JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 4703–4709 **4703**

Effects of Lactulose Supplementation on the Growth of Bifidobacteria and Biotransformation of Isoflavone Glycosides to Isoflavone Aglycones in Soymilk

THUY T. PHAM AND NAGENDRA P. SHAH*

School of Molecular Sciences, Faculty of Health, Engineering and Science, Victoria University, Werribee Campus, P.O. Box 14428, Melbourne, Victoria 8001, Australia

Bifidobacterium animalis subsp. *lactis* bb12 and *B. longum* 20099 were used to hydrolyze isoflavone glycosides (IG) to biologically active forms, which are isoflavone aglycones (IA), in soymilk (SM) prepared from soy protein isolate (SPI) and SM supplemented with 0.5% (w/v) of lactulose (SML). Supplementation of lactulose significantly ($p < 0.05$) enhanced the viable counts of *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 up to 2.34 and 2.15 log CFU/mL, respectively. *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099 utilized 3.32 and 3.75 mg/mL of lactulose at 24 h of incubation, respectively. Supplementation of lactulose also appeared to be a key factor in decreasing the pH of SML. The biotransformation of IG to IA was enhanced significantly by 6.8−17.1% and 12.8−13.5% in SML by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively. However, the presence of lactulose in SML showed the stimulating effect on *B. longum* 20099 only after 12 h of incubation. Isoflavone aglycones ranged from 69.5 to 77.1% of total isoflavone compounds in SML after incubation.

KEYWORDS: Soymilk; isoflavone aglycones; lactulose; isoflavone glycosides; *Bifidobacterium*; biotransformation

INTRODUCTION

Lactulose is produced during the heat treatment of lactose as a result of an isomerization reaction (Lobry de Bruyn−Alberda van Ekenstein rearrangement), which transforms β-D-galactose 1 → 4 α-D glucose of lactose to β-D-galactose 1 → 4 α-D fructose (*1*). Lactulose has been considered as a bifidogenic factor, which is able to proliferate healthy intestinal microflora (*2, 3*). Lactulose was also reported to enhance the β-glucosidase and β-galactosidase activities of intestinal microflora (*4*). Both of these enzymes were shown to hydrolyze isoflavone glycosides (IG), which are inactive phytochemical compounds, to isoflavone aglycones (IA), which are biologically active forms (*5*). Isoflavone aglycones are claimed to have estrogenic property and can be used as "natural components" to replenish the estrogen in woman at menopausal and postmenopausal age (*6–8*). Although isoflavone compounds are found abundantly in soy products, soy protein isolate (SPI) is usually employed as a source of isoflavone (*9*). In addition, SPI contains approximately 85−90% protein and has a highest score of protein digestibility corrected amino acid of between 0.95 and 1.00 (*10*). Hence, SPI is widely used in the food industry. However, SPI provides only a small amount of IA including daidzein, glycitein, genistein, biochanin A, and formononetin of the total isoflavone components in SPI (**Figure 1**) (*5*). Several methods including basic-, acidic-, and enzymatic hydrolysis are reported to convert IG to IA (*5, 11, 12*). In the past few years, β-glucosidase producing probiotic organisms have also been used to produce IA in fermented soymilk (*13–16*). These bacteria, in addition to providing this enzyme, can contribute health benefits to people consuming fermented soymilk (*17*). However, the rate of the biotransformation of IG to IA is usually low. Tsangalis et al. (*13*) reported that *B. longum* transformed only 9.8% of the total isoflavone glycosides to aglycones in soymilk after 24 h of fermentation at 37 °C. Furthermore, soymilk prepared from SPI did not support the growth of *Bifidobacterium animalis* subsp. *lactis* (*18, 19*). The low level of simple carbon available in SPI (1%) may be the reason, since the main carbohydrates including sucrose, raffinose, and stachyose are removed during processing (*20*). Therefore, it is expected that the growth of probiotic organisms could be enhanced in soymilk if it is supplemented with a carbon source such as lactulose. Lactulose is also expected to stimulate the production of β-glucosidase and β-galactosidase resulting in more efficient biotransformation of IG to IA (*4*). Therefore, the objectives of this study were to investigate the influence of the supplementation of lactulose on the growth of bifidobacteria and their biotransformation ability of IG to IA in soymilk prepared from SPI.

MATERIALS AND METHODS

Isoflavone Compounds and Other Chemicals. Genistein, daidzein, glycitein, flavone, Carrez I, Carrez II, L-cysteine·HCl, D-glucose, and

* To whom correspondence should be addressed. Phone: + 61 3 9919 8289. Fax: + 61 3 9919 8284. E-mail: Nagendra.Shah@vu.edu.au.

10.1021/jf072716k CCC: $40.75
Published on Web 05/24/2008

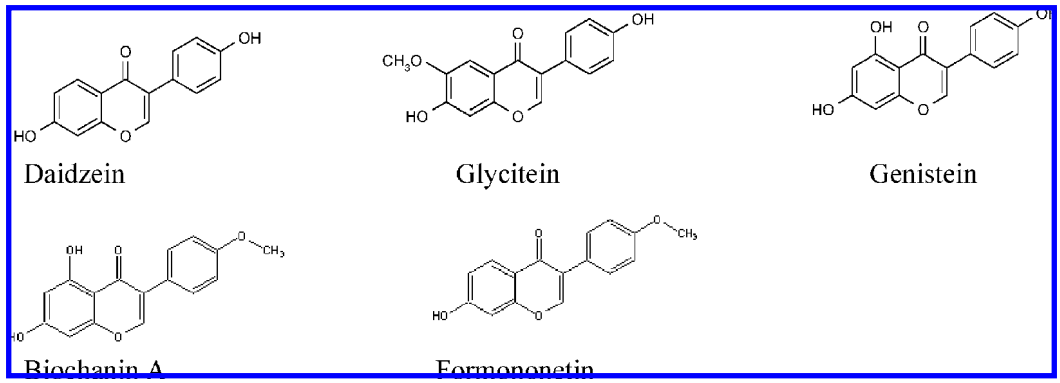

Figure 1. Chemical structure of isoflavone aglycones. Source: Hughes et al. Phytoestrogens and Health; Food Standards Agency: London, 2003; p 444.

lactulose were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Daidzin, glycitin, genistin, formononetin, and biochanin A were obtained from Indofine Chemical Company, Inc. (Summerville, NJ). Malonyl- and acetyl-β-glycosides (malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin) were obtained from LC Laboratories (Woburn, MA). Acetonitrile, methanol, ethanol, and phosphoric acid used for HPLC were of analytical grade. Soy protein isolate SUPRO 590 was from The Solae Co. (Chatswood, NSW, Australia).

