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Effects of Skim Milk Powder Supplementation to Soy Yogurts on Biotransformation of Isoflavone Glycosides to Biologically Active Forms during Storage

T. T. Pham, and N. P. Shah

***Abstract*—**Three batches of yogurts were made with soy protein isolate (SPI) supplemented with 2% (S2), 4% (S4) or 6% (S6) of skim milk powder (SMP). The fourth batch (control; S0) was prepared from SPI without SMP supplementation. *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (Lb 11842) and *Streptococcus thermophilus* ST 1342 (ST 1342) were used as the starter culture. Biotransformation of the inactive forms, isoflavone glycosides (IG) to biologically active forms, isoflavone aglycones (IA), was determined during 28 d storage. The viability of both microorganisms was significantly higher ($P < 0.05$) in S2, S4, and S6 than that in S0. The ratio of lactic acid/acetic acid in S0 was in the range of 15.53 – 22.31 compared to 7.24 – 12.81 in S2, S4 and S6. The biotransformation of IG to IA in S2, S4 and S6 was also enhanced by 9.9 -13.3% compared to S0.

***Keywords*—**Isoflavone aglycones, isoflavone glycosides, skim milk powder and soy yogurt.

I. Introduction

SOY food products are perceived as healthy food and are considered an important part of the diet. More than 50% consumers in the USA agreed that soy foods are healthy foods [1]. Twenty one per cent of Australian population has been reported to consume soy based products such as soy bread, soy-based infant formula, soy-cheese and soy yoghurt [2]. Among the soy food products, soy yogurt has received a lot of attention because of its health benefits. In addition to the high quality protein in soy yogurt, it contains a considerable amount of isoflavone compounds which have been known as a natural substance to replenish the female hormone oestrogen in order to relieve the menopausal symptoms [3;4]. However, compared to cow-milk yogurt, soy yogurt has some disadvantages. From a nutritional standpoint, soy yogurt is deficient in calcium content [5]. Moreover, soymilk which is utilized to make soy yogurt, does not support the growth of micro-organisms including bifidobacteria and lactobacilli [6-9]. As a result, the biotransformation level of the non-active isoflavone glycosides (IG), which dominantly exist in soymilk, to the biologically active forms, isoflavone aglycones (IA), by such microorganisms is low. It is undesirable since IA are the only isoflavone compounds that possess the estrogenic activity and other health benefits [4]. Therefore, supplementation of soymilk with skim milk powder (SMP) is expected to improve the nutritional values as well as the health benefits of soy yogurt. The supplementation is expected to improve the growth of the yogurt starter culture and enhance the biotransformation of the dominant IG to IA. Pham and Shah [6;8] reported that the supplementation of SMP to soymilk increased the biotransformation of IG to IA by 12.6% and 23.7% by bifidobacteria and lactobacilli, respectively. Moreover, the SMP supplemented soy yogurt (SMPY) could be an excellent source of protein, calcium and the biologically active IA.

To date, a number of studies have reported the sensory and rheological characteristics of soy yogurt as well as the survival of bacteria in the product [10-13]. However, there is very little information on the effects of the supplementation of SMP to soy yogurt on organic acid production and the survival of the yogurt starter culture during the storage period. Furthermore, the biotransformation of IG to IA in soy yogurt as well as in SMPY during the storage has not been investigated. Hence, the objective of this study was to investigate the influence of the supplementation of SMP on the survival of the yogurt starter including *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, organic acid production and biotransformation of IG to IA in both soy yogurt and SMPY at the end of fermentation as well as during 28 days storage period.

II. Materials and Methods

A. Chemicals

Genistein, daidzein, glycitein, flavone, Carrez I, Carrez II, lactic acid, glacial acetic acid, and D-glucose 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, USA). Malonyl- and acetyl- β glycosides (malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl

T. T. Pham and N. P. Shah are in School of Biomedical and Health Sciences, Faculty of Health, Engineering and Science, Victoria University, PO Box 14428, Melbourne, Victoria 8001, Australia (phone: + 61 3 9919 8152, fax: + 61 3 9919 8284, e-mail: Thuy.pham14@live.vu.edu.au).

daidzin, acetyl glycitin, acetyl genistin) were obtained from LC Labs (Woburn, MA, USA). Sulphuric acid was from Merck (Merck KGaA, Darmstadt, Germany). Soy protein isolate (SPI) SUPRO 590 was from The Solae Co. (Chatswood, NSW, Australia). Reinforced clostridial agar and M17 agar were from Amyl Media (Danenong, Vic, Australia). Skim milk powder was from Murray Goulburn Co-operative Ltd. (Brunswick, Vic, Australia). The water used was milli-Q grade. Acetonitrile, methanol, ethanol and phosphoric acid used for HPLC were of analytical grade.

