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Assay of β-Glucosidase Activity of Bifidobacteria and the Hydrolysis of Isoflavone Glycosides by *Bifidobacterium* sp. Int-57 in Soymilk Fermentation

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Abstract The isoflavone glycosides are hydrolyzed by β-glucosidase from gut microbes to the bioactive aglycones. However, the specific bacteria from the human intestinal tract that are involved in the metabolism of these compounds are not known. This study was undertaken to develop a fermented soymilk which converts isoflavones to the more bioactive aglycones form using a *Bifidobacterium* strain. The β-glucosidase activity of 15 *Bifidobacterium* strains were measured during cell growth. Among them, *Bifidobacterium* sp. Int-57 was selected for this study, because it has the highest β-glucosidase activity. Growth, acid development, β-glucosidase activity, and the hydrolysis of daidzin and genistin were investigated in four soymilks inoculated with *Bifidobacterium* sp. Int-57. After 12 h of fermentation, the counts of viable *Bifidobacterium* sp. Int-57 in all the soymilks reached a level of more than 10^8 cfu/ml, which was then maintained. The pH of soymilks started to decrease rapidly after 6 h of fermentation and leveled off after 18 h. The titratable acidity of BL#1 soymilk, BL#2 soymilk, and JP#1 soymilk increased from 0.18 to 1.21, 1.15, and 1.08% over the fermentation period, respectively. After 24 h of fermentation, the β-glucosidase activity in BL#1 soymilk, BL#2 soymilk, JP#1 soymilk, and JP#2 soymilk increased to 59.528, 40.643, 70.844, and 56.962 mU/ml, respectively. The isoflavone glycosides, daidzin and genistin, in soymilks were hydrolyzed completely in the relatively short fermentation time of 18 h. These results show that *Bifidobacterium* sp. Int-57 can be used as a potential starter culture for developing fermented soymilk which has completely hydrolyzed isoflavone glycosides.

Key words: *Bifidobacterium* sp. Int-57, β-glucosidase activity, daidzin, genistin, soymilk

Soybeans are a predominant dietary source of isoflavones, which are heterocyclic phenols closely related in structure to the estrogenic steroids and present in soybean mainly as the 7-*O*-glycosides. Isoflavones have wide ranging beneficial effects upon health, for example, in the prevention of breast, bowel, prostate, and other cancers [1], cardiovascular disease [20], and osteoporosis [4]. It is known that colonic bacteria play an important role in the metabolism of isoflavone glycosides [16]. *In vitro* studies of germ-free or antibiotic-treated experimental animals and the *in vivo* incubation of conjugated isoflavones with fecal material have shown that the isoflavone glycosides undergo hydrolysis by β-glucosidase derived from fecal bacteria, leading to the production of isoflavone aglycones, which can be absorbed from intestinal lumen or further metabolized by anaerobic intestinal microflora [7, 14, 16]. Furthermore, the isoflavone glycosides, daidzin and genistin, are less bioactive than their respective aglycones, daidzein and genistein, and are very poorly absorbed compared with their corresponding aglycones [24]. Therefore, bacteria with β-glucosidase activity are potentially important in the production of compounds with higher estrogenicity and better absorption, facilitating the bioavailability of isoflavones [13]. However, the specific bacteria in the human intestinal tract that are involved in the metabolism of these compounds are not known.

Soymilk is the water extract of soybeans, which are known to contain high quality proteins, but no cholesterol or lactose. Therefore, soymilk has been administered to people who are lactase-deficient [21]. However, the unfavorable beany flavor and the high content of indigestible α-D-galactosyl oligosaccharides, the flatulence factors, limit the consumption of soybean as a raw food material [11]. Fermentation methods have been examined to overcome these limitations and to improve the nutritional value of soymilk [8, 10, 12, 15, 21]. New varieties of yellow and black soybeans have been developed in National Crop Experiment Station, Rural Development Administration in Korea. Jinpum#1kong (JP#1) lacks two of the three lipoxygenase isozymes (L-2 and L-3) and Jinpum#2kong (JP#2) lacks all the lipoxygenase isozymes (L-1, L-2, and L-3), which affect the production of the beany flavor in soybeans and

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their products [17]. Black#1kong (BL#1) and Black#2kong (BL#2) for cooking with rice had been known to have a stronger antioxidative effect than yellow soybeans [6].