Cultures and Fermentation of Soymilk Supplemented with Lactulose (SML) and Soymilk (SM) by Bifidobacteria. Frozen pure cultures of *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099 were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia). The two probiotic organisms were activated in De Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, U.K.) (pH adjusted to 6.7 $\pm$ 0.1 using 5 M NaOH) by growing successively twice at 37 °C for 20 h. The third transfer was carried out separately in SML prepared from SPI, lactulose, water (4.0, 0.5, 95.5 w/w), or in SM prepared from SPI and water (4.0, 96.0 w/w). One liter of sterile SML and SM was individually inoculated with 1% (v/v) of the active culture of probiotic organisms and anaerobically incubated at 37 °C for 24 h. Aliquots (100 mL) were withdrawn aseptically at 0, 6, 12, 18, and 24 h of incubation for enumeration of viable probiotic populations, determination of pH, and quantification of lactulose. The rest of the samples was freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavones.

Determination of pH. The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (model 8417, Hanna Instruments, Singapore) at 20 °C after calibrating with fresh pH 4.0 and 7.0 standard buffers.

Determination of Lactulose Contents. Quantification of lactulose was based on Chavez-Servin et al. (*21*) with some modifications. Briefly, one milliliter of SM or SML was dissolved in 10 mL of ethanol−water (50:50, v/v) and placed in a 60 °C water bath (model NB 6T-10935, Thermoline Australia, Scientific Equipments, Smithfield, NSW, Australia) until dissolved completely. To this, 250 μL of each of Carrez I and Carrez II solutions and 5 mL of acetonitrile were added to precipitate proteins in the samples. The solution was made up to 50 mL using ethanol−water (50:50, v/v), filtered through Advance No. 1 filter paper, a C18 Sep-pak Plus cartridge (Waters, Milford, MA), and a 0.45 μm nylon filter (Phenomenex, Lane Cove, NSW, Australia), and then injected into the HPLC system. The instrument and HPLC conditions included an Alltech Alltima (Deerfield, IL) Prevail-Carbohydrate ES (4.6 mm $\times$ 250 mm), a 5 μm particle size column, and a Hewlett-Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with an auto sampler, a quaternary pump, an Alltech light-scattering detector Varex MK III ELSD, a vacuum degasser, and a thermostatically controlled column compartment. The column temperature was kept at 25 °C. The injection volume was 20 μL. The mobile phase for isocratic HPLC was acetonitrile−water (70:30, v/v). The flow rate was 0.8 mL/min. Standard solutions for calibration curve were based on five lactulose working solutions prepared by diluting pure lactulose with methanol (50%, v/v) at various concentrations between 50 μg/mL to 500 μg/mL (*18*).

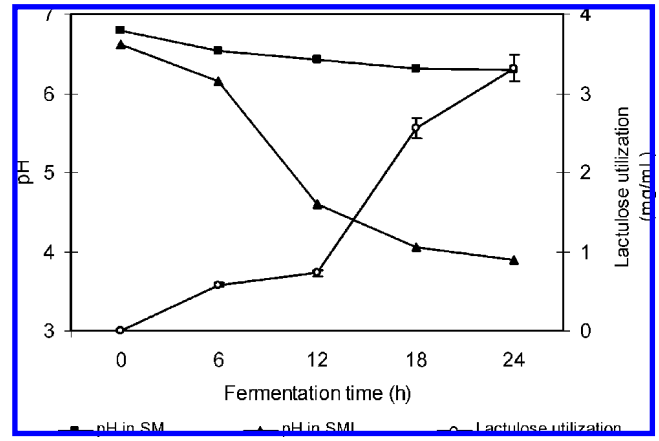

Figure 2. Changes in pH in SM and SML and lactulose utilization in SML by *B. animalis* subsp. *lactis* bb12 at 37 °C during 24 h of fermentation. Results expressed as mean $\pm$ standard error ($n = 3$).

Enumeration of Viable Micro-organisms. MRS agar supplemented with 1% (w/v) of D-glucose was used for enumeration. Filter-sterilized L-cysteine·HCl solution was also added to the final concentration of 0.05% (w/v) for bifidobacterial growth. One milliliter of serial dilutions at 0, 6, 12, 18, and 24 h of incubation was aseptically spread on to the plates and incubated at 37 °C for 3 days in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD) with a gas generating kit (Oxoid Ltd., Hampshire, U.K.). Colony counts between 25 and 250 were enumerated.

Determination of Isoflavone Contents. Extraction of isoflavone and HPLC analysis were performed in triplicate based on Griffith and Collison (*22*) and Nakamura et al. (*23*) with some modifications as described in Pham and Shah (*18*). Briefly, 1 g of freeze-dried sample was added to 10 mL of methanol (80%, v/v) and 1 mL of acetonitrile (100%, v/v) with stirring using a vortex mixer (Chiltern Scientific, Auckland, New Zealand). Then, 100 μL each of Carrez I and Carrez II solutions was added to the samples and mixed thoroughly. One hundred microliter of flavone (1 mg/mL) as the internal standard was added followed by thorough shaking. The samples were left in a water bath (model NB 6T-10935, Thermoline Australia) at 50 °C for 120 min until the proteins precipitated. The samples were then filtered through a Whatman No. 3 filter paper and a 0.45 μM Phenomenex nylon filter (Lane Cove, NSW, Australia) into a HPLC vial and then injected into the HPLC system within 4 h to avoid the degradation of malonyl- and acetyl glycosides (*22*).