B. Cultures and Fermentation of Soy Yogurts

Pure frozen culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (Lb 11842) was obtained from Australian Starter Culture Research Centre (Werribee, Vic, Australia). Pure frozen culture of *Streptococcus thermophilus* ST 1342 (ST 1342) was from the Victoria University Culture Collection (Werribee, Vic, Australia). The two organisms were separately activated in de Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) by growing successively twice at 37 ^{o}C for 20 h. The experimental plan for the four batches of yogurt is shown in Table I. Two litres of soymilk with or without SMP were heat treated in a water bath (model NB 6T-10935, Thermoline Scientific, Australia) at 85 ^{o}C for 30 min, followed by cooling to 42 ^{o}C and were aseptically inoculated with 1% each of Lb 11842 and ST 1342. Inoculated mixes were then poured into 50 mL sterile cups with lids and incubated at 42 ^{o}C until the pH of the products reached 4.50 ± 0.10. The finished yogurts were immediately cooled in an ice bath and then stored at 4 ^{o}C for 28 d.

TABLE I
THE EXPERIMENTAL PLAN FOR MAKING YOGURTS

Yogurt batches	Composition		
	D-glucose (%) (w/v)	SMP (%) (w/v)	SPI (%) (w/v)
Soy yogurt S0	1.0	0.0	11.0
Soy yogurt supplemented with SMP (SMPY)			
S2	0.0	2.0	10.0
S4	0.0	4.0	8.0
S6	0.0	6.0	6.0

C. Determination of pH

The pH of the yogurts was monitored at the end of the fermentation (0 d), and at 7 d interval during 28 d of the storage 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.

D. Enumeration of Viable Micro-Organisms

One gram sample from each yogurt batch was taken at 0, 7, 14, 21 and 28 d of storage and serial dilutions were prepared in 0.15% (w/v) peptone and water. The colonies of Lb 11842 and ST 1342 were enumerated as described previously using the pour plate technique [14]. Briefly, M17 agar was used for the selective enumeration of ST 1342 and the plates were incubated aerobically at 37 ^{o}C for 72 h. Reinforced clostridial agar (pH 5.3) was used for the enumeration of Lb 11842 and the microorganism was incubated anaerobically at 37 ^{o}C for 72 h. Plates showing colonies between 25 to 250 were enumerated and recorded as colony forming unit (CFU) per gram of the product.

E. Determination of Organic Acids

Lactic and acetic acids were determined using the method described by Donkor et al. [15] with some modifications. Briefly, 0.5 gram of the yogurt sample was mixed with 25 µL of 15.5 M nitric acid and then diluted with 0.8 mL of 5 mM H2S04. The mixture was centrifuged at 14,000 x g for 30 min using an Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Australia) to remove proteins. The supernatant was filtered through a 0.45 µm membrane filter (Phenomenex, Lane Cove, NSW, Australia) into a HPLC vial. The HPLC systems were Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) and an Aminex HPX-87H, 300 x 7.8 mm ion-exchange column (Bio-Rad Life Science Group, Hercules, CA, USA). The column was maintained at 65 ^{o}C by a column heater serial No. 2451 (Timberline Instrument Inc., Boulder, CO, USA). Sulphuric acid (5 mM) was used as a mobile phase at a flow rate of 0.6 mL/min. The level of organic acids was quantified based on standard curves prepared using standard solutions.

F. Determination of Isoflavone Contents

Extraction of isoflavone and HPLC analysis were based on Griffith & Collison [16] and Nakamura et al. [17] with some modifications. Briefly, approximately 50 g sample of S0, S2, S4 and S6 were taken separately immediately after the heat treatment to quantity isoflavone compounds before fermentation. Similarly, 50 gram yogurt samples of each batch were taken at the end of the fermentation (0 d) and at 7, 14, 21 and 28 d of the storage and freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavones. One gram 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 microlitres each of Carrez I and Carrez II solutions were added to the samples and mixed thoroughly to precipitate proteins. One hundred microlitres 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 Scientific, Australia) at 50 ^{o}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 the HPLC vial then injected into HPLC system within 4 h to avoid the degradation of malonyl- and acetyl glycosides [16].