Bifidobacteria are the natural inhabitants in the gut of warm-blooded animals. They have health-promoting properties by maintaining an improved intestinal bacterial composition, the reduction of serum cholesterol, inhibition of the growth of potential pathogens, stimulation of the immune response, and possible anticarcinogenic activity [5]. In addition, these bacteria appear to be promising starter cultures for the production of good tasting fermented soymilk products that contain reduced quantities of antinutritional factors [18]. For these reasons, the recent incorporation of bifidobacteria in various dairy products has become increasingly popular. However, there is a lack of detailed information in the literature about the behavior of bifidobacteria in soymilk.

The objective of this study was to develop a fermented soymilk containing isoflavones in more bioactive aglycones form and to improve acceptability. The screening of the *Bifidobacterium* strain with the highest β-glucosidase activity from the commercial and isolated *Bifidobacterium* strains was conducted. In addition, the suitability of this strain as a starter culture for the fermentation of soymilk and its capacity to hydrolyze the conjugated isoflavones present in the soymilk were evaluated.

MATERIALS AND METHODS

Microoganisms and Culture Conditions

Five commercial *Bifidobacterium* strains (*Bifidobacterium animalis* ATCC 25527, *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707, *B. bifidum* ATCC 29521, and *B. infantis* ATCC 15697) were purchased from the American Type Culture Collection (ATCC), and the healthy human feces origin *Bifidobacterium* isolates (*Bifidobacterium* sp. Int-57, *B. bifidum* BGN2, *B. bifidum* BGN3, *B. bifidum* BGN4, *Bifidobacterium* sp. RD13, *Bifidobacterium* sp. SJ32, *Bifidobacterium* sp. RD54, *Bifidobacterium* sp. RD3, *Bifidobacterium* sp. RD65, and *Bifidobacterium* sp. SH5) were obtained from Professor Dr. G. E. Ji, Department of Food and Nutrition, Seoul National University (Seoul, Korea).

After two successive transfers of the nine *Bifidobacterium* strains in the modified BHI broth (Difco, Detroit, MI, U.S.A.) supplemented with 0.05% L-cysteine-HCl (w/w) (Sigma Chemical Co., St. Louis, MO, U.S.A.) at 37°C for 24 h anaerobically, the activated cultures were again inoculated into modified BHI broth at 37°C for 48 h. At 6 h intervals, samples were collected and analyzed for their growth and β-glucosidase activity.

Assay of β-Glucosidase Activity

The bacterial strain with the highest β-glucosidase activity was selected for further study of the metabolism of isoflavone glycosides in soymilk. The β-glucosidase activity was assayed by determining the rate of hydrolysis of *p*-nitrophenyl-β-glucopyranoside (Sigma, St. Louis, MO, U.S.A.). Cells from the culture were harvested by centrifugation at 11,000 rpm for 15 min at 4°C, washed twice with 0.2 M sodium phosphate buffer at pH 6.0, and resuspended in the same buffer. β-Glucosidase activity was measured by adding acetone-toluene (9:1, v/v) solution and 1 mM *p*-nitrophenyl-β-glucopyranoside, and incubating the mixture at 45°C. The reaction was terminated by adding 0.5 M sodium carbonate solution. The amount of *p*-nitrophenol released in the supernatant was measured at 400 nm using a model DU® 530 spectrophotometer (Beckman, 4300 N. Harbor Boulevard, Fullerton, U.S.A.). One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate per min. Each experiment was repeated three times and the values reported represent mean values of these results.

Preparation and Fermentation of Soymilk

Two yellow soybeans, Jinpum#1kong (JP#1) and Jinpum#2kong (JP#2), and two black soybeans, Black#1kong (BL#1) and Black#2kong (BL#2), were obtained from the National Crop Experiment Station, Rural Development Administration in Korea.

Whole soybeans were washed and soaked in distilled water for 8 h at 20°C. Water was decanted, and the soaked soybeans were mixed with 10 times their weight of distilled water and blended for 3 min in an Osterizer blender (U.S.A.). The resultant slurry was then filtered through a double-layered cheesecloth to yield the soymilk, which was dispensed into containers and autoclaved for 15 min at 121°C.

