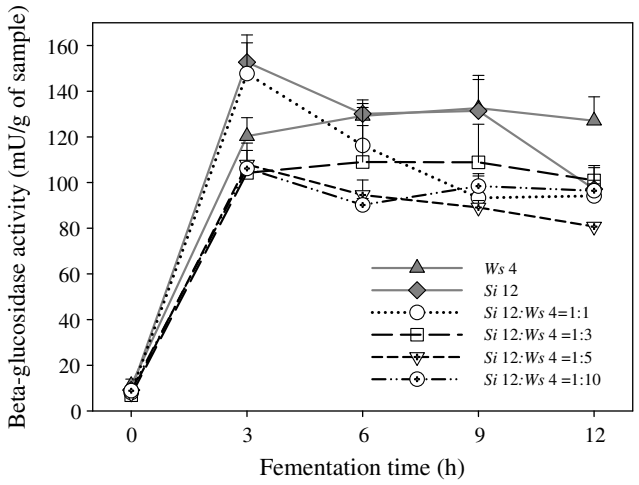

Fig. 4. Changes in β-glucosidase activity of soymilk fermented with LAB at 37 °C. *Si* 12 and *Ws* 4 indicates *S. infantarius* 12 and *Weissella* sp 4, respectively.

Fig. 4 shows changes in β-glu activity of soymilk inoculated with either single or mixed cultures of *Ws* 4 and *Si* 12 during 12 h of fermentation at 37 °C. β-Glu activities of all soymilks rapidly increased during the first 3 h of fermentation, reaching to the range of 104.2 ± 3.6–152.7 ± 8.5 mU/g of soymilk. During the rest of fermentation period, β-glu activity in soymilk inoculated with *Si* 12 rapidly decreased with fermentation time while that with *Ws* 4 gradually increased up to 9 h of fermentation. As a result, the maximum activity in soymilk inoculated with *Si* 12 was observed at 3 h (152.7 ± 8.5 mU/g of soymilk) while at 9 h for *Ws* 4 (132.6 ± 14.3 mU/g of soymilk). On the other hand, soymilk fermented with mixed cultures showed the maximum activities at 3 or 6 h depending on the mixing ratios. In overall, soymilk fermented with mixed cultures showed relatively low β-glu activities compared to those with single cultures, *Si* 12 and *Ws* 4. Mutual interaction between *Si* 12 and *Ws* 4 might be the reason for lowering β-glu activity, but the exact reason remains to be investigated.

In general, an increase and a decrease in β-glu activity during fermentation have been reported to correspond well to growth phases of organisms in soymilk (Otieno et al., 2006; Pyo et al., 2005). In the present study, a sharp increase in β-glu activity in soymilk fermented with different cultures agreed well with exponential growth phase of the starter.

3.4. Changes in isoflavone glucoside and aglycone contents of soymilk by lactic fermentation

Si 12 and *Ws* 4 in single or mixed cultures with various mixing ratios were tested for capacities of hydrolyzing isoflavone glucosides to bioactive aglycone forms in soymilk. This study focused on hydrolysis of daidzin and genistin to corresponding aglycones, daidzein and genistein. Changes in these four isoflavone contents in soymilk were monitored during 12 h of fermentation at 37 °C and then percent hydrolysis of daidzin and genistin at the respective fermentation stages was calculated as described in Table 1.