The HPLC system included an Alltech Alltima (Deerfield, IL, USA) HP C18 HL (4.6 x 250) mm with 5 µm particle size and an Alltima HP C18HL (7.5 x 4.6) mm with a 5 µm particle size as a guard column, a Hewlett Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with

an auto sampler, a quaternary pump, a diode array ultraviolet detector, a vacuum degasser and a thermostatically controlled column compartment. The column temperature was kept at 25 ^{o}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 5 working solutions at concentrations ranging from 1 µg/mL 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 soy yogurt samples was determined by AACC 40-40 [18]. The biotransformation of IG to aglycones was defined as percentage of IG hydrolyzed and was calculated as follows:

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

G. Statistical analysis of data

The fermentation trial was carried out in duplicate and all analyses were performed in triplicate. The data were analysed using one-way analysis of variance (ANOVA) at 95% confidence intervals using Microsoft Excel Statpro as described by Allbright et al. [19]. ANOVA data with a $P < 0.05$ was classified as statistically significant.

III. Results and Discussion

A. The Organic Acids Produced in S0 and SMPY during the Storage

Figure 1 presents the amount of lactic and acetic acid in the yogurts and Fig. 2 shows the pH changes in the yogurts at the end of fermentation and during the storage period. In addition to lactic and acetic acids, other organic acids including propionic, citric, and butyric are also produced in soy yogurt in a small quantity [20;21]. After 8-10 h of fermentation, the pH of all the yogurts dropped to 4.60 (Fig. 2). The supplementation of glucose to S0 allowed the pH in the yogurt to fall to the same pH range of 4.50 - 4.60. The fermentation time required for S0 was the shortest (8 h) although the amount of organic acids at the end of fermentation was the lowest compared to other SMPY (Fig. 1).

This is possibly due to lower buffering capacity of SPI than SMP [22]. As shown in Fig. 1, there was less lactic acid produced while more acetic acid was generated in SMPY.

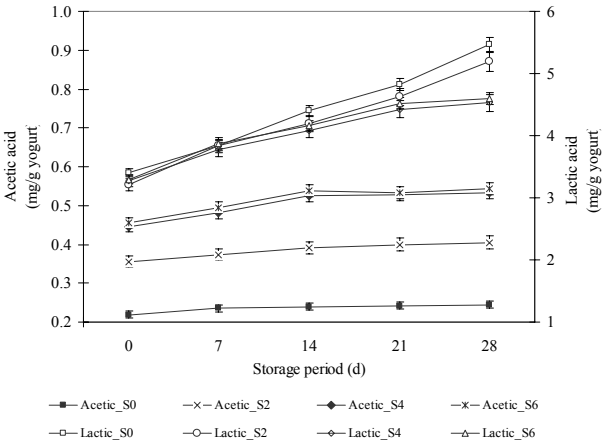

Fig. 1 Lactic and acetic acids (mg/g) produced in soy yogurt (S0, S2, S4 and S6) during the storage period of 28 d at 4 ^{o}C

Results are expressed as mean ± standard error (n = 6). S0: soy yogurt without SMP supplementation. S2: soy yogurt with 2% SMP supplementation. S4: soy yogurt with 4% SMP supplementation. S6: soy yogurt with 6% SMP supplementation.

The ratio of lactic/acetic acid in S0 was in the range of 15.53 – 20.31 compared to 7.24 – 11.83 in SMPY during the storage period. The difference in the ratio of lactic acid/acetic acid between S0 and the SMPY may due to the different carbon source. The main carbon source in SMPY was lactose compared to glucose in S0. Our previous study also found that the ratio of lactic/acetic acid changed in soymilk supplemented with SMP fermented by lactic acid bacteria compared with that in soymilk alone [9]. This was in agreement with the study of Donkor et al. [20], who also reported that the amount of lactic and acetic acids varied in yogurt supplemented with different sources of carbon including inulin and Hi-maize. There was no significant difference ($P > 0.05$) in the amount of both lactic and acetic produced in S4 and S6 at the end of fermentation as well as during storage. In general, both amount of lactic and acetic acid increased during the storage period (Fig. 1) resulting in a decrease in the pH values (Fig. 2). However, acetic and lactic acid produced were stable from 14 d and 21 d, respectively.