For the fermentation, soymilk was inoculated with a culture of *Bifidobacterium* sp. Int-57 to obtain an initial population of 10^6 cfu/ml. Incubation was carried out at 37°C and samples were taken at 6-h intervals over a 24-h period.

Viable cell counts of *Bifidobacterium* sp. Int-57 were determined in triplicate by using the pour plate method on BL agar (Difco, Detroit, MI, U.S.A.) supplemented with 5% horse blood (v/v) (Komed Co., Ltd., Sungnam-city, Kyungki-do, Korea). Plates were incubated under anaerobic conditions using an anaerobic jar (Difco, Detroit, MI, U.S.A.) method and gas pack system (AnaeroGen™ Compact, Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 2–3 days.

The Determination of pH and Titratable Acidity

The pH of the sample was measured with a pH meter (DP-215 M, Dong Woo Medical, Seoul, Korea), and titratable acidity was determined by titration with 0.1 N NaOH solution and expressed as percent lactic acid [3]. Each experiment was repeated three times, and the results were represented as the means of triplicate tests.

HPLC Quantification of Isoflavone Glycosides

Authentic standards of genistin, daidzein, and genistein were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and daidzin was obtained from Indofine (Somerville, NJ, U.S.A.). Each culture was extracted with 80% ethanol solution for 24 h at 50°C, and allowed to settle for a few minutes. The supernatant was then filtered with a syringe filter (Millex LCR 13 mm NS, MSCLRO, 0.45 μm, Millipore Co., Bedford, MA, U.S.A.) and the filtrate was used for HPLC analysis. Isoflavones analysis was performed by reversed-phase HPLC (HP 1090 Series II, Hewlett Packard Co., Waldbronn, Germany) equipped with a C18 column (RP C18 90A Phamaceutical, Vydac™, U.S.A.) connecting a guard column packed with μ Bondapak C18 (Waters guard-Pak™ precolumn, Milford, MA, U.S.A.). The mobile phase was composed of 0.1% acetic acid in acetonitrile (solvent A) and 0.1% acetic acid in third-distilled water (solvent B). Following the injection of 20 μl of sample, solvent A was increased from 15% to 35% over 50 min, and then held at 35% for 10 min. The solvent flow rate was 1 ml/min and the eluted isoflavones were detected at 254 nm. Results shown represent the means of three independent experiments.

Results and Discussions

Screening the *Bifidobacterium* Strain with the Highest β-Glucosidase Activity

The β-glucosidase activities of 15 *Bifidobacterium* strains were determined using the substrate *p*-nitrophenyl-β-D-glycopyranoside (data not shown). The β-glucosidase activity in modified BHI medium reached a maximum after 18–36 h of cultivation, which corresponded to the stationary phase of growth based on the O.D. 600 (optical density at 600 nm) of the cultures, and then decreased during the death phase. *Bifidobacterium* sp. Int-57, *Bifidobacterium* sp. RD13, and *Bifidobacterium* sp. SJ32 showed higher β-glucosidase activity than the other *Bifidobacterium* strains; in particular, *Bifidobacterium* sp. Int-57 showed the highest activity, whereas *B. longum* ATCC 15707 had the lowest β-glucosidase activity among the 9 *Bifidobacterium* strains tested. As shown in Fig. 1, the β-glucosidase activity of *Bifidobacterium* sp. Int-57 was 24.40 mU/ ml after 6 h of cultivation, and increased abruptly during the exponential phase, and then decreased rapidly after reaching its maximum level (45.70 mU/ml after 30 h of fermentation). However, *Bifidobacterium* sp. Int-57 maintained a high β-glucosidase activity at the end of the cultivation period (15.17 mU/ml). The β-glucosidase activity of *Bifidobacterium* sp. RD13 and *Bifidobacterium* sp. SJ32 reached a maximum after 36 h of incubation, i.e., 25.33 mU/ml and 25.08 mU/ml, and thereafter reduced to 11.24 mU/ml and 10.66 mU/ml at the end of the cultivation period, respectively. Some