The HPLC system included an Alltech Alltima (Deerfield, IL) HP C18 HL (4.6 mm $\times$ 250 mm), a 5 μm particle size column, and an Alltima HP C18HL (7.5 mm $\times$ 4.6 mm) as well as a 5 μm guard column, a Hewlett-Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with an auto sampler, a quaternary pump,

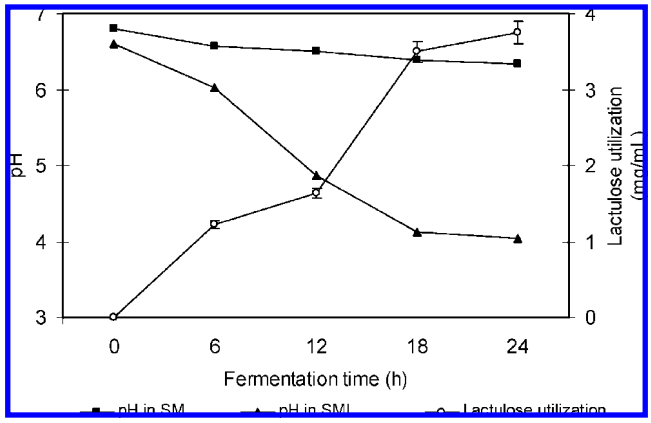

Figure 3. Changes in pH in SM and SML and lactulose utilization in SML by *B. longum* 20099 at 37 °C during 24 h of fermentation. Results expressed as mean ± standard error ($n = 3$).

a diode array ultraviolet detector, a vacuum degasser, and a thermostatically controlled column compartment. The column temperature was kept at 25 °C. The mobile phase consisted of solvent A (water−phosphoric acid, 1000:1, v/v) and solvent B (water−acetonitrile−phosphoric acid, 200:800:1, v/v/v). The gradient was as follows: solvent A 100% (0 min) → 80% (5 min) → 0% (50 min) →100% (55 min) →100% (60 min). The flow rate was 0.8 mL/min. The diode array UV detector was set at 259 nm. Stock solutions for 14 isoflavone standards were prepared by dissolving 1 mg of the crystalline pure compound in 10 mL of 100% methanol. Each solution was diluted with methanol (100%) to five working solutions at concentrations ranging from 1 to 40 μg/mL, in order to prepare standard curves.

Retention time and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavones. Isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). The moisture content of the freeze-dried soymilk samples was determined by AACC 40-40 (*24*). The biotransformation of IG to aglycones was defined as the percentage of IG hydrolyzed and was calculated as follows:

$$\%\ \text{IG hydrolysis} = \frac{\text{initial IG} - \text{residual IG}}{\text{initial IG}} \times 100$$

Statistical Analysis of Data. The fermentation trials and all analyses were performed in triplicate, and the data were analyzed using one-way analysis of variance (ANOVA) at 95% confidence intervals using Microsoft Excel Statpro, as described by Allbright et al. (*25*). ANOVA data with a $p < 0.05$ was classified as statistically significant.

RESULTS AND DISCUSSION

Lactulose Utilization by Bifidobacteria and the pH Changes in SM and SML During Incubation. Figures 2 and **3** present the lactulose utilization by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SML and the changes in pH values during the incubation, respectively. Lactulose utilization by the two probiotic organisms increased steadily during 24 h of incubation. At the end of the incubation, *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 used 68.9 and 77.8% of the initial lactulose, respectively. The pH of SM decreased slightly from 6.80 to 6.30 by *B. animalis* subsp. *lactis* bb12 and to 6.34 by *B. longum* 20099 during 24 h incubation. This result is in agreement with Tsangalis et al. (*26*), who reported that the pH of SM prepared from SPI remained high at 5.99 after 24 h of fermentation of SM by *B. animalis*. The high pH of fermented SM may be due to the lack of fermentation as a result of low levels of sugars in SPI (*27*). Garbutt (*28*) indicated that sugars metabolized by fermentative organisms make the medium more acidic; however, the medium remains alkaline if amino acids are used as a carbon source. High pH is undesirable for

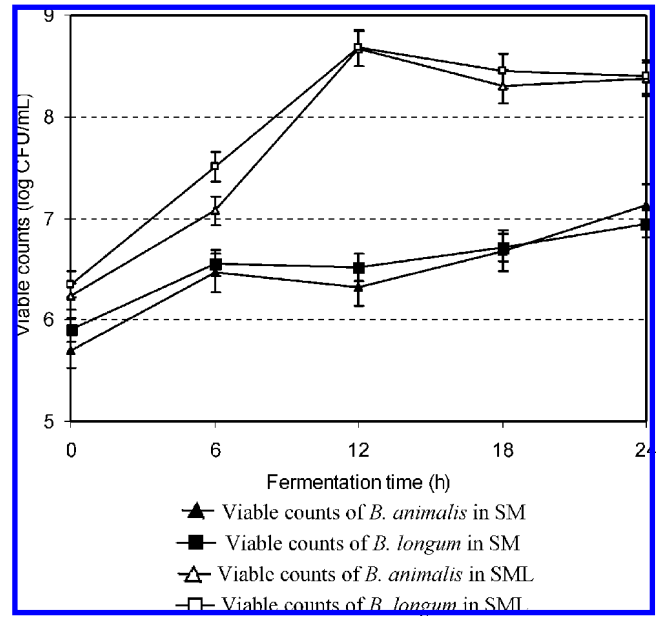

Figure 4. Viable counts of *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SM and SML during fermentation for 24 h at 37 °C. Results expressed as mean ± standard error ($n = 3$).

fermented product as spoilage may occur. However, it appeared that lactulose played a key role in lowering the pH of SML. The pH decreased rapidly from 6.61 to 3.90 and to 4.04 in SML by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively. Dubey and Mistry (*29*) reported that the supplementation of lactulose to a soy-based formula enhanced the production of lactic and acetic acids by bifidobacteria. In our study, although *B. longum* 20099 utilized higher level of lactulose than *B. animalis* subsp. *lactis* bb12, the pH remained higher than the medium fermented by *B. animalis* subsp. *lactis* bb12. Kontula et al. (*30*) indicated that the end products of the lactulose fermentation by lactic acid bacteria are not only organic acids but also CO2 and ethanol, which may also affect the final pH.