B. The viability of ST 1342 and Lb 11842 in S0 and SMPY during Storage

Table II shows the viable counts of ST 1342 and Lb 11842 at the end of the fermentation and during the storage. The viable count of both ST 1342 and Lb 11842 in S0 was significantly lower ($P < 0.05$) than that in SMPY during the entire storage. It appears that the supplementation of SMP enhanced the viability of both ST 1342 and Lb 11842. This is possibly due to the presence of nutrients in SMP, particularly

TABLE II
VIABILITY OF MICROORGANISMS (LOG CFU/G) IN THE YOGURTS DURING 28 D STORAGE AT 4 °C

Microorganisms	Storage time (d)				
	0	7	14	21	28
Lb 11842_S0	8.14 ± 0.07 ^{Aa}	8.06 ± 0.04 ^{Aab}	8.01 ± 0.05 ^{Ab}	7.93 ± 0.10 ^{Ab}	7.92 ± 0.08 ^{Ab}
Lb 11842_S2	8.55 ± 0.09 ^{Ba}	8.50 ± 0.11 ^{Ba}	8.43 ± 0.09 ^{Bab}	8.30 ± 0.08 ^{Bb}	8.29 ± 0.08 ^{Bb}
Lb 11842_S4	8.53 ± 0.07 ^{Ba}	8.46 ± 0.07 ^{Ba}	8.35 ± 0.06 ^{Bab}	8.26 ± 0.07 ^{Bbc}	8.21 ± 0.08 ^{Bc}
Lb 11842_S6	8.53 ± 0.12 ^{Ba}	8.49 ± 0.06 ^{Bab}	8.35 ± 0.06 ^{Bbc}	8.25 ± 0.07 ^{Bc}	8.21 ± 0.10 ^{Bc}
ST 1342_S0	9.12 ± 0.11 ^{Aa}	9.10 ± 0.06 ^{Aa}	9.05 ± 0.09 ^{Aa}	8.77 ± 0.05 ^{Ab}	8.75 ± 0.06 ^{Ab}
ST 1342_S2	9.68 ± 0.06 ^{Ba}	9.52 ± 0.07 ^{Bb}	9.31 ± 0.05 ^{Bc}	9.15 ± 0.08 ^{Bd}	9.00 ± 0.07 ^{Be}
ST 1342_S4	9.68 ± 0.07 ^{Ba}	9.49 ± 0.06 ^{Bb}	9.36 ± 0.07 ^{Bc}	9.32 ± 0.07 ^{Cc}	9.14 ± 0.06 ^{Cd}
ST 1342_S6	9.64 ± 0.07 ^{Ba}	9.42 ± 0.07 ^{Bb}	9.36 ± 0.06 ^{Bb}	9.34 ± 0.06 ^{Cbc}	9.24 ± 0.08 ^{Dc}

Results are expressed as mean ± standard error (n = 6). Mean values in the same row with the same lowercase letter superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular micro-organism with the same uppercase letter superscripts are not significantly different (P > 0.05). S0: Soy yogurt without SMP supplementation. S2: Soy yogurt with 2% SMP supplementation. S2: Soy yogurt with 2% SMP supplementation. S4: Soy yogurt with 4% SMP supplementation. S6: Soy yogurt with 6% SMP supplementation

lactose as a carbon source, which is generally deficient in SPI. As SPI contains less than 1% of carbon source, soymilk prepared from SPI alone generally did not support the growth of bifidobacteria and lactobacilli [9;23;24]. However, the viable counts of Lb 11842 and ST 1342 in S0 remained relatively high, which was 7.92 log CFU/g and 8.75 CFU/g, respectively, at the end of the storage (Table II).