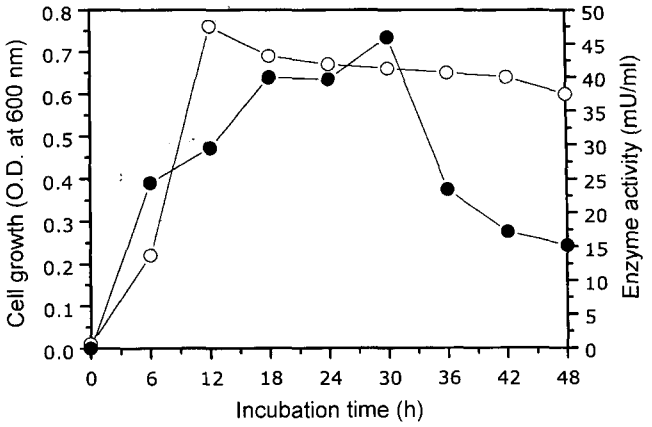

Fig. 1. Cell growth and β-glucosidase activity of *Bifidobacterium* sp. Int-57 at 37°C.
Symbols: ●, β-glucosidase activity; ○, cell growth.

bifidobacteria had been reported to possess higher β-glucosidase activity than other intestinal bacteria, such as *Staphylococcus*, *Bacteroides*, *Eubacterium*, *E. coli*, *Lactobacillus*, and *Streptococcus*, which are also resident flora of the intestinal tract [9]. On the other hand, although Choi *et al.* [9] reported that the levels of β-glucosidase activity of *Bifidobacterium* sp. Int-57 are higher than the other *Bifidobacterium* strains when grown in BHI medium, *Bifidobacterium* sp. Int-57 was 3.14 times less active than the strain used in the present study. This discrepancy in the extent of β-glucosidase activity observed in soymilk may be attributed to the differences in the growth medium, i.e., Choi *et al.* [9] used only BHI medium, whereas we used the modified BHI medium supplemented with 0.05% L-cysteine-HCl (w/w). These results demonstrated that the activation level of β-glucosidase in *Bifidobacterium* sp. Int-57 is significantly depended on the type of growth medium used. Therefore, *Bifidobacterium* sp. Int-57 was selected for further study on its suitability as a starter culture for soymilk fermentation, and its capacity to metabolize the isoflavone glycosides present in soymilk.

Cell Growth and Acid Development of *Bifidobacterium* sp. Int-57 in Soymiks

The growth patterns of *Bifidobacterium* sp. Int-57 in four soymilks (BL#1 soymilk, BL#2 soymilk, JP#1 soymilk, and JP#2 soymilk) are shown in Fig. 2(a). After 12 h of fermentation, the viable population of *Bifidobacterium* sp. Int-57 in all the soymilks reached a level of more than 10^8 cfu/ml, and remained unchanged until after 24 h of fermentation. Shuler-Malyoth *et al.* [22] indicated that a good cultured milk should contain 10^6–10^8 cfu bifidobacteria per ml to be of dietetic-therapeutic benefit. Hence, the results of growth rates of Int-57 in soymilks indicated the potential usefulness of this strain, exclusively for the preparation of fermented soymilk. In BL#2 soymilk, *Bifidobacterium* sp.