The contents of isoflavone glucosides in soymilk fermented with either single or mixed cultures significantly decreased with fermentation time ($p < 0.05$). Rates of hydrolysis of isoflavone glucosides varied depending on the types of cultures. *Si* 12 showed very low rates of hydrolysis of isoflavone glucosides in soymilk compared to *Ws* 4. As a result, after 3 h of fermentation, only 8.9% and 7.8% of the initial daidzin and genistin contents, respectively, were hydrolyzed by *Si* 12 while about 95% of the initial daidzin and genistin was hydrolyzed by *Ws* 4 (Table 1). Additionally, at the end of fermentation, hydrolysis of daidzin and genistin in soymilk by *Si* 12 was limited to about 23% and 33%, respectively, while these glucosides almost completely hydrolyzed by *Ws* 4 (99%). For mixed cultures, degree of hydrolysis of isoflavone glucosides were slightly improved as the portion of *Ws* 4 to *Si* 12 in mixed cultures increased from 1 to 10 under the conditions of the present study. Accordingly, the highest percent hydrolysis of daidzin and genistin was observed in soymilk fermented with 1:10 mixed culture and followed by 1:5, 1:3, and lastly 1:1 mixed cultures. This phenomenon was clearly observed at the early stage of fermentation. At 3 h, percent hydrolysis of genistin by 1:10, 1:5, 1:3, and 1:1 mixed cultures was 96.2%, 91.8%, 88.0%, and 38.3%, respectively. However, there was no remarkable difference in the daidzin and genistin levels of soymilk fermented with the mixed cultures at 12 h of fermentation.

Due to hydrolysis of daidzin and genistin, the contents of corresponding aglycones, daidzein and genistein, in soymilk significantly increased during fermentation ($p < 0.05$). The daidzein and genistein levels were significantly affected by the type of LAB species and mixing ratios of mixed cultures during the first 6 h of fermentation ($p < 0.05$). However, the effects of different cultures on the aglycone contents in soymilk were not great when fermentation time exceeded 9 h. After 12 h, as shown in Table 1, the contents of daidzein (1535 ± 59 μg/100 g of sample) and genistein (2072 ± 57 μg/100 g of sample) in soymilk fermented with *Ws* 4 were about 3 times higher than those with *Si* 12.

Comparing percent hydrolysis of isoflavone glucosides at the equivalent stage of fermentation, rates of hydrolysis of isoflavone glucosides by *Ws* 4, 1:3, 1:5 and 1:10 mixed cultures were higher than those reported in the previous studies (Choi, Woo, & Noh, 1999; Chun et al., 2007; Jeon, Ji, & Hwang, 2002; Otieno et al., 2006). Jeon et al. (2002) reported a 100% conversion of daidzin and genistin in soymilk fermented with *Bifidobacterium* sp. Int-57, but it was achieved after 18 h of fermentation. We previously reported that *Lactobacillus paraplantarum* KM preferentially metabolized genistin rather than daidzin in soymilk, resulting in 90% and 100% hydrolysis of the daidzin and genistin, respectively, during 6 h of fermentation (Chun et al., 2007). On the other hand, in the present study, *Ws*

Table 1
Changes in the contents of isoflavones of soymilk fermented with LAB at 37 °C (μg/100 g of sample)