TABLE III
BIOTRANSFORMATION OF IG TO IA IN SOY YOGURT WITHOUT SMP SUPPLEMENTATION (S0) DURING THE STORAGE AT 4 °C FOR 28 D

Isoflavones mg/100 g freeze-dried sample)	Before fermentation	Storage time (d)				
		0	7	14	21	28
Daidzin	12.87 ± 0.83 ^{a}	1.71 ± 0.21 ^{b}	1.46 ± 0.18 ^{b}	1.39 ± 0.15 ^{b}	1.44 ± 0.16 ^{b}	1.37 ± 0.12 ^{b}
Glycitin	5.63 ± 0.35 ^{a}	3.40 ± 0.19 ^{b}	2.45 ± 0.12 ^{c}	2.36 ± 0.12 ^{c}	2.43 ± 0.14 ^{c}	2.23 ± 0.11 ^{c}
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.40 ± 1.27 ^{a}	3.84 ± 0.14 ^{b}	3.67 ± 0.12 ^{b}	3.29 ± 0.17 ^{b}	2.95 ± 0.15 ^{b}	2.87 ± 0.14 ^{b}
Malonyl glycitin	2.68 ± 0.14 ^{a}	2.09 ± 0.11 ^{b}	2.05 ± 0.12 ^{b}	2.09 ± 0.13 ^{b}	2.04 ± 0.14 ^{b}	2.02 ± 0.12 ^{b}
Malonyl genistin	61.62 ± 2.43 ^{a}	25.62 ± 1.24 ^{b}	24.53 ± 1.57 ^{b}	23.91 ± 1.87 ^{b}	23.41 ± 1.24 ^{b}	23.20 ± 1.65 ^{b}
Acetyl daidzin	5.79 ± 0.42	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	25.29 ± 1.22 ^{a}	16.56 ± 0.81 ^{b}	16.16 ± 0.92 ^{b}	16.08 ± 0.81 ^{b}	16.29 ± 0.75 ^{b}	16.02 ± 0.84 ^{b}
Total IG	136.28 ± 3.38 ^{a}	53.22 ± 2.00 ^{b}	50.32 ± 2.43 ^{bc}	49.12 ± 2.65 ^{bc}	48.56 ± 1.98 ^{c}	47.71 ± 2.48 ^{c}
Daidzein	ND	18.68 ± 1.02 ^{a}	18.99 ± 1.11 ^{a}	19.21 ± 1.00 ^{a}	19.83 ± 1.15 ^{a}	19.85 ± 1.07 ^{a}
Glycitein	ND	1.38 ± 0.14 ^{a}	1.88 ± 0.12 ^{b}	2.00 ± 0.15 ^{b}	2.06 ± 0.15 ^{b}	2.03 ± 0.13 ^{b}
Genistein	4.13 ± 0.25 ^{a}	27.97 ± 1.56 ^{b}	28.54 ± 1.53 ^{b}	28.75 ± 1.46 ^{b}	28.82 ± 1.11 ^{b}	29.04 ± 1.21 ^{b}
Total IA	4.13 ± 0.25 ^{a}	48.03 ± 2.72 ^{b}	49.41 ± 0.54 ^{b}	49.96 ± 0.61 ^{b}	50.71 ± 2.11 ^{b}	50.92 ± 2.15 ^{b}
IG hydrolyzed (%)		61.0	63.1	64.0	64.4	65.0

Results are expressed as mean ± standard error (n = 6). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. IA: Isoflavone aglycones. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit).

This is possibly due to the supplementation of 1% glucose which also supported the growth of ST 1342 and Lb 11842, but not as well as that with SMP supplementation. This is in agreement with our previous studies [6;8], in which the supplementation of SMP to soymilk increased the viable counts of bifidobacteria and lactobacilli. Although ST 1342 and Lb 11842 are not classified as probiotic organisms, these bacteria can improve lactose digestion and may help promote a healthy immune system. Hence, it is desirable that they remain alive in at a high concentration during storage in order to have beneficial effects [25]

In general, the viable counts of microorganisms decreased in both S0 and SMPY as the amount of organic acids increased (Fig. 1) during the storage reducing pH values to 4.10 – 4.60 (Fig. 2), which were unfavourable for their growth [26]. However, our results contradicted with those of Donkor et al. [15] which showed that the viable count of both St. *thermophilus* and Lb. *bulgaricus* increased during the storage at 4 °C for the first 14 d. In our study, the viable counts of Lb 11842 in all the three batches of SMPY were not significantly different (P > 0.05) during the entire storage. This means the effect of the SMP supplementation on survival of Lb 11842 at 2% was similar to that at 4% or 6%. Generally, the viability of ST 1342 was higher than that of Lb 11842. This is in agreement with the results of Donkor et al. [15] who also found that St. *thermophilus* had the higher viable counts than Lb. *bulgaricus* in soy yogurt. However, during the storage period, the viable counts of ST 1342 declined by of 0.37 – 0.54 log CFU/g while those of Lb 11842 decreased only up to 0.32 log CFU/g (Table II).

