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Hydrolysis of Isoflavones and Consumption of Oligosaccharides during Lactic Acid Fermentation of Soybean Milk

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Abstract

Aiming to select suitable strains of lactic acid bacteria for producing yogurt-like functional food from soybean milk, the fermentation properties of 14 lactic acid bacterial strains belonging to genera *Bifidobacterium*, *Lactobacillus*, *Lactococcus* and *Streptococcus* were evaluated using soybean milk as the culture medium. All 14 strains examined were able to grow in soybean milk. Twelve out of the 14 strains lowered the pH of the medium and produced lactic acid. Tested strains showed a variety of consumption patterns of oligosaccharides and hydrolyzing capacity of isoflavone glucosides. Three strains, *Bifidobacterium breve* JCM 1192, *B. bifidum* JCM 1255 and *Lb. casei* subsp. *rhamnosus* IFO 3425 exhibited the most promising results. These strains could hydrolyze both daidzin and genistin to corresponding aglycones, and consume raffinose and stachyose in addition to lowering the pH and producing lactic acid.

Discipline: Food
Additional key words: daidzin, genistin, raffinose, stachyose, *Bifidobacterium, Lactobacillus, Streptococcus*

Introduction

In recent years, the health benefits of soybean-based products have been widely recognized all over the world. Epidemiological studies and clinical trials have revealed that isoflavones and oligosaccharides in soybean are effective for the prevention of various chronic diseases and hormone-associated health disorders[16]. Isoflavones are a kind of flavonoid present in *Leguminosae*, exhibiting many biological functions. Genistein, daidzein and their derivatives such as glucoside forms are major isoflavones in soybean. They have an estrogen-like activity that has been shown to be effective in preventing osteoporosis[19], in controlling and preventing cancer[10,13], in lowering total and LDL cholesterol[12] and also have some benefits in the postmenopausal hormone replacement therapy[5,18]. Dominant oligosaccharides of soybean are sucrose, raffinose and stachyose. The latter two are α-galactosyl oligosaccharides, and called prebiotics which are defined as nondigestible food components that give beneficial effect to the health by selectively activating probiotics, beneficial enterobacteria such as bifidobacteria[8].

Brazil is the second largest soybean producer in the world. However, its consumption as a food product is not very popular among the Brazilian population. One of the reasons is its off-flavor of low-molecular-weight-aldehydes which are produced during processing. The other reason is that soy-based food gives some people an uncomfortable feeling due to the presence of nondigestible oligosaccharides and trypsin inhibitor. Therefore, to

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introduce soy-based products to people who are not familiar with the specific flavor and taste, it is required to develop a method to produce good tasting soy-based food. Lactic acid fermentation would be a promising way, because some lactic acid bacteria are effective to reduce the off-flavor. Moreover, since the soybean benefits are more easily achieved with the isoflavones in their aglycone forms, fermented soybean products could provide a better source of food factors. For these reasons, soybean milk fermentation has also been studied and nowadays we have many reports using different microorganisms and different methods to obtain a good yogurt-like product (low pH, high lactic acid concentration and high viable microorganism number) from soybean milk[2,4,9,11]. In this way, the objective of this study was to evaluate not only growth properties of lactic acid bacteria in soybean milk, but to produce lactic acid and to lower the pH and also to consume sugars and to transform the isoflavones to their aglycone forms.

Materials and methods

1. Bacterial strains

Streptococcus thermophilus MAFF 400301 was obtained from the MAFF Genebank (Ibaraki, Japan). *Bifidobacterium breve* JCM 1192, *B. longum* JCM 1217, *B. infantis* JCM 1222, *B. bifidum* JCM 1255, *B. adolescentis* JCM 1275, *Lactobacillus casei* subsp. *casei* JCM 1134 and *Lactococcus lactis* subsp. *lactis* JCM 5805 were obtained from the Japan Collection of Microorganisms, (RIKEN, the Institute of Physical and Chemical Research, Saitama, Japan). *Lb. casei* subsp. *rhamnosus* IFO 3425, *Lb. delbrueckii* sbsp. *bulgaricus* IFO 139537 and *Streptococcus thermophilus* IFO 13957 were supplied from Institute of Fermentation Osaka (IFO), Japan. The other 4 strains, named CYSa, CYSb, CYLa, and CYLb were isolated from commercial yogurt using plate count agar containing bromocresol purple (BCP Agar, Nissui Co, Japan). CYSa and CYSb were *Streptcoccus*-like lactic acid bacteria; Gram positive, catalase-negative and cocci. CYLa and CYLb were *Lactobacillus*-like bacteria; Gram positive, catalase-negative and rod.