Viable Counts of Bifidobacteria in SML and SM during Incubation. Figure 4 shows the viable counts of the bifidobacteria in SML and SM. *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099 showed a similar level of growth in both SM and SML during the incubation. The viable counts of both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SM increased slightly from 5.70 to 7.12 and 5.90 to 6.94 log CFU/mL, respectively, after 24 h of incubation. This suggests that SM did not support their growth well, possibly because of the lack of simple sugars in SM (*10, 20*). However, the growth of the probiotic organisms was significantly increased ($p < 0.05$) on supplementation with lactulose, as indicated by cell counts. During 24 h of incubation, the viable counts of both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 increased to 8.37 and 8.40 log CFU/mL, respectively. It appeared that lactulose was favored by the probiotic organisms as they grew well in SML (**Figures 2**, **3**, and **4**). Lactulose also increased the viability of some *Lactobacillus* strains including *L. casei* and *L. zeae* in the study of Desai et al. (*31*). Salminen and Salminen (*2*) and Kontula et al. (*30*) also reported that lactulose promoted the growth of *L. acidophilus*. It has been suggested that in order to provide health benefits, the viable number of probiotic organisms must be above 10^7 cfu/g of a fermented product (*32*).

Biotransformation of IG to Aglycones in SML and SM by Bifidobacteria. The moisture content of the freeze-dried samples ranged from 1.9 to 2.0%. There was no significant