TABLE IV
BIOTRANSFORMATION OF IG TO IA IN SOY YOGURT WITH 2% SMP SUPPLEMENTATION (S2) DURING THE STORAGE AT 4°C FOR 28 D

Isoflavones mg/100 g freeze-dried sample)	Before fermentation	Storage time (d)				
		0	7	14	21	28
Daidzin	11.75 ± 0.66	ND	ND	ND	ND	ND
Glycitin	5.07 ± 0.34	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	20.50 ± 1.12 [a]	1.57 ± 0.10 [b]	1.55 ± 0.11 [b]	0.86 ± 0.12 [b]	1.15 ± 0.19 [b]	0.84 ± 0.14 [b]
Malonyl glycitin	2.50 ± 0.14 [a]	2.40 ± 0.12 [a]	2.25 ± 0.17 [abc]	2.18 ± 0.15 [b]	2.23 ± 0.14 [b]	2.10 ± 0.12 [bc]
Malonyl genistin	56.00 ± 2.68 [a]	20.36 ± 1.32 [b]	20.42 ± 1.53 [b]	20.57 ± 1.22 [b]	20.36 ± 1.21 [b]	20.23 ± 1.35 [b]
Acetyl daidzin	5.37 ± 0.32	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	22.99 ± 0.57 [a]	11.83 ± 0.75 [b]	11.71 ± 0.85 [b]	11.26 ± 0.78 [b]	11.49 ± 0.85 [b]	11.19 ± 0.76 [b]
Total IG	124.18 ± 4.69 [a]	36.16 ± 1.85 [b]	35.93 ± 2.66 [b]	34.87 ± 2.27 [b]	35.23 ± 1.73 [b]	34.36 ± 2.37 [b]
Daidzein	ND	19.13 ± 0.98 [a]	19.15 ± 1.12 [a]	19.49 ± 1.05 [a]	19.42 ± 1.07 [a]	19.53 ± 0.87 [a]
Glycitein	ND	2.76 ± 0.20 [a]	2.83 ± 0.15 [a]	2.89 ± 0.17 [a]	2.83 ± 0.18 [a]	2.97 ± 0.19 [a]
Genistein	3.72 ± 0.17 [a]	28.95 ± 1.23 [b]	28.95 ± 1.43 [b]	28.98 ± 1.52 [b]	29.05 ± 1.62 [b]	29.12 ± 1.75 [b]
Total IA	3.72 ± 0.17 [a]	50.84 ± 2.41 [b]	50.93 ± 2.70 [b]	51.36 ± 2.74 [b]	51.30 ± 2.87 [b]	51.62 ± 2.81 [b]
IG hydrolyzed (%)		70.9	71.1	71.9	71.6	72.3

Results are expressed as mean ± standard error (n = 6). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. IA: Isoflavone aglycones. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit).