Isoflavones	Time[B] (h)	Isoflavone contents in soymilk fermented with[A]					
		Ws 4	*Si* 12	*Si* 12:*Ws* 4 = 1:1	*Si* 12:*Ws* 4 = 1:3	*Si* 12:*Ws* 4 = 1:5	*Si* 12:*Ws* 4 = 1:10
Daidzin	0	1456 ± 42^{aA} (0.0)[C]	1456 ± 42^{aA} (0.0)	1456 ± 42^{aA} (0.0)	1456 ± 42^{aA} (0.0)	1456 ± 42^{aA}(0.0)	1456 ± 42^{aA} (0.0)
	3	72 ± 3^{bC} (95.1)	1327 ± 44^{bA} (8.9)	528 ± 27^{bB} (63.8)	78 ± 2^{bC} (94.6)	34 ± 1^{bD} (97.7)	33 ± 1^{bD} (97.8)
	6	15 ± 1^{cC} (99.0)	1161 ± 14^{cA} (20.3)	95 ± 9^{cB} (93.5)	20 ± 0^{cC} (98.6)	19 ± 1^{bC} (98.7)	16 ± 2^{bC} (98.9)
	9	13 ± 1^{cC} (99.1)	1131 ± 12^{cA} (22.3)	73 ± 7^{cdB} (95.0)	19 ± 0^{cC} (98.7)	18 ± 1^{bC} (98.8)	14 ± 1^{bC} (99.1)
	12	12 ± 1^{cC} (99.2)	1121 ± 14^{cA} (23.0)	34 ± 2^{dB} (97.7)	19 ± 0^{cC} (98.7)	18 ± 2^{bC} (98.8)	13 ± 1^{bC} (99.1)
Genistin	0	1792 ± 57^{aA} (0.0)	1792 ± 57^{aA} (0.0)	1792 ± 57^{aA} (0.0)	1792 ± 57^{aA} (0.0)	1792 ± 57^{aA} (0.0)	1792 ± 57^{aA} (0.0)
	3	99 ± 2^{bDE} (94.5)	1651 ± 43^{bA} (7.8)	1105 ± 49^{bB} (38.3)	216 ± 12^{bC} (88.0)	146 ± 12^{bD} (91.8)	68 ± 13^{bE} (96.2)
	6	36 ± 2^{cC} (97.9)	1474 ± 27^{cA} (17.7)	292 ± 28^{cB} (83.7)	27 ± 0^{cC} (98.5)	27 ± 2^{cC} (98.5)	27 ± 1^{cC} (98.5)
	9	25 ± 0^{cC} (98.6)	1419 ± 9^{dA} (20.8)	146 ± 20^{dB} (91.8)	28 ± 4^{cC} (98.5)	27 ± 1^{cC} (98.5)	24 ± 1^{cC} (98.7)
	12	12 ± 1^{cD} (99.3)	1211 ± 14^{cA} (32.4)	58 ± 9^{eB} (96.8)	27 ± 2^{cC} (98.5)	24 ± 3^{cCD} (98.6)	24 ± 1^{cCD} (98.6)
Daidzein	0	261 ± 28^{cA}	261 ± 28^{cA}	261 ± 28^{dA}	261 ± 28^{cA}	261 ± 28^{cA}	261 ± 28^{dA}
	3	1430 ± 18^{bA}	275 ± 8^{cD}	1084 ± 59^{cC}	1306 ± 53^{bB}	1376 ± 32^{bAB}	1347 ± 32^{cB}
	6	1495 ± 46^{aA}	412 ± 29^{bD}	1355 ± 9^{bC}	1456 ± 58^{aAB}	1408 ± 46^{bBC}	1477 ± 17^{bAB}
	9	1494 ± 43^{aA}	496 ± 17^{aB}	1498 ± 68^{aA}	1489 ± 31^{aA}	1503 ± 23^{aA}	1502 ± 32^{abA}
	12	1535 ± 59^{aA}	499 ± 28^{aB}	1484 ± 25^{aA}	1511 ± 39^{aA}	1521 ± 25^{aA}	1531 ± 33^{aA}
Genistein	0	315 ± 13^{cA}	315 ± 13^{dA}	315 ± 13^{dA}	315 ± 13^{dA}	315 ± 13^{dA}	315 ± 13^{dA}
	3	1930 ± 15^{bA}	300 ± 22^{dE}	846 ± 30^{cD}	1715 ± 47^{cC}	1804 ± 24^{cB}	1864 ± 36^{cB}
	6	2034 ± 54^{aA}	445 ± 5^{cE}	1572 ± 26^{bD}	1932 ± 33^{bC}	1961 ± 48^{bBC}	2003 ± 37^{bAB}
	9	2056 ± 50^{aA}	609 ± 24^{bC}	1900 ± 53^{aB}	2027 ± 55^{aA}	2042 ± 41^{aA}	2084 ± 38^{aA}
	12	2072 ± 57^{aA}	665 ± 48^{aC}	1918 ± 42^{aB}	2037 ± 53^{aA}	2058 ± 46^{aA}	2097 ± 32^{aA}

[A] Mean ± standard deviation. Values at 0 h indicate the isoflavone contents of non-fermented soymilk before inoculation of lactic acid bacteria. Values in the same column of each isoflavone with different superscript small letters are statistically different ($p < 0.05$). a > b > c > d. Values in the same row with different superscript capital letters are statistically different ($p < 0.05$). A > B > C > D > E. *Si* 12 and *Ws* 4 indicate *S. infantarius* 12 and *Weissella* sp. 4, respectively.

[B] Fermentation time (h).