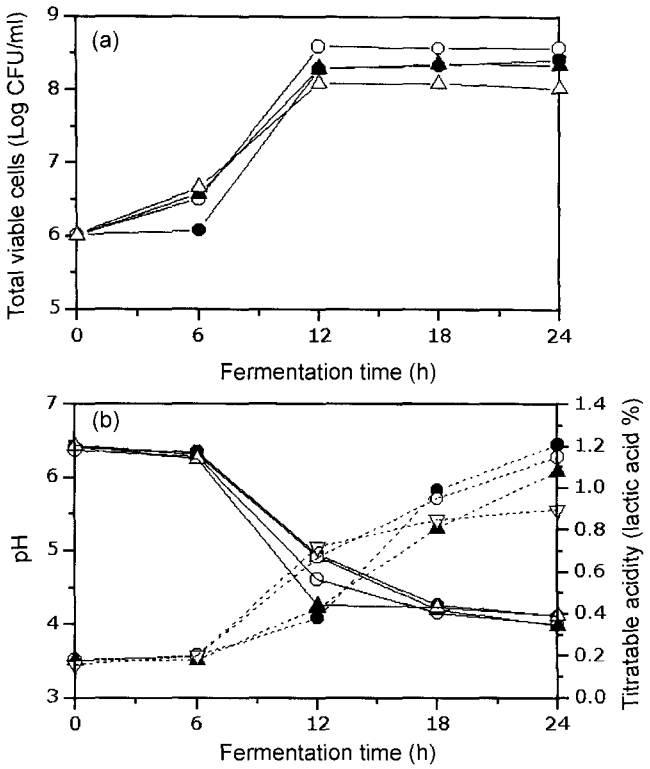

Fig. 2. The changes of (a) the cell growth and (b) pH (—) and titratable acidity (····) in soymilks inoculated with *Bifidobacterium* sp. Int-57 incubated at 37°C for 24 h.
Symbols: ●, BL#1 soymilk; ○, BL#2 soymilk; ▲, JP#1 soymilk; △, JP#2 soymilk.

Int-57 increased in population by 2.59 orders of magnitude after 12 h of fermentation, whereas the corresponding values attained by BL#1 soymilk and JP#1 soymilk were 2.41 and 2.35 after 24 h and 18 h of fermentation, respectively. Among the soymilks tested, JP#2 soymilk resulted in the lowest viable cell counts of *Bifidobacterium* sp. Int-57, but the final bacterial counts were similar to those of other soymilks after 24 h of fermentation. Similar observations were noted by Kamaly [15], who indicated that *B. longum* and *B. bifidum* in soymilk increased in population by 1.8 and 2.4 orders of magnitude after 24 h of fermentation.

Figure 2(b) shows the change of pH and acidity during the fermentation of soymilks with *Bifidobacterium* sp. Int-57. There was no appreciable difference in the pH of soymilks. The pH of soymilks started to decrease rapidly after only 6 h of fermentation and leveled off after 18 h. After fermentation, the reduction of pH 2.45 units was a maximum in JP#1 soymilk, followed by BL#2 soymilk, JP#2 soymilk, and BL#1 soymilk, of 2.38, 2.32, and 2.29 pH units, respectively. These were relatively high pH reductions compared with a previous study [15]. Kamaly [15] reported that pH changes by *B. longum* and *B. bifidum* in soymilk were 1.01 and 1.30 pH units, respectively. While Angeles and Marth [2] reported that coagulation of sterilized soymilk occurred at pH about 5.7, Park and Lee [19] reported that commercial yogurt have the pH range of 4.2 to 4.4. In the present study, the pH of soymilks reached that of commercial yogurt after 18 h of fermentation, with an exception of JP#2 soymilk, in which the pH started to drop drastically after only 12 h of fermentation to 4.26, and then stabilized. The acidity of BL#1 soymilk, BL#2 soymilk, and JP#1 soymilk increased from 0.18% at the beginning to 1.21, 1.15, and 1.08% at the end of fermentation, respectively. Moreover, a relatively lower titratable acidity of 0.90% was noted in the JP#2 soymilk after 24 h of fermentation. However, the titratable acidity of 24-h fermented soymilks was higher than that obtained previously [12]. Hou *et al.* [12] found that the titratable acidity of soymilks fermented with *B. infantis* CCRC 14633 and *B. longum* B6 increased from 0.17% to 0.25% and 0.30%, respectively, during 24 h of fermentation. The relatively marked acid development in the present study compared with previous studies [12, 15] might have been due to differences in the *Bifidobacterium* species and the composition of soybean cultivars used. In addition, as previously noted [22], the coagulum formed from soymilk was characterized by a fragile and weak curd.

β-Glucosidase Activity and Hydrolysis of Isoflavone Glycosides in the Fermentation of Soymilks

The behaviors of *Bifidobacterium* sp. Int-57 in soymilks were studied with respect to changes in their compositions of isoflavones and β-glucosidase activities during fermentation. As shown in Fig. 3, the β-glucosidase activity in soymilks increased dramatically after 12 h, which corresponded to the stationary phase of growth, based on the viable cell counts in the soymilks (Fig. 1). After 24 h fermentation, the β-glucosidase activity in BL#1 soymilk, BL#2 soymilk, JP#1 soymilk, and JP#2 soymilk increased to 59.528, 40.643, 70.844, and 56.962 mU/ml, respectively.