TABLE V
BIOTRANSFORMATION OF IG TO IA IN SOY YOGURT WITH 4% SMP SUPPLEMENTATION (S4) DURING THE STORAGE AT 4°C FOR 28 D

Isoflavones mg/100 g freeze-dried sample)	Before fermentation	Storage time (d)				
		0	7	14	21	28
Daidzin	9.36 ± 0.62	ND	ND	ND	ND	ND
Glycitin	4.09 ± 0.33	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	16.43 ± 1.02 [a]	0.98 ± 0.12 [b]	1.12 ± 0.11 [b]	0.93 ± 0.10 [b]	0.73 ± 0.11 [b]	0.71 ± 0.14 [b]
Malonyl glycitin	2.01 ± 0.16 [a]	1.98 ± 0.17 [a]	1.92 ± 0.17 [a]	1.86 ± 0.15 [a]	1.85 ± 0.19 [a]	1.85 ± 0.12 [a]
Malonyl genistin	44.85 ± 2.01 [a]	19.05 ± 2.11 [b]	19.11 ± 1.85 [b]	18.46 ± 1.74 [b]	18.40 ± 1.45 [b]	18.25 ± 1.24 [b]
Acetyl daidzin	4.27 ± 0.32	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	18.21 ± 1.24[a]	3.52 ± 0.22 [b]	3.32 ± 0.17 [b]	3.02 ± 0.24 [b]	3.04 ± 0.32 [b]	3.00 ± 0.16 [b]
Total IG	99.22 ± 5.70 [a]	25.53 ± 2.62 [b]	25.47 ± 2.30 [b]	24.27 ± 2.23 [b]	24.02 ± 2.07 [b]	23.81 ± 1.66 [b]
Daidzein	ND	15.46 ± 0.99 [a]	15.56 ± 1.05 [a]	15.60 ± 1.12 [a]	15.65 ± 1.07 [a]	15.72 ± 1.14 [a]
Glycitein	ND	2.35 ± 0.21 [a]	2.37 ± 0.24 [a]	2.40 ± 0.28 [a]	2.34 ± 0.29 [a]	2.44 ± 0.21 [a]
Genistein	3.04 ± 0.15 [a]	24.07 ± 1.52 [b]	24.08 ± 1.75 [b]	24.95 ± 1.96 [b]	25.33 ± 1.66 [b]	25.46± 1.53 [b]
Total IA	3.04 ± 0.15 [a]	41.88 ± 2.72 [b]	42.01 ± 3.04 [b]	42.95 ± 3.36 [b]	43.32 ± 3.02 [b]	43.62 ± 2.88 [b]
IG hydrolyzed (%)		74.3	74.3	75.5	75.8	76.0

Results are expressed as mean ± standard error (n = 6). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. IA: Isoflavone aglycones. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit).

C. The biotransformation of IG to IA in S0 and SMPY

Tables 3 to 6 present the biotransformation of IG to IA in S0, S2, S4 and S6, respectively, at the end of the fermentation and during storage. The moisture content of the freeze-dried yogurt samples ranged from 1.9 - 2.0%. There were no significant differences (P > 0.05) in the moisture contents of the freeze-dried samples. Therefore, it is assumed that there was no effect of the moisture content on the quantification of isoflavone compounds. The isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). The HPLC chromatogram and the retention times of 14 standard isoflavone compounds and of flavone as the internal standard are shown in Fig. 3. The detection limit of HPLC method was approximately 10-3 mg/mL.

In general, there were only 7 IG found in all types of soymilk (S0, S2, S4 and S6) before fermentation. Two IA including biochanin A and formononetin were not detected in the yogurts at the end of the fermentation as well as during the storage period. This also suggests their glycosides forms (sissotrin and ononin, respectively) were not available in SPI. The initial isoflavone content before fermentation decreased steadily in S0, S2, S4 and S6 due to the increasing addition of SMP and the decreasing amount of SPI (Table I).

As shown in the tables, the biotransformation of IG to IA occurred during the fermentation in all the type of yogurts, especially in those containing SMP. The level of biotransformation of IG to IA in SMPY was 9.9, 13.3, and 12.9% higher in S2 (Table IV), S4 (Table V) and S6 (Table

VI), respectively, than that in S0 (Tables III) at the end of the fermentation (0 d). Daidzin and glycitin were hydrolysed completely while they were still present in S0 at the end of the fermentation. It was apparent that the supplementation of SMP enhanced the level of biotransformation of IG to IA. This is in agreement with our previous studies, Pham and Shah [6,8], which showed that the presence of SMP in soymilk significantly increased the level of the biotransformation of IG to IA by probiotic organisms including bifidobacteria and lactobacilli. The enhancing effect of the supplementation of SMP on the biotransformation is possibly due to the presence of lactose in SMP. To utilize lactose, microorganisms must produce β-D-galactosidase which breaks lactose molecules to D-glucose and D-galactose. According to Pham and Shah [27], β-galactosidase is also able to hydrolyse IG to IA. In addition, the improving the viability of Lb 11842 and ST 1342 of SMP in SMPY could augment the biotransformation of IG to IA.

