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Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842

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Abstract: Crude enzyme extracts from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5, and 1.0 g/L to hydrolyse glycitin (isoflavone glycosides) to its biologically active form (isoflavone aglycones; IA) in soymilk (SM) prepared from soy protein isolate (SPI) supplemented with 2.0% (w/v) of D-glucose. Enumeration of microbial populations, measurement of pH and quantification of isoflavones was carried out at 0 h, 6 h and 12 h of fermentation. The quantification of isoflavone compounds in SM was carried out using HPLC. The biotransformation of glycitin was higher at the enzyme level of 1.0 g/L from *B. animalis* Bb12 at 12 h than that at 0.5 g/L or 0.1 g/L, and the level of biotransformation was 74.4%, while 75.23% of glycitin was biotransformed with the enzyme extracted from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at the same level of enzyme. The decrease in pH by *B. animalis* Bb12 was lowest with 1.0 g/L and highest with the control (4.69). Similarly, the decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with 1.0 g/L (5.19) and highest with the control (5.86). The final viable population of the *B. animalis* Bb12 ranged from 5.94 to 7.49 log CFU/mL and that of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 4.42 to 6.70 log CFU/mL and the organisms showed the highest viable population of 6.70 log CFU/mL at 12 h with 1.0 g/L crude enzyme.

Keywords: Aglycones, glycosides, soy protein isolate, biotransformation, isoflavones, *Lactobacillus, Bifidobacterium*, extracted crude enzyme

Introduction

Soy protein isolate (SPI) is made from defatted soy meal by removing fat and carbohydrates, resulting in a product containing 90% protein. Soymilk made from soy protein isolate (SPI) has no undesirable flavour (Shurtleff and Aoyagi, 1984). However, soymilk made from SPI has reduced level of biologically active isoflavone due to losses during protein isolation (Wang and Murphy, 1996). Soymilk generally contains a total of 4 to 12 mg isoflavones per 100 g (King and Bignell, 2000; Tsangalis *et al.,* 2002) but is subject to considerable variation in isoflavone content and composition (Murphy *et al.,* 1999; King and Bignell, 2000).

The phytoestrogens found abundantly in soybeans consist of the di-phenolic, isomeric family of compounds named isoflavones. Soybean and soy-food derived isoflavones are found in 4 chemical forms, including aglycones, malonyl-, acetyl-, and glucoside conjugates. The biologically active, estrogen-like isoflavone isomers are the aglycone configurations of genistein, daidzein, and glycitein (Setchell and Cassidy, 1999). Aglycone isomers are able to bind to estrogen receptor sites and hence mimic the functions of estradiol in the human body (Setchell, 1998; Setchell and Cassidy, 1999). From reviews of epidemiological (Cassidy, 1996; Setchell, 1998) and small-scale human clinical studies, isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders common in current Western civilizations. Genistein has been demonstrated to promote the health of human beings by reducing the occurrence of specific chronic diseases, namely, cancer and atherosclerosis (Lee *et al.,* 1991; Witztum, 1994). Daidzein and genistein have been documented to have beneficial effects on osteoporosis (Anderson *et al.,* 1987). Soy isoflavones have also been shown to relieve menopause symptoms (Aldercreutz *et al.,* 1992). Asian populations with their high intake (50 to 70 mg/d) of soy-derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms, and mortality from cardiovascular disease and cancer. According to Murphy *et al.* (1999) and Tsangalis *et al.* (2002) of the total concentration of isoflavones in soymilk greater than 90% of the isomers exist as glucosidic forms. Izumi *et al.* (2000) found that aglycone forms were absorbed faster and in greater amounts than their glucosides in humans. Furthermore, Setchell *et al.* (2002) reported that isoflavone glucosides were not absorbed through the human gut wall, and their bioavailability required initial hydrolysis of the sugar moiety by intestinal β-glucosidases. This suggests that consuming isoflavone aglycone-rich soy foods may be more effective in preventing chronic diseases.