2. Soybean milk preparation

Soybean milk was prepared from a variety *Tachinagaha* harvested in Ibaraki, Japan in 1999. The grains (100 g) were mixed with 500 g of water, heated in a microwave oven (500 W) at 100ºC for 30 sec to inactivate lipoxygenase and avoid off-flavor formation. The grains and water were ground for 2 min in a pasteurized kitchen mixer and filtered using three layers of plastic mesh (60% polyester, 40% polyethylene terephthalate). The obtained soybean milk was pasteurized at 85ºC for 20 min twice with an interval of incubation at 37ºC for 4 h. The obtained soybean milk was used for the fermentation test after adjusting total solid content to 10%.

3. Culture of microorganisms

Lactic acid bacterial strains except for bifidobacteria were activated by transferring from skim milk (10%, w/v) into Tomato Juice Broth (TJB) or Tomato Juice Agar (TJA) followed by incubation at 37ºC for 24 h[1]. Bifidobacteria were activated in GAM broth (Nissui Co., Japan) at 37ºC for 24 h. Bacteria (inoculum size; 2.5×10^6 CFU/mL) except for bifidobacteria were cultured in the soybean milk (20 mL) in a 50 mL plastic tube and incubated at 37ºC for 24 h, followed by 15 days of storage at 4ºC[7]. Bifidobacteria were incubated in an Anaero Pack system (Mitsubishi Gas Chemical, Japan) at 37ºC for 24 h, followed by 15 days of storage at 4ºC.

4. Microbial analysis

Serial dilutions of a sample were prepared in autoclaved 0.85% sodium chloride and 1 mL of appropriate diluted suspension was mixed with molten Standard Methods Agar (Nissui Co., Japan) for counting total viable bacteria, BCP Agar for the lactic acid bacteria except for bifidobacteria and GAM Agar for the bifidobacteria. Standard Methods Agar plates and BCP Agar plates were incubated at 37ºC for 48 h. GAM plates were incubated in an Anaero Pack system at 37ºC for 48 h.

5. Physical and chemical analyses

The total solid content of the soybean milk was determined by weighing 10 g of soybean milk and drying at 105ºC for 6 h. For the titratable acidity measurement, 10 g of soybean milk or culture broth were weighed in an erlenmeyer flask and 20 mL of CO_2-free water was added. The titration was done with standardized 0.1N NaOH using phenolphthalein as an indicator. The titratable acidity was expressed as lactic acid.

Oligosaccharides in the culture broth (200 mg) were extracted by 80% ethanol (0.8 mL). After centrifugation, the supernatant was filtered through 0.22 μm membrane filter. The filtrate was analyzed with an HPLC (CCPS isocratic system, Tosoh, Japan) equipped with an evaporative light scattering detector (Sedex 55, France) and a TSK gel Amide 80 column (4.6 × 250 mm, Tosoh). The mobile phase used was 70% acetonitrile (isocratic elution) at a flow rate of 1.0 mL/min. Separation of oligosaccharides was performed at 80ºC. Authentic sucrose, raffinose and stachyose were products of Wako Pure Chemicals (Osaka, Japan).

Isoflavones in the culture broth (200 mg) were

extracted by 0.8 mL of 80% acetonitrile containing 0.5% formic acid and quercetin (18 mg, Sigma, St. Louis, USA) as an internal standard. After centrifugation, the supernatant was filtered through 0.22 μm membrane filter. The filtrate was analyzed with an HPLC (CCPM II system, Tosoh) equipped with a photodiode array detector and a Tosoh TSK-gel super ODS column (4.6 × 100 mm). The mobile phase system consisted of a linear gradient of 0.5% formic acid (solution A) and acetonitrile (solution B), starting with 5% B and reaching 45% B after 20 min.

Results and discussion

The composition of isoflavones in soymilk largely changed after a sterilizing treatment at 121ºC for 20 min (data not shown). Therefore, a milder pasteurizing treatment (heating at 85ºC for 20 min twice with an interval of incubation at 37ºC for 4 h) which did not give a significant change in the composition of isoflavone in soybean milk was employed in this study. No microbial contamination was detected after this treatment by the counting method described in materials and methods.