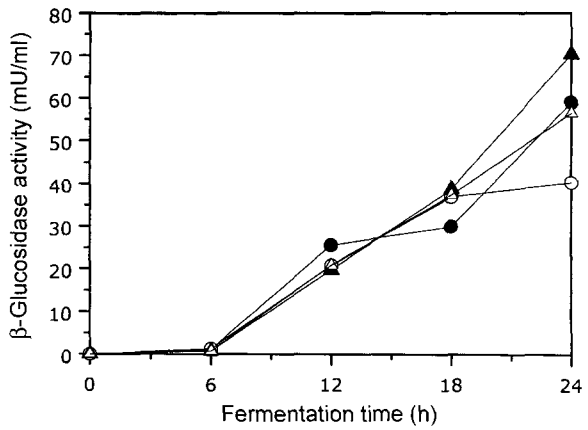

Fig. 3. The changes of β-glucosidase activity in soymilks inoculated with *Bifidobacterium* sp. Int-57 at 37°C for 24 h.
Symbols: ●, BL#1 soymilk; ○, BL#2 soymilk; ▲, JP#1 soymilk; △, JP#2 soymilk.

Table 1. Changes of daidzin and genistin contents in soymilks fermented with *Bifidobacterium* sp. Int-57 at 37°C.

	Fermentation time (h)	Isoflavone glycosides (μg/ml)		Isoflavone aglycones (μg/ml)	
		Daidzin	Genistin	Daidzein	Genistein
BL#1 soymilk	0	21.25	38.90	9.28	3.33
	18	n.d.*	n.d.	21.23	19.87
BL#2 soymilk	0	47.40	94.08	11.73	8.15
	18	n.d.	n.d.	42.30	56.26
JP#1 soymilk	0	98.76	129.61	21.52	15.36
	18	n.d.	n.d.	57.81	55.15
JP#2 soymilk	0	59.89	85.43	13.14	8.00
	18	n.d.	n.d.	48.39	45.94

*n.d.: not detected.

The concentrations of daidzin and genistin in soymilks are listed in Table 1. Isoflavone glycosides, daidzin and genistin, in soymilks were hydrolyzed completely in a relatively short fermentation time of 18 h. Thus, the reduction in the contents of daidzin and genistin and the increase in the contents of their respective aglycones in the present study may be attributed to the hydrolytic reaction catalyzed by β-glucosidase produced by *Bifidobacterium* sp. Int-57. These results confirm an earlier study by Choi *et al.* [8], who reported that 99.8–104.7% of daidzin and 95.3–106.1% of genistin were hydrolyzed during fermentation in soymilks inoculated with *Lactobacillus bulgaricus* KCTC 3188, *L. casei* KCTC 3109, *L. delbrueckii* subsp. *delbrueckii* KCTC 1047, *L. delbrueckii* subsp. *lactis* KCTC 1058, and *L. lactis* KCTC 2181. However, the rates of increase in daidzein and genistein were not proportional to the decrease of their respective glycosides contents. On the basis of a previous report [13], some of hydrolyzed daidzein and genistein might have been converted to other metabolites. Hur *et al.* [13] found that *E. coli* HGH21 and the Gram-positive strain HGH6 converted daidzin and genistin to their respective aglycones, and strain HGH6 was found to be involved in further modification of the isoflavonoid C-ring. This strain reduced a double bond between C-2 and C-3 of the C-ring of both daidzein and genistein to a single bond, which resulted in the formation of dihydrodaidzein and dihydrogenistein, respectively. These metabolites were found after incubation of daidzein and genistein with colonic microflora and also found in the urine of individuals consuming soy isoflavones [7, 14].

In conclusion, the results obtained in the present experiment show that *Bifidobacterium* sp. Int-57 is suitable as a starter culture for soymilk fermentation and that it plays an important role in the generation of biologically active isoflavone aglycones. Furthermore, JP#1 lacks two of the three lipoxygenase isozymes (L-2 and L-3) and JP#2 lacks all of the lipoxygenase isozymes (L-1, L-2 and L-3), which produce the beany flavor in soybeans and their products. Therefore, it is possible to produce a fermented soymilk which is enriched in isoflavone aglycones, thereby improving the acceptability of JP#1 or JP#2 soymilk by inoculating with *Bifidobacterium* sp. Int-57.

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