[C] Numbers in brackets indicate% hydrolysis of daidzin and genistin in soymilk during fermentation with different cultures. Values were calculated by 100 × (the isoflavone content in non-fermented soymilk–isoflavone content in fermented soymilk/ the isoflavone content in non-fermented soymilk).

4 showed 99% hydrolysis of both daidzin and genistin in soymilk to daidzein and genistein, respectively, in 6 h.

A rapid decrease in the isoflavone glucoside contents and an increase in the corresponding aglycone contents corresponded well to the early fermentation period when a sharp increase in β-glu activity of soymilk was observed (Fig. 3). It was in agreement with the reports of Matsuda et al. (1994), Pyo et al. (2005), Chien et al. (2006), and Otieno et al. (2006). Unexpectedly, in the present study, *Si* 12 showed very low capacity of hydrolyzing isoflavone glucosides compared to *Ws* 4 even though its β-glu activity was the highest during the 9 h of fermentation. It might be due to different characteristics between the β-glu produced by *Si* 12 and *Ws* 4 in soymilk. Some studies reported various hydrolyzing capacities of β-glu for isoflavone glucosides depending on microorganisms, growth medium, the type of substrates, and presence of isozymes (Choi et al., 1999; Matsuda et al., 1994; Matsuura & Obata, 1993; Pyo et al., 2005). Matsuura and Obata (1993) who isolated three isoforms of β-glu (A, B, and C) from soybeans showed their different activities for substrates. Relative activity for daidzin, genistin, and *p*-NPG was 100, 103, and 126, respectively, for β-glu B and 100, 136, and 213, respectively, for β-glu C (Matsuura & Obata, 1993). It indicated that genistin could be more easily hydrolyzed by β-glu, especially β-glu C, in the soybeans than daidzin. Matsuda et al. (1994) also reported different hydrolyzing activities of β-glu from *L. casei* subsp. *rhamnosus* for daidzin (62.0%), genistin (56.7%), and *p*-NPG (100%). They, in the same study, also noted that β-glu from almond could not hydrolyze soybean isoflavone glucosides (daidzin and genistin) (Matsuda et al., 1994). Considering these reports, it was speculated that *Si* 12 might produce a kind of β-glu which was relatively insufficient in hydrolyzing daidzin and genistin in soymilk but efficiently hydrolyzed β-glucosidic bond found in *p*-NPG, an artificial substrate used for assay of β-glu activity.

The biologically active estrogen-like isoflavones are the aglycone forms such as daidzein and genistein. In particular, genistein has been reported to be the most effective isoflavone for inhibiting cell growth of human prostate cancer cells (Onozawa et al., 1998) while daidzein showed higher bioavailabilities than genistein in adult woman (Xu, Wang, Murphy, Cook, & Hendrich, 1994). If isoflavone glucosides are effectively utilized in vivo, sugar moiety is first converted into aglycones by intestinal β-glu (Setchell et al., 2002). It suggests that fermented soymilk containing more aglycones is desirable since bioactive isoflavones could be absorbed directly through the intestinal epithelium without the time delay required for hydrolytic cleavage of the glucoside moiety. Results reported in the present study shows that *Ws* 4 and mixed cultures with 1:3, 1:5 and 1:10 ratios are desirable starters for development of bioactive fermented soymilk products in that they can effectively con-

vert most of the diadzin and genistin in soymilk to bioactive daidzein and genistein, respectively, in 6 h. However, under the conditions in the present study, the 12 h of fermentation period was not enough for single culture of *Ws* 4 to reach pH of 4.2–4.4, the general pH range of commercial yoghurts, while mixed cultures with *Si* 12 of 1:3, 1:5, and 1:10 ratios reached those pH values in 9 h with a 98%–99% conversion of isoflavone glucosides to aglycone forms. It shows that mixed cultures with 1:3, 1:5, and 1:10 ratios would be more effective starters for production of bioactive fermented soymilk with more aglycones and appropriate acidity in a short time.

Acknowledgements

This research was supported by funds received from the Technology Development Program for the Ministry of Agriculture and Forestry, Republic of Korea. Chun, J., was supported by the 2nd stage Brain Korea 21 program from the Ministry of Education & Human Resources Development, Republic of Korea.

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