As shown in Table 1, all 14 strains of lactic acid bacteria tested in this study could grow in the soybean milk medium with a maximum viable cell count greater than 10^8 CFU/mL. Growth curves varied significantly between bacterial species as shown in Figs. 1-3. *Lb. delbrueckii* subsp. *bulgaricus* IFO 13953 (Fig. 3) and two *Lactobacillus*-like strains, CYLa and CYLb, showed maximum viable cell counts at 6 h and a constant decline in viable cell counts until 24 h. *B. breve* JCM 1192 (Fig. 1), *B. infantis* JCM 1222 and *B. longum* JCM 1217 grew logarithmically throughout 24 h of anaerobic culture, but these strains were not able to survive during the 15 days of storage at 4ºC. *S. thermophilus* MAFF 400301 and *Streptcoccus*-like lactic acid bacteria CYSa and CYSb exhibited quick growth until 6 h and kept constant viable

Table 1. Profiles of lactic acid bacteria on soybean milk fermentation

Strain	Growth[a)]	pH lowering[b)]	Lactic acid production[c)]	Sugar consumption[d)]	Isoflavone hydrolysis[e)]
Bifidobacterium adolescentis JCM 1275	++++	+++	+++	SO	G
Bifidobacterium bifidum JCM 1255	++++	+++	++++	O	DG
Bifidobacterium breve JCM 1192	++++	+++	+++	O	DG
Bifidobacterium infantis JCM 1222	++++	+++	+++	O	G
Bifidobacterium longum JCM 1217	++++	+++	+++	N	G
Lactobacillus casei subsp. *casei* JCM 1134	++++	–	–	N	DG
Lactobacillus casei subsp. *rhamnosus* IFO 3425	++++	+++	+++	O	DG
Lactobacillus delbrueckii subsp. *bulgaricus* IFO 13953	++++	++++	++++	SO	N
CYLa	++++	++++	++++	SO	N
CYLb	++++	++++	++++	SO	N
Lactococcus lactis subsp. *lactis* JCM 5805	++++	–	–	N	DG
Streptococcus thermophilus MAFF 400301	++++	+++	+++	S	N
CYSa	++++	+++	++++	S	N
CYSb	++++	+++	++++	S	N

a): ++++, more than 10^8 CFU/mL.
b): ++++, lower than pH 4.0; +++, pH 4.0–4.74; –, higher than pH 4.74.
c): ++++, more than 1.0 g/100 g; +++, less than 1.0 g/100 g; –, not detected.
d): S, consumed only sucrose; SO, consumed sucrose, raffinose and/or stachyose; O, consumed raffinose and/or stachyose but not sucrose; N, none.
e): G, hydrolyzed only genistin; DG, both daidzin and genistin; N, none.

cell counts until 24 h.

Lowering pH of culture media and production of lactic acid are essential for manufacturing yogurt. In the soybean milk, most strains tested could lower pH and produce lactic acid. However, *Lb. casei* subsp. *casei* JCM 1134 and *Lc. lactis* subsp. *lactis* JCM 5805 showed neither lowering pH of the culture medium nor production of lactic acid, but these strains could grow in the soybean milk. *Lb. delbrueckii* subsp. *bulgaricus* IFO 13953 (Fig. 3), CYLa and CYLb produced significantly high amounts of lactic acid. Lactic acid concentrations of the culture broth of *Lb. delbrueckii* subsp. *bulgaricus* IFO 13953, CYLa and CYLb at 24 h were 1.50, 1.58 and 1.59 g per 100 g of culture medium at 24 h, respectively. After 15 days of storage at 4ºC, there was no change in pH or in lactic acid concentration for any culture broth.

Tested lactic acid bacterial strains showed a variety of consumption patterns of oligosaccharides. Hence, they could be classified into four groups: strains which consumed only sucrose (type S), strains which consumed only raffinose and/or stachyose (type O), strains which consumed sucrose, raffinose and/or stachyose (type SO) and strains which could not consume any of those oligosaccharides (type N). *S. thermophilus* MAFF 400301, CYSa and CYSb were classified into type S. *B. bifidum* JCM 1255, *B. breve* JCM 1192 (Fig. 1), *B. infantis* JCM