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β-Galactosidase (β-gal) is an essential enzyme used for effective conversion of isoflavone glycoside to aglycones (Pandjaitan *et al.*, 2000). β-Gal has superior activity for hydrolysing acetyl-glycoside and malonyl-glycoside isoflavones. If β-gal can effectively convert acetyl-glycoside and malonyl-glycoside to their aglycones, it can lead to an enhancement of isoflavone aglycones in soy protein isolate. Shah and Lankaputhra (2002) have reported that the genus *Bifidobacterium* constitutes a major part of the natural microflora of the human intestinal tract. Tsangalis *et al.* (2004) revealed that *B. animalis* Bb12 hydrolysed isoflavone glucosides into aglycones when grown in soymilk increasing the concentration of aglycones from 8 to 50% of total isoflavones. In human clinical studies, *B. animalis* Bb-12 has shown to effectively modulate intestinal microflora (Playne, 2002). Our objective was to examine the effectiveness of crude enzyme extract from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in biotransformation of glycitin, an isoflavone glycoside (IG) to their aglycones in soymilk made from SPI.

Materials and Methods

Isoflavone compounds and other chemicals

Genistein, daidzein, and flavone were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Glycitin, Formononetin, and Biochanin A were obtained from Indofine Chemical Co. (Summerville, N.J., U.S.A.). 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).

Bacterial growth and media

The organisms were activated by two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) incubated at 37ºC for *B. animalis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The activated cultures were again inoculated into MRS broth and inoculated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* spp. *bulgaricus* ATCC 11842 for 18 h. The third transfer was carried out in 4% (w/v) SPI containing 2.0% (w/v) D-glucose (Prahran Health Foods, Prahran, Vic., Australia) prepared as per Tsangalis *et al*., (2002). For production of extract crude enzyme, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 × g for 20 min at 10ºC). The supernatant was discarded and cell pellets were collected. A total of 5 mL of 0.03 M sodium phosphate buffer (pH 6.8) was added and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme.

Enumeration of viable microorganisms

One millilitre sample was used for the enumeration of populations of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. MRS agar supplemented with 1% (w/v) D-glucose was used for enumeration of the organisms. Peptone and water at 0.15% (w/v) diluent was used to perform serial dilutions. One millilitre of serially diluted samples at 0, 6, and 12 h was aseptically spread onto the plates and incubated at 37°C for *B. animalis* Bb12 and 45°C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (Merk Pty Limited, Kilsyth, Vic, Australia) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

Isoflavone standard solution and calibration curves

Stock solutions of isoflavone standards such as glycitin, daidzein, genistein, biochanin A and formononetin were prepared by dissolving 1 g of crystalline pure compound in 10 mL of 100% methanol. Each solution was diluted with methanol (100%) to 5 working solutions at concentration ranging from 1 to 40 µg/mL in order to prepare a standard curve. Retention time and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavones.

Determination of isoflavone content

Extraction of isoflavones for HPLC analysis

The extraction of isoflavone aglycones and glucoside isomers and HPLC analysis was performed in triplicate based on Griffith and Collison (2001) and

Nakamura and *et al.* (2001) with some modifications as described in Pham and Shah (2007). Briefly, 10 mL of methanol (80%, v/v) and 1 mL of acetonitrile (100%, v/v) were added to 1 g of freeze-dried sample with stirring using a vortex mixer (Chiltern Scientific, Auckland, New Zealand). In addition, 100 μL each of Carrez I and Carrez II solutions were added to the samples and mixed thoroughly. Furthermore, 100 μL 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 2 h until the proteins precipitated. The samples were then filtered through a Whatman No. 3 filter paper and a 0.45 μM Phenomenex nylon filter into an HPLC vial then injected into HPLC system within 4 h to avoid the degradation (Griffith and Collison, 2001). The HPLC system included an Alltech Alltima HP C18 HL (4.6 × 250 mm), a 5-μm particle size column and an Alltima HP C18HL (7.5 × 4.6 mm), a 5 μm guard column, Hewlett Packard 1100 series HPLC with an autosampler, a quaternary pump, a diode array ultraviolet detector, a vacuum degasser, and a thermostatically controlled column compartment. 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. A diode array UV detector was set at 259 nm.