TABLE VI
BIOTRANSFORMATION OF IG TO IA IN SOY YOGURT WITH 6% SMP SUPPLEMENTATION (S6) DURING THE STORAGE AT 4°C FOR 28 D

Isoflavones mg/100 g freeze-dried sample)	Before fermentation	Storage time (d)				
		0	7	14	21	28
Daidzin	7.00 ± 0.52	ND	ND	ND	ND	ND
Glycitin	3.04 ± 0.31	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	12.35 ± 0.75 [a]	1.07 ± 0.10 [b]	1.05 ± 0.12 [b]	0.86 ± 0.09 [b]	0.69 ± 0.08 [b]	0.66 ± 0.09 [b]
Malonyl glycitin	1.62 ± 0.49 [a]	1.39 ± 0.92 [b]	1.44 ± 0.12 [b]	1.36 ± 0.15 [b]	1.28 ± 0.18 [b]	1.31 ± 0.14 [b]
Malonyl genistin	33.52 ± 1.99 [a]	14.85 ± 0.18 [b]	14.58 ± 0.84 [b]	13.84 ± 0.84 [b]	13.78 ± 0.14 [b]	13.76 ± 0.13 [b]
Acetyl daidzin	3.33 ± 0.29	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	13.81 ± 0.85 [a]	2.21 ± 0.19 [b]	2.15 ± 0.21 [b]	2.06 ± 0.18 [b]	1.88 ± 0.15 [b]	1.69 ± 0.12 [b]
Total IG	74.67 ± 5.20 [a]	19.52 ± 1.39 [b]	19.22 ± 1.29 [b]	18.12 ± 1.26 [b]	17.63 ± 0.55 [b]	17.42 ± 0.48 [b]
Daidzein	ND	11.58 ± 0.75	11.47 ± 0.65	11.57 ± 0.60	11.66 ± 0.84	11.67 ± 0.82
Glycitein	ND	1.73 ± 0.17	1.71 ± 0.18	1.75 ± 0.14	1.79 ± 0.12	1.77 ± 0.14
Genistein	2.20 ± 0.16 [a]	18.23 ± 1.02 [b]	18.36 ± 1.11 [b]	18.96 ± 0.75 [b]	19.02 ± 0.84 [b]	19.15 ± 0.85 [b]
Total IA	2.20 ± 0.16 [a]	31.54 ± 2.40 [b]	31.54 ± 1.94 [b]	32.28 ± 1.49 [b]	32.47 ± 2.10 [b]	32.59 ± 2.61 [b]
IG hydrolyzed (%)		73.9	74.3	75.8	76.4	76.7

Results are expressed as mean ± standard error (n = 6). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. IA: Isoflavone aglycones. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit).

Comparing the conversion of IG to IA among the SMPY, 2% of SMP supplemented soy yogurt (S2) had the lowest biotransformation level of 70.9% at the end of fermentation (Table IV). The biotransformation level in S6 of 73.9% (Table 6) was slightly lower than that in S4 (74.3%) (Table V). Therefore, an increase in the level of SMP supplementation to soymilk may not enhance the level of biotransformation once the SMP supplementation reached at 4%.

In general, the biotransformation level of IG to IA increased slightly in all the yogurt batches during the storage period. There were no significant differences (P > 0.05) in the total IG residual during the entire 28 d of storage. Similarly, total IA produced were not significantly different (P > 0.05) during storage in all the yogurt batches (Tables III to VI). The reason is possibly due to the inactive status of two enzymes including β-glucosidase and β-galactosidase, which play the key roles in the hydrolysis of IG to IA, in an acidic condition (pH 4.10–4.60) of the soy yogurts. This also suggests that both IG and IA were considerably stable in the acidic pH range of 4.10 – 4.60.

IV. CONCLUSION

The viability of the yogurt starter including Lb 11842 and ST 1342 was significantly enhanced (P < 0.05) in soy yogurt supplemented with SMP during fermentation as well as during the 28 d storage. The biotransformation of IG to IA was extensively enhanced by up to 13.3% due to the supplementation of SMP. The optimum level of the SMP supplementation was 4%, which gave the highest level of biotransformation and greatest viability of Lb 11842 and ST 1342. The ratio of lactic/acetic acid decreased as the level of supplementation of SMP increased in SMPY.

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Thuy T. Pham obtained her BE Degree with first class honours from Hanoi University of Technology in 2000. In 2006, she was offered a scholarship to pursue PhD research at Victoria University. Her PhD research project title is: "Biotransformation of soy isoflavone to biologically active forms in soymilk by probiotic organisms as affected by supplementation with prebiotics". Till date, she has published 8 papers in international and high impact journals and 5 conference papers. Her research interests are probiotic organisms, dairy products and fermented soy products.