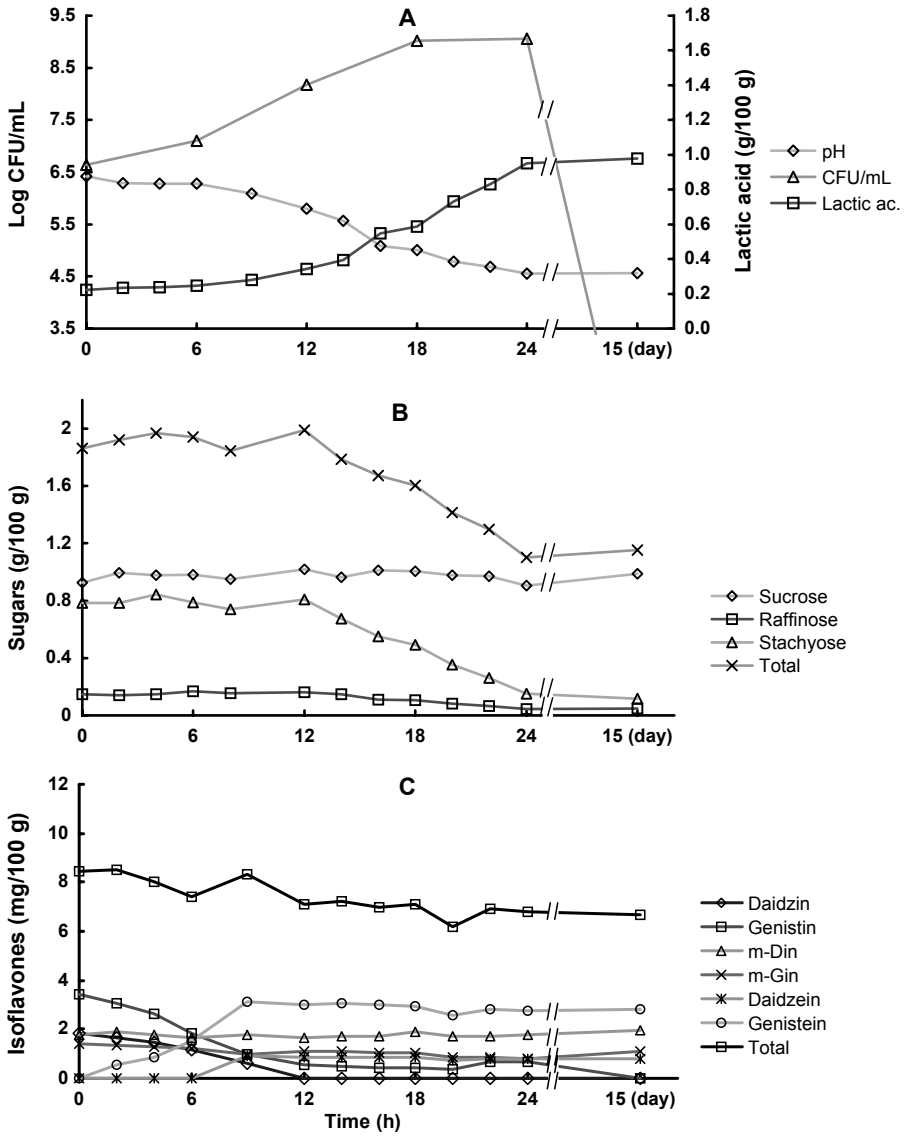

Fig. 1. Fermentation of soybean milk with *Bifidobacterium breve* JCM 1192
A: CFU/mL, pH and lactic acid (titratable acidity).
B: Sugar concentration.
C: Isoflavone concentration.

1222 and *Lb. casei* subsp. *rhamnosus* IFO 3425 were type O. *B. adolescentis* JCM 1275 (Fig. 2), *Lb. delbrueckii* subsp. *bulgaricus* JCM 13953 (Fig. 3), CYLa and CYLb were type SO. *B. longum* JCM 1217, *Lb. casei* subsp. *casei* JCM 1134 and *Lc. lactis* subsp. *lactis* JCM 5805 were type N. Most bifidobacteria tested in the present study could utilize raffinose and/or stachyose. This data well agreed with some previous reports[6,15]. Strains classified into type O and SO are effective to remove nondigestible sugars from soybean milk. In addition, many of these strains are probiotics which improve microbial flora of human intestine[8].

Tested lactic acid bacterial strains could be classified into three groups based on their hydrolyzing capacity of isoflavones: strains which hydrolyze mainly genistin (type G), strains which hydrolyze both daidzin and genistin (type DG) and strains which did not hydrolyze isoflavones (type N). *B. adolescentis* JCM 1275 (Fig. 2), *B. infantis* JCM 1222 and B. *longum* JCM 1217 were classified into type G. *B. bifidum* JCM 1255, *B. breve* JCM 1192 (Fig. 1), *Lb. casei* subsp. *casei* JCM 1134, *Lb. casei* subsp. *rhamnosus* IFO 3425 and *Lc. lactis* subsp. *lactis* JCM 5805 were type DG. Out of the 15 strains tested, 6 strains could not hydrolyze isoflavones (type N): *Lb. delbrueckii* subsp. *bulgaricus* IFO 13953 (Fig. 3), CYLa, CYLb, *S. thermophilus* MAFF 400301, CYSa and CYSb.