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 (AACC 2000) methods. The biotransformation of IG to IA was defined as percentage of IG hydrolyzed and was calculated as follows:

$$\text{Percent glycitin (IG) hydrolysis} = \frac{\text{initial glycitin} - \text{residual glycitin}}{\text{initial glycitin}} \times 100$$

Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

Results and Discussion

pH changes during incubation

The effect of change of pH in soy protein isolate during incubation as affected by growing *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 1 and 2. In general, pH value of 1.0 g/L crude enzyme extract (CEE) was lower ($p>0.05$) compared with other crude enzyme concentrations for both organisms. The pH value was significantly ($p<0.05$) higher in control (without crude enzyme extract) and 0.1g/L of CEE than the others in both organisms. Both organisms showed no significantly different ($p>0.05$) pH values at 0 h and 6 h at different crude enzyme concentrations including control and a significant difference ($p<0.05$) was found at 12 h between 1.0 g/L and 0.5g/L CEE than the others. The decrease in pH by *B. animalis* Bb12 was lowest with 1.0 g/L (4.35) followed by 0.5 g/L (4.45) and the highest with the control (4.69). The pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82 percent in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE respectively, at 12 h as compared with 0 h (Figure 1). Similarly, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with 1.0 g/L (5.19) followed by 0.5 g/L (5.52) and highest with the control (5.86). At 12 h, the pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE respectively, at 12 h as compared with 0 h (Figure 2). The drop in pH correlated with an increase in population of the two organisms (Tables 1 and 2).

Table 1. Viable microbial counts (log CFU/mL) of *B. animalis* Bb12 in soymilk during 12 h fermentation at 37◦C

	Enzyme concentrations (g/L)			
	Control	0.1 g/L	0.5 g/L	1.0 g/L
0 h	5.9 ± 0.01^{b}	6.0 ± 0.02^{c}	6.1 ± 0.03^{c}	6.2 ± 0.06^{c}
6 h	6.5 ± 0.03^{b}	6.4 ± 0.27^{b}	6.8 ± 0.03^{b}	6.9 ± 0.01^{b}
12 h	6.9 ± 0.02^{a}	7.2 ± 0.01^{a}	7.3 ± 0.02^{a}	7.4 ± 0.03^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05).

Table 2. Viable microbial counts (log CFU/mL) of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 fermentation at 37◦C in soymilk during 12 h

	Enzyme concentration (g/L)			
	Control	0.1 g/L	0.5 g/L	1.0 g/L
0 h	4.4 ± 0.03^{b}	4.4 ± 0.06^{b}	4.7 ± 0.04^{b}	4.9 ± 0.01^{b}
6 h	6.0 ± 0.10^{a}	6.0 ± 0.22^{a}	6.3 ± 0.10^{a}	6.5 ± 0.09^{a}
12 h	6.1 ± 0.19^{a}	6.2 ± 0.10^{a}	6.4 ± 0.21^{a}	6.7 ± 0.13^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Mean values in the same column for a particular organism with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05).

Viable counts of Lactobacillus *and* Bifidobacterium *during incubation*

Tables 1 and 2 demonstrate the viable count of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in soymilk prepared from soy protein isolate. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

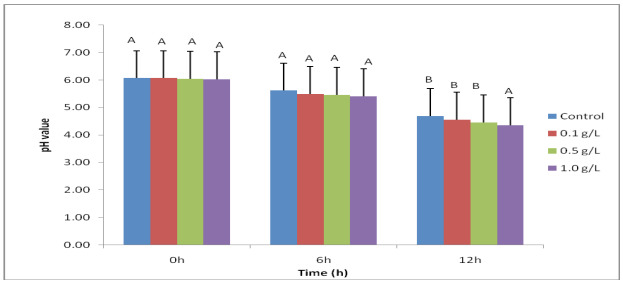

Figure 1. pH value of soymilk during 12 h fermentation by *B. animalis* Bb12 at 37◦C