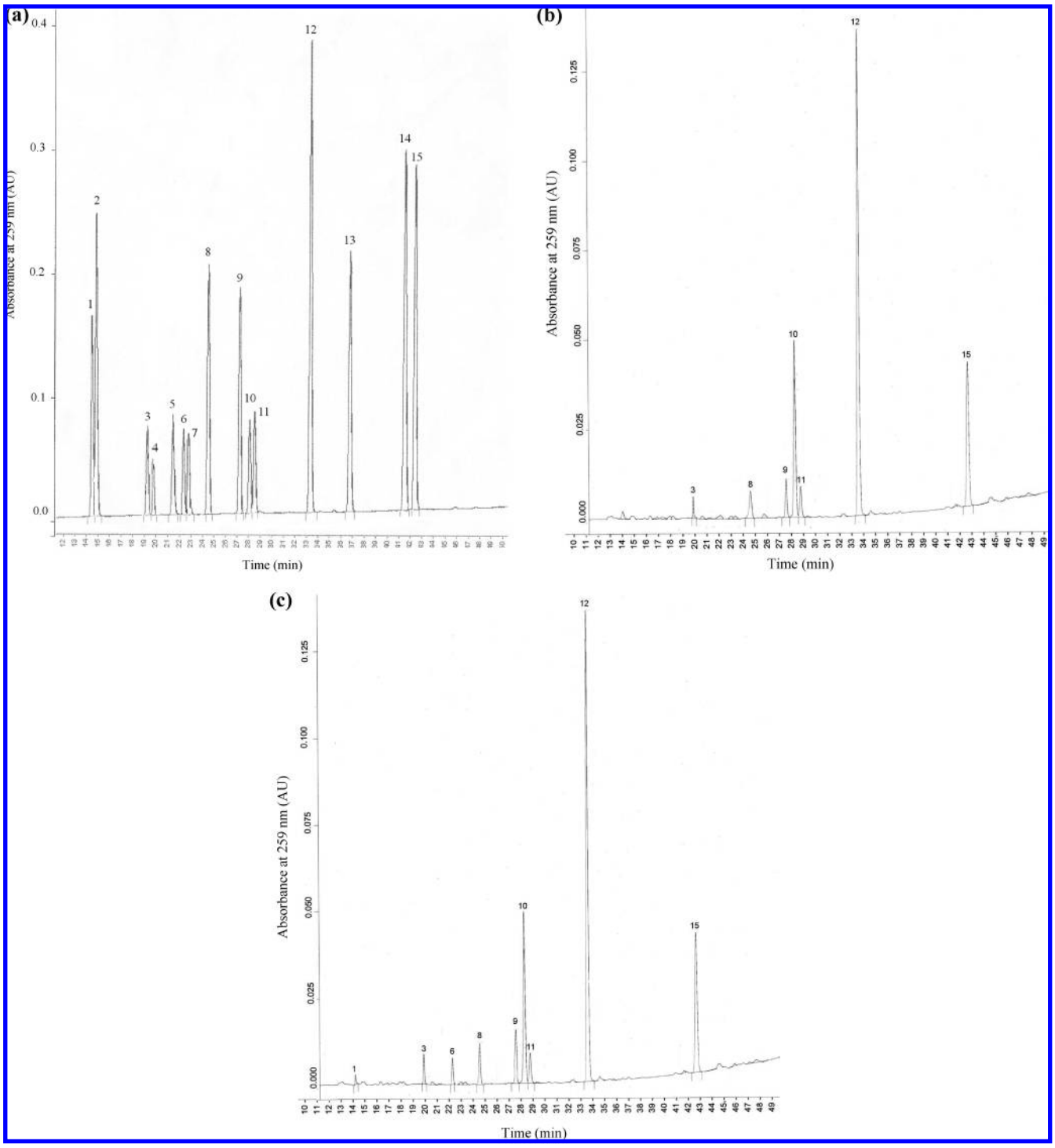

Figure 5. Chromatograms of isoflavone compounds obtained in an HPLC system coupled to a diode array detector. (**5a**) Chromatograms of 14 isoflavone standards and flavone. The peaks are as follows: 1, daidzin (20 μg/mL); 2, glycitin (40 μg/mL); 3, malonyl daidzin (8 μg/mL); 4, malonyl glycitin (4 μg/mL); 5, genistin (10 μg/mL); 6, acetyl daidzin (10 μg/mL); 7, acetyl glycitin (8 μg/mL); 8, malonyl genistin (24 μg/mL); 9, acetyl genistin (20 μg/mL); 10, daizein (8 μg/mL); 11, glycitein (8 μg/mL); 12, genistein (32 μg/mL); 13, biochanin A (24 μg/mL); 14, formononetin (24 μg/mL); and 15, flavone (20 μg/mL). (**5b**) Chromatograms of isoflavone compounds in SML at 24 h of fermentation at 37 °C by *B. animalis* subsp. *lactis* bb12. The peaks are as follows: 3, malonyl daidzin; 8, malonyl genistin; 9, acetyl genistin; 10, daizein; 11, glycitein; 12, genistein; and 15, flavone. (**5c**) Chromatograms of isoflavone compounds in SM at 24 h of fermentation at 37 °C by *B. animalis* subsp. *lactis* bb12. The peaks are as follows: 1, daidzin; 3, malonyl daidzin; 6, acetyl daidzin; 8, malonyl genistin; 9, acetyl genistin; 10, daizein; 11, glycitein; 12, genistein; and 15, flavone.

difference in the moisture content of the freeze-dried samples ($p > 0.05$). Therefore, we assumed that there was no effect of the moisture content on the quantification of isoflavone compounds. The HPLC chromatogram and the retention times of 14 standard isoflavone compounds and the internal standard are shown in **Figure 5**. The flavone as the internal standard eluted at 42 min and segregated from isoflavone compounds without overlapping. The detection limit of HPLC analysis was approximately 10^{-8} g/mL. There were only 7 IG found in SM at 0 h (**Tables 1** and **2**). Genistein was the only IA detected in SM at 0 h and was at a very low concentration (4.50 mg/100 g of freeze-dried SM). Biochanin A and formononetin were not

Table 1. Biotransformation of IG to IA in SML and SM by *B. animalis* subsp. *lactis* bb12 at 37 °C during 24 h Incubation[1]

isoflavone (mg/100 g of freeze-dried sample)	SML					SML				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
daidzin	12.52 ± 1.07[a]	3.98 ± 0.32[b]	1.89 ± 0.15[c]	ND	ND	14.03 ± 0.70[a]	9.34 ± 0.75[b]	6.01 ± 0.54[c]	3.21 ± 0.25[d]	2.98 ± 0.21[d]
glycitin	5.36 ± 0.34[a]	5.01 ± 0.54[a]	1.88 ± 0.26[b]	1.69 ± 0.23[b]	ND	6.13 ± 0.10[a]	5.50 ± 0.60[a]	3.20 ± 0.36[b]	1.51 ± 0.24[c]	ND
genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
malonyl daidzin	22.09 ± 1.58[a]	9.52 ± 0.52[b]	7.21 ± 0.25[bc]	5.11 ± 0.29[cd]	4.05 ± 0.31[d]	24.49 ± 1.69[a]	17.04 ± 1.04[b]	8.31 ± 0.71[c]	6.50 ± 0.42[cd]	5.80 ± 0.35[d]
malonyl glycitin	2.62 ± 0.25	ND	ND	ND	ND	3.02 ± 0.07	ND	ND	ND	ND
malonyl genistin	57.83 ± 4.25[a]	29.86 ± 2.12[b]	10.21 ± 0.56[c]	9.99 ± 0.74[c]	9.52 ± 0.45[c]	67.23 ± 2.02[a]	29.35 ± 1.37[b]	22.61 ± 1.57[c]	20.25 ± 2.12[c]	18.33 ± 1.04[c]
acetyl daidzin	5.71 ± 0.65[a]	5.61 ± 0.62[a]	3.02 ± 0.28[b]	3.00 ± 0.32[b]	ND	6.41 ± 0.19[a]	6.20 ± 0.51[a]	4.21 ± 0.36[b]	3.50 ± 0.24[bc]	2.98 ± 0.20[c]
acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
acetyl genistin	24.01 ± 1.98[a]	11.57 ± 0.88[b]	5.33 ± 0.41[c]	5.26 ± 0.39[c]	5.21 ± 0.52[c]	27.50 ± 1.63[a]	17.60 ± 1.08[b]	14.87 ± 1.21[b]	13.85 ± 1.17[b]	13.25 ± 1.11[b]
total IG	130.14 ± 6.18[a]	65.55 ± 2.08[b]	29.54 ± 1.09[c]	25.05 ± 1.33[cd]	18.78 ± 1.71[d]	148.81 ± 2.88[a]	85.03 ± 4.15[b]	59.21 ± 1.61[c]	48.82 ± 1.62[d]	43.34 ± 2.01[d]
daidzein	ND	12.21 ± 1.00[a]	17.25 ± 1.02[b]	18.78 ± 1.32[b]	19.45 ± 1.56[b]	ND	6.32 ± 0.45[a]	16.87 ± 1.25[b]	17.75 ± 1.32[b]	18.42 ± 1.24[b]
glycitein	ND	1.36 ± 0.16[a]	3.22 ± 0.25[b]	3.35 ± 0.32[b]	4.01 ± 0.45[b]	ND	2.12 ± 0.22[a]	3.25 ± 0.25[a]	3.95 ± 0.31[a]	4.25 ± 0.54[a]
genistein	3.95 ± 0.45[a]	26.75 ± 1.85[b]	39.25 ± 2.54[c]	39.65 ± 3.11[c]	39.75 ± 2.96[c]	4.50 ± 0.32[a]	30.31 ± 2.46[b]	37.97 ± 2.11[c]	38.03 ± 2.58[c]	39.21 ± 2.59[c]
total IA	3.95 ± 0.45[a]	40.32 ± 3.01[b]	59.72 ± 3.81[c]	61.78 ± 4.11[c]	63.21 ± 4.97[c]	4.50 ± 0.32[a]	38.75 ± 3.13[b]	58.09 ± 3.61[c]	59.73 ± 1.57[c]	61.88 ± 3.29[c]
IG hydrolyzed (%)	0.0	49.6	77.3	80.8	85.6	0.0	42.9	60.2	67.2	70.9