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

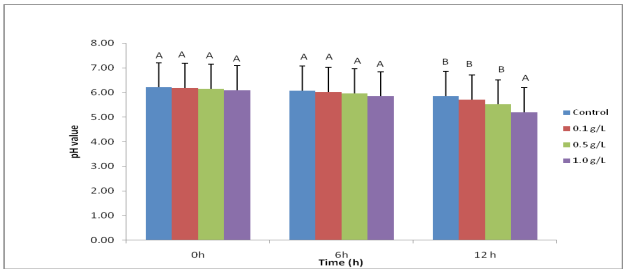

Figure 2. pH value of soymilk during 12 h fermentation by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at 45◦C

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

produced higher ($p<0.05$) viable counts at 1.0 g/L enzyme concentration during the entire incubation. *B. animalis* Bb12 showed a significant difference ($p>0.05$) in the viable count at 0 h, 6 h and 12 h at different enzyme concentrations including control and a no significant difference ($p<0.05$) was found within crude enzyme concentrations at 0 h, 6 h and 12 h (Table 1). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different viable counts at 0 h and 12 h; however, there was no significant difference ($p<0.05$) in viable counts between 0 and 6 h (Table 2). Moreover, there was no significant difference ($p<0.05$) within enzyme concentrations including control at 0 h, 6 h and 12 h. The final viable population of the *B. animalis* Bb12 ranged from 6.9 to 7.4 log CFU/mL and the organism showed the highest viable population of 7.4 log CFU/mL at 12 h with 1.0 g/L CEE followed by 0.5 g/L of crude enzyme at 7.3 log CFU/mL and lowest with control at 6.9 log CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 17.31, 20.36, 20.51 and 20.10% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE, respectively, at 12 h as compared with 0 h (Table 1). Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.1 to 6.7 log CFU/mL and the organism showed the highest viable population of 6.7 log CFU/mL at 12 h with 1.0 g/L crude enzyme followed by 0.5 g/L crude enzyme 6.4 log CFU/mL and lowest with control 6.1 CFU/mL. The viable count increased ($p<0.05$) by 40.18, 40.44, 36.56, and 35.84% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE, respectively, at 12 h as compared with 0 h (Table 2). Soymilk could not appeared to support the growth of *Bifidobacterium and Lactobacillus*, possibly due to the low amount (less than 1%) of simple carbon compounds in SPI, including sucrose, raffinose, and stachyose, which have been removed during processing (Nutrition Data, 2007). According to Shah (2006), the mild acidic condition of soymilk during fermentation (pH 6.15 to 6.80) was still in a favourable range for the growth of *Bifidobacterium* could be responsible for maintaining the viability of the probiotic organism. Supplementation of carbon sources to soymilk especially D-glucose stimulated the growth of *Bifidobacterium*, which could explain why there was an increase in the viable population of the organisms (Briczinski *et al.*, 2006).

Biotransformation of IG to IA by Lactobacillus *and* Bifidobacterium *in soymilk*

Tables 3 and 4 show the biotransformation of IG (glycitin) to IA (daidzein and genistein) in soymilk by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and their hydrolytic potential during fermentation at 37°C for *B. animalis* Bb12, and 45°C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The concentration of glycitin was recorded in decreasing trend whereas concentrations of daidzein and genistein were in increasing trend in soymilk during hydrolysis by microbial enzymes. The percent of moisture content of freeze dried samples ranged from 1.78 to 2.2. The isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). There were no significant differences ($P > 0.05$) in the moisture contents of the freeze-dried samples. Therefore, it is believed that there was no effect of the moisture content on the quantification of isoflavone compounds.

In general, there was only one IG (glycitin) and four IA (daidzein, genistein, biochanin A and formononetin) were used to determine the quantification of IG and IA in the soymilk sample at 0 h, 6 h and 12 h. Isoflavone concentrations of glycitin, daidzein and genistein were detected in different CEE concentrations including 0.1 g/litre, 0.5 g/litre and 1.0 g/litre and control at entire incubation. Biochanin A and Formononetin were not detected in soymilk in different CEE concentration and control. This also suggests their glycosides forms (sissotrin and ononin, respectively) were not available in SPI.

Statistically, *B. animalis* Bb12 showed a significant difference ($p<0.05$) in the glycitin and

daidzein at 0 h, 6 h and 12 h and there was no significant difference between different CEE concentrations; however genistein showed a significant difference ($p<0.05$) at both incubations as well as different CEE concentrations and control at 0 h, 6 h and 12 h. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference in glycitin at 0 h, 6 h and 12 h; however, there was no significant difference in hydrolysis ($p>0.05$) between different CEE concentrations and control. Moreover, daidzein and genistein showed a significant difference ($p<0.05$) in both organisms in all incubations as well as different CEE concentrations and control at 0 h, 6 h and 12 h.

B. animalis Bb12 produced glycitin at 9.41, 11.80, 8.11 and 13.83 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 6.32, 8.42, 8.11 and 5.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h and 3.47, 4.98, 5.42 and 3.52 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of glycitin was found at 13.83 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 0 h. Similarly, daidzein was produced at 23.92, 22.64, 25.57 and 26.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 0 h; 25.23, 30.36, 45.49 and 42.33 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 48.05, 52.16, 49.97and 47.81 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of IA in daidzein was found 52.16 mg/100 g of freeze-dried in 0.1 g/litre of CEE at 12 h. Likewise, genistein was produced 17.60, 18.68, 20.71 and 20.42 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 25.26, 27.67, 31.71 and 29.22 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 6 h; and 31.1, 32.43, 30.82 and 35.31 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of IA in genistein was found 35.31 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 12 h.

On the other hand, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced glycitin 7.42, 9.15, 10.55, and 14.26 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 6.04, 5.41, 4.51, 6.51 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 4.76, 4.56, 2.8, and 3.53 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at12 h. The higher level of glycitin was found 14.26 mg/100 g of freeze-dried in 1.0 g/litre at 0 h. Likewise, daidzein was produced 21.93, 21.03, 20.14 and 22.77 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 23.15, 24.04, 23.43 and 21.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 19.2, 25.2, 22.8 and 22.60 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of daidzein was found 25.2 mg/100 g of freeze-dried in 0.1g/litre of crude enzyme at 12 h. Similarly, genistein was produced 11.67, 14.59, 15.24 and 15.46 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively at 0 h; 14.04, 15.16, 16.38 and 15.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 16.34, 17.4, 17.8 and 17.56 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of genistein was found 17.80 mg/100 g of freeze-dried in 0.5 g/litre of CEE at 12 h.

The biotransformation of glycitin occurred higher in *B. animalis* Bb12 (74.44%) followed by (62.15%) with 1.0 g/litre CEE at 12 h and 6 h, respectively and lowest percent hydrolysis (28.66%) with 0.1 g/litre of CEE at 6 h (Table 3). However, the biotransformation of glycitin was higher for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (at 75.23%) followed by 73.46% with 1.0 g/litre and 0.5 g/litre CEE, respectively, at 12 h and lowest percent of hydrolysed (18.55%) with control at 6 h (Table 4). D-glucose appeared to have stimulating effect on the biotransformation by the organism at 12 h. The results suggest that D-glucose allowed the growth of these two organisms (data not shown). The biotransformation of glycitin might be a consequence of high level of viable cells in soymilk. During hydrolysis, the concentration of glycosides such as glycitin reduced while the concentration of aglycones such as genistein and daidzein increased in soymilk fermented by both organisms. The conversion of individual forms of isoflavone glycosides to respective aglycones has been reported by Otieno and Shah (2006a). In addition, low pH condition in soymilk may have also contributed to the increase in the biotransformation level. Delmonte *et al.* (2006) and Mathias *et al.* (2006) reported that some IG was partly hydrolyzed to IA in a low pH condition.