[1] Results expressed as mean ± standard error ($n = 3$). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different ($p > 0.05$). Abbreviations used: IG, isoflavone glycosides; ND, not detected (the isoflavone content that was in 1 g of freeze-dried soymilk used to extract isoflavones with a sample injection volume of 20 μL was lower than the detection limit of the method); SML, soymilk supplemented with lactulose; SM, soymilk; IG, isoflavone glycosides; IA, isoflavone aglycones.

Table 2. Biotransformation of IG to IA in SML and SM by *B. longum* 20099 at 37 °C during 24 h Incubation[1]

isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
daidzin	12.52 ± 1.07[a]	9.27 ± 1.06[b]	4.05 ± 0.42[c]	2.05 ± 0.42[c]	ND	14.03 ± 0.70[a]	6.59 ± 0.42[b]	3.20 ± 0.24[c]	1.50 ± 0.15[d]	ND
glycitin	5.36 ± 0.34[a]	5.00 ± 0.66[a]	2.01 ± 0.19[b]	1.79 ± 0.21[b]	1.59 ± 0.19[b]	6.13 ± 0.10[a]	5.41 ± 0.62[a]	3.06 ± 0.39[b]	2.35 ± 0.32[bc]	1.92 ± 0.20[c]
genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
malonyl daidzin	22.09 ± 1.58[a]	16.83 ± 1.12[b]	8.21 ± 0.75[c]	5.11 ± 0.75[c]	5.08 ± 0.68[c]	24.49 ± 1.69[a]	17.51 ± 0.37[b]	10.65 ± 0.32[c]	6.21 ± 0.70[d]	4.61 ± 0.43[d]
malonyl glycitin	2.62 ± 0.25[a]	1.63 ± 0.25[b]	ND	ND	ND	3.02 ± 0.07	ND	ND	ND	ND
malonyl genistin	57.83 ± 4.25[a]	43.92 ± 2.54[b]	14.29 ± 0.98[c]	13.25 ± 0.89[c]	11.08 ± 0.78[c]	67.23 ± 2.02[a]	31.49 ± 1.75[b]	29.87 ± 1.54[bc]	28.85 ± 1.65[bc]	26.90 ± 1.82[c]
acetyl daidzin	5.71 ± 0.65[a]	5.21 ± 0.54[ab]	4.99 ± 0.51[b]	3.20 ± 0.34[c]	3.15 ± 0.49[c]	6.41 ± 0.19[a]	5.31 ± 0.62[a]	5.11 ± 0.59[a]	3.86 ± 0.53[ab]	3.19 ± 0.23[b]
acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
acetyl genistin	24.01 ± 1.98[a]	14.09 ± 1.01[b]	6.32 ± 0.35[c]	6.21 ± 0.58[c]	6.25 ± 0.41[c]	27.50 ± 1.63[a]	16.50 ± 1.06[b]	13.45 ± 1.19[b]	13.39 ± 1.08[b]	13.54 ± 0.99[b]
total of IG	130.14 ± 6.18[a]	95.95 ± 4.66[b]	39.87 ± 1.66[c]	31.61 ± 1.19[c]	27.15 ± 1.78[c]	148.81 ± 2.88[a]	82.81 ± 2.02[b]	65.34 ± 2.45[c]	56.16 ± 2.27[d]	50.16 ± 2.81[d]
daidzein	ND	4.68 ± 0.65[a]	13.52 ± 1.05[b]	17.80 ± 1.11[c]	19.03 ± 1.02[c]	ND	7.89 ± 1.25[a]	14.89 ± 1.54[a]	18.67 ± 1.29[a]	20.40 ± 1.47[a]
glycitein	ND	0.69 ± 0.15[a]	3.19 ± 0.29[b]	3.33 ± 0.21[b]	3.51 ± 0.29[b]	ND	1.96 ± 0.28[a]	3.45 ± 0.21[a]	3.92 ± 0.27[a]	3.98 ± 0.33[a]
genistein	3.95 ± 0.45[a]	16.92 ± 1.22[b]	37.37 ± 3.01[c]	38.23 ± 2.65[c]	39.41 ± 3.14[c]	4.50 ± 0.32[a]	29.80 ± 1.38[b]	33.83 ± 2.14[bc]	33.52 ± 1.78[bc]	35.27 ± 2.50[c]
total of IA	3.95 ± 0.45[a]	22.29 ± 0.42[b]	54.08 ± 4.35[c]	59.36 ± 3.97[c]	61.95 ± 4.45[c]	4.50 ± 0.32[a]	39.65 ± 2.91[b]	52.17 ± 0.39[bc]	56.11 ± 2.80[bc]	59.65 ± 3.64[c]
IG hydrolyzed (%)	0.0	26.3	69.4	75.7	79.1	0.0	44.4	56.1	62.3	66.3

[1] Results expressed as mean ± standard error ($n = 3$). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different ($p > 0.05$). Abbreviations used: IG, isoflavone glycosides; ND, not detected (the isoflavone content that was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 μL was lower than the detection limit of the method); SML, soymilk supplemented with lactulose; SM, soymilk; IG, isoflavone glycosides; IA, isoflavone aglycones.

detected during the fermentation by both strains of *Bifidobacterium*. This suggests that ononin (biochanin A glycoside) and sissotrin (formononetin glycoside) were not available in SPI. The initial IG in SM and SML at 0 h were 148.81 and 130.14 mg/100 g of freeze-dried matter, respectively. The lower initial level of the isoflavone compounds in SML was due to the supplementation of lactulose.

The biotransformation of IG to IA in SML and SM by *B. animalis* subsp. *lactis* bb12 is shown in **Table 1**. In general, the biotransformation of IG to IA occurred rapidly in the first 12 h of incubation. In the following 12 h of incubation, the level of biotransformation increased slowly. There was no significant difference ($p > 0.05$) between the IA produced at 12, 18, and 24 h of incubation in both SM and SML by *B. animalis* subsp. *lactis* bb12. Acetyl daidzin appeared to be more stable than daidzin during the fermentation. At 18 h of incubation, daidzin was completely hydrolyzed, compared to 47.5% of acetyl daidzin converting to daidzein in SML. Similarly, at 18 h of incubation in SM, 77.1% and 45.4% of daidzin and acetyl daidzin were hydrolyzed, respectively. Mathias et al. (*12*) reported that acetyl daidzin was fairly stable in a low pH condition. Our study showed that supplementation of lactulose extensively enhanced the biotransformation level of IG to IA during incubation. The level of biotransformation in SML ranged from 49.6% to 85.6%, which was 6.7−14.7% higher than that in SM. At the end of incubation, IA comprised 77.1% (63.21 mg/100 g of freeze-dried sample) compared to 58.8% (61.88 mg/100 g of freeze-dried sample) of the total isoflavone compounds in SML and SM, respectively. Daidzin and acetyl daidzin were hydrolyzed entirely in SML; however, they were still present in SM after 24 h of incubation.

Table 2 shows the biotransformation of IG to IA in SML and SM by *B. longum* 20099. Similar to *B. animalis* subsp. *lactis* bb12, the biotransformation of IG to IA occurred rapidly in the first 12 h of incubation. Although the initial level of glycitin was lower than that of daidzin, it was still detected at 24 h of incubation, while daidzin was completely hydrolyzed in both SML and SM. This suggests that *B. longum* 20099 transformed daidzin more efficiently than glycitin. The supplementation of lactulose increased the biotransformation of IG to IA by *B. longum* 20099 from 12.8 to13.4%. However, the stimulating effect was only observed from 12 h of incubation (**Table 2**). At 6 h of incubation, IG were transformed to IA at the lower level in SML (26.3%) compared to that in SM (44.4%). At the end of incubation, IA increased from 2.9 to 69.5% of the total isoflavones in SML compared to 54.3% in SM. As regards the residual IG after 24 h of incubation, *B. animalis* subsp. *lactis* bb12 hydrolyzed β-glycosides genistin better than *B. longum* 20099 did in both SM and SML, while *B. longum* 20099 hydrolyzed daidzin more effectively than *B. animalis* subsp. *lactis* bb12 in SM. In general, *B. animalis* subsp. *lactis* bb12 exhibited better biotransformation of IG to IA than *B. longum* 20099 in both SML and SM (**Tables 1** and **2**). On the other hand, the lactulose utilization did not show any relationship with the level of biotransformation of IG to IA. *Bifidobacterium animalis* subsp. *lactis* bb12 utilized a lower level of lactulose than that of *B. longum* 20099, but the biotransformation level was higher during the incubation.

Tsangalis et al. (*13*) reported that *Bifidobacterium pseudolongum* converted 57.8% of IG to aglycones in SM after 24 h of incubation while, in the study of Chien et al. (*15*), *B. longum* hydrolyzed only 6.4% of IG to IA after 32 h of incubation. Therefore, the biotransformation level appeared to vary widely among probiotic organisms. Juskiewicz and Zdunczyk (*4*) suggested that the β-glucosidase and β-galactosidase activities of microorganisms from the gut of rats were enhanced extensively when they were fed a diet rich in lactulose. In addition, to utilize lactulose, the two strains of *Bifidobacterium* must have produced β-D-galactosidase to hydrolyze a lactulose molecule into two simple sugars including galactose and fructose. Hence, the presence of lactulose in SML may have enhanced the two enzymes produced by *Bifidobacterium*, and as the result, the biotransformation of IG to IA was enhanced.

Conclusion. Lactulose appeared to be a favorable carbon source for both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, as the supplementation of lactulose supported their growth. The viable counts of bifidobacteria in SML were significantly higher ($p < 0.05$) than those in SM during the entire incubation, although the presence of lactulose plays a key role in decreasing the pH values in media. The lowering of pH of SML due to supplementation of lactulose may have enhanced the biotransformation of IG to IA. The biotransformation increased up to 17.1% by the probiotic organisms in the presence of lactulose after 12 h of incubation. The fermentation of both SML and SM could be completed in 18 h, since not much biotransformation occurred beyond this period.

ABBREVIATIONS USED

SML, Soymilk supplemented with lactulose; SM, soymilk; SPI, soy protein isolate; IG, isoflavone glycosides; IA, isoflavone aglycones.

LITERATURE CITED

(1) Chavez-Servin, J. L.; Romeu-Nadal, M.; Castellote, A. I.; Lopez-Sabater, M. C. Evolution of free mono and di-saccharide content of milk-based formula powder during storage. *Food Chem.* **2006**, *97*, 103–108.

(2) Salminen, S.; Salminen, E. Lactulose, lactic acid bacteria, intestinal microecology and mucosal protection. *Scand. J. Gastroenterol., Suppl.* **1997**, *222*, 45–48.

(3) Gonzales, A. S. P.; Naranjo, G. B.; Malec, L. S.; Vigo, M. S. Available lysine, protein digestibility and lactulose in commercial infant formulas. *Int. Dairy J.* **2003**, *13*, 95–99.

(4) Juskiewicz, J.; Zdunczyk, Z. Lactulose-induced diarrhoea in rats: effects on caecal development and activities of microbial enzymes. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2002**, *133*, 411–417.

(5) Pham, T. T.; Shah, N. P. Hydrolysis of isoflavone glycosides in soymilk by beta-galactosidase and beta-glucosidase. *J. Food Biochem.* Accepted for publication.

(6) Setchell, K. D. R. Soy isoflavones—Benefits and risks from nature's selective estrogen receptor modulators (SRRMs). *J. Am. Coll. Nutr.* **2001**, *20*, 354–365.

(7) Setchell, K. D. R.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131*, 1362–1375.

(8) Setchell, K. D. R.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *139*, 758–767.

(9) Soyfoods Association of North America Soy Protein Isolate. http://www.soyfoods.org/products/soy-fact-sheets/soy-protein-isolate/ (accessed August 15, 2007).

(10) Riaz, M. N. *Soy application in food*; Taylor & Francis Group: Boca Raton, FL, 2006; p 288.

(11) Delmonte, P.; Perry, J.; Rader, J. I. Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: extraction followed by basic or acid hydrolysis. *J. Chromatogr., A* **2006**, *1107*, 59–69.

(12) Mathias, K.; Ismail, B.; Corvalan, C. M.; Hayes, K. D. Heat and pH effects on the conjugated form of genistin and daidzin isoflavones. *J. Agric. Food Chem.* **2006**, *54*, 7495–7502.
(13) Tsangalis, D.; Ashston, J. F.; McGill, A. E. J.; Shah, N. P. Enzymic transformation of isoflavone phytoestrogens in soymilk by beta-glucosidase producing bifidobacteria. *J. Food Sci.* **2002**, *67*, 3104–3113.
(14) Wei, Q.-K.; Chen, T.-R.; Chen, J.-T. Using of *Lactobacillus* and *Bifidobacterium* to product the isoflavone aglycones in fermented soymilk. *Int. J. Food Microbiol.* **2007**, *117*, 120–124.
(15) Chien, H.-L.; Huang, H. Y.; Chou, C. C. Transformation of isoflavone phytoestrogens during the fermentations of soymilk with lactic acid bacteria and bifidobacteria. *Food Microbiol.* **2006**, *23*, 772–778.
(16) Otieno, D. O.; Ashton, J. F.; Shah, N. P. Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobaterium animalis*, *Lactobacillus acidophilus*, and *Lactobacillus casei*. *Food Res. Int.* **2006**, *39*, 394–407.
(17) Shah, N. *Probiotics and fermented milks in: Manufacturing yogurt and fermented milks*; Blackwell Publishing Ltd.: Oxford, U.K., 2006; pp 341−354.
(18) Pham, T. T.; Shah, N. P. Biotransformation of isoflavone glycosides by Bifidobacterium animalis in soymilk supplemented with skim milk powder. *J. Food. Sci.* **2007b**, *72*, M316–M324.
(19) Kamaly, K. M. Bifidobacteria fermentation of soy bean milk. *Food Res. Int.* **1997**, *30*, 675–682.
(20) Snyder, H. E.; Kwon, T. W. *Soybean utilization*; Van Nostrand Reinhold: New York, 1987; p 346.
(21) Chavez-Servin, J. L.; Castellote, A. I.; Lopez-Sabater, M. C. Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. *J.Chromatogr., A* **2004**, *1043*, 211–215.
(22) Griffith, A. P.; Collison, M. W. Improved methods for the extraction and analysis of isoflavones from soy- containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography -mass spectrometry. *J. Chromatogr., A* **2001**, *913*, 397–413.
(23) Nakamura, Y.; Kaihara, A.; Yoshii, K.; Tsumura, Y.; Ishimitsu, S.; Tonogai, Y. Content and composition of isoflavonoids in mature or immature beans and bean sprouts consumed in Japan. *J. Health Sci.* **2001**, *47*, 394–406.
(24) American Association of Cereal Chemists Approved Methods Comittee. *Approved methods of the American Association of Cereal Chemists*, 10th ed.; American Association of Cereal Chemists: St. Paul, MN, 2000.
(25) Allbright, S.; Winston, W.; Zappe, C. *Data analysis and decision making with Microsoft Excel*; Brooks/Cole Publishing Co.: Pacific Grove, CA, 1999; p 996.
(26) Tsangalis, D.; Ashton, J. F.; Stojanovska, L.; Wilcox, G.; Shah, N. P. Delopment of an isoflavone aglycone-enriched soymilk using germ, soy protein isolate and bifidobacteria. *Food Res. Int.* **2004**, *37*, 301–312.
(27) Nutrition Data Soy Protein Isolate. http://www.nutritiondata.com/facts-C00001−01c218n.html (accessed August 15, 2007).
(28) Garbutt, J. *Essentials of Food Microbiology*; Arnold: London, 1997.
(29) Dubey, U. K.; Mistry, V. V. Effect of bifidogenic factors on growth characteristics of bifidobacteria in infant formulas. *J. Dairy Sci.* **1996**, *79*, 1156–1163.
(30) Kontula, P.; Suihko, M.-L.; Wright, A. V.; Mattila-Sandholm, T. The effect of lactose derivatives on intestinal lactic acid bacteria. *J. Dairy Sci.* **1999**, *82*, 249–256.
(31) Desai, A. R.; Powell, I. B.; Shah, N. P. Survival and activity of probiotic lactobacilli in skim milk containing prebiotics. *J. Food. Sci.* **2004**, *69*, FMS57–FMS60.
(32) Ouwehan, A. C.; Salminen, S. J. The health effects of cultured milk products with viable and non-viable bacteria. *Int. Dairy J.* **1998**, *8*, 749–758.

Received for review September 12, 2007. Revised manuscript received February 10, 2008. Accepted April 2, 2008.

JF072716K