Table 3. Biotransformation of IG to IA in soymilk by *B. animalis* Bb12

Isoflavone(mg/ 100 g) of freeze dried sample		Control			Enzyme concentrations (g/L) 0.1			0.5			1.0		
		0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
	Glycitin	9.41± 0.01^{a}	6.32 ± 0.01^{b}	3.47 ± 0.01^{c}	11.80± 0.01^{a}	8.42 ± 0.01^{b}	4.98 ± 0.01^{c}	13.23 ± 0.01^{a}	8.11 ± 0.01^{b}	5.42± 0.01^{c}	13.83 ± 0.01^{c}	5.23 ± 0.01^{b}	3.53 ± 0.01^{c}
	% of glycitin (IG) hydrolysed	**0**	**32.86**	**63.14**	**0.0**	**28.66**	**57.81**	**0.0**	**38.70**	**59.01**	**0.0**	**62.15**	**74.44**
	Daidzein	23.92 ± 0.02^{c}	25.53± 0.03^{b}	48.05 ± 0.08^{a}	22.64± 0.02^{c}	30.36 ± 0.02^{b}	52.16 ± 0.01^{a}	25.57 ± 0.01^{c}	45.49 ± 0.01^{b}	49.97 ± 0.01^{a}	26.23 ± 0.03^{c}	42.33 ± 0.01^{b}	47.81 ± 0.12^{a}
	Genistein	17.60 ± 0.01^{c}	25.26± 0.01^{b}	31.1 ± 0.01^{a}	18.68± 0.03^{c}	27.67 ± 0.01^{b}	32.43 ± 0.03^{a}	20.71 ± 0.05^{b}	31.71 ± 0.05^{a}	30.82 ± 0.05^{a}	20.42 ± 0.03^{c}	29.22 ± 0.02^{b}	35.31 ± 0.01^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05).
Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

Table 4. Biotransformation of IG to IA in soymilk by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Isoflavone(mg/ 100 g) of freeze dried sample		control			Enzyme concentration (g/L) 0.1			0.5			1.0		
		0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
	Glycitin	7.42± 0.02^{a}	6.04 ± 0.01^{b}	4.76 ± 0.01^{b}	9.15± 0.02^{a}	5.41± 0.00^{b}	4.56 ± 0.01^{c}	10.55± 0.01^{a}	4.51 ± 0.00^{b}	2.8 ± 0.01^{c}	14.26 ± 0.01^{a}	6.51 ± 0.00^{b}	3.53 ± 0.01^{c}
	% of glycitin (IG) hydrolysed	**0.0**	**18.55**	**35.80**	**0.0**	**40.88**	**50.16**	**0.0**	**57.22**	**73.46**	**0.0**	**54.34**	**75.23**
	Daidzein	21.93 ± 0.01^{b}	23.15 ± 0.01^{a}	19.2 ± 0.01^{c}	21.03± 0.01^{c}	24.04± 0.01^{b}	25.2 ± 0.01^{a}	20.14± 0.01^{c}	23.43 ± 0.01^{a}	22.8 ± 0.01^{b}	22.77 ± 0.01^{a}	21.50 ± 0.01^{c}	22.60 ± 0.01^{b}
	Genistein	11.67 ± 0.01^{c}	14.04 ± 0.01^{b}	16.34 ± 0.01^{a}	14.59± 0.02^{b}	15.16± 0.28^{b}	17.4 ± 0.02^{a}	15.24± 0.01^{c}	16.38 ± 0.01^{b}	17.8 ± 0.01^{a}	15.46 ± 0.01^{c}	15.5 ± 0.02^{b}	17.56 ± 0.01^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05).
Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

Conclusions

The result of this study demonstrated that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 are capable of hydrolysing glycitin to biologically active forms in soymilk (SM) prepared from soy protein isolate (SPI) and soymilk supplemented with 2.0% (w/v) of D-glucose at different concentrations of CEE. The increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of daidzein and genistein in fermented soymilk. Increased daidzein and genistein content in fermented soymilk is likely to improve the biological functionality of soymilk. The crude enzyme extract played a greater role in biotransformation. The biotransformation of glycitin occurred lower in *B. animalis* Bb12 (74.44 %) than the *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (75.23 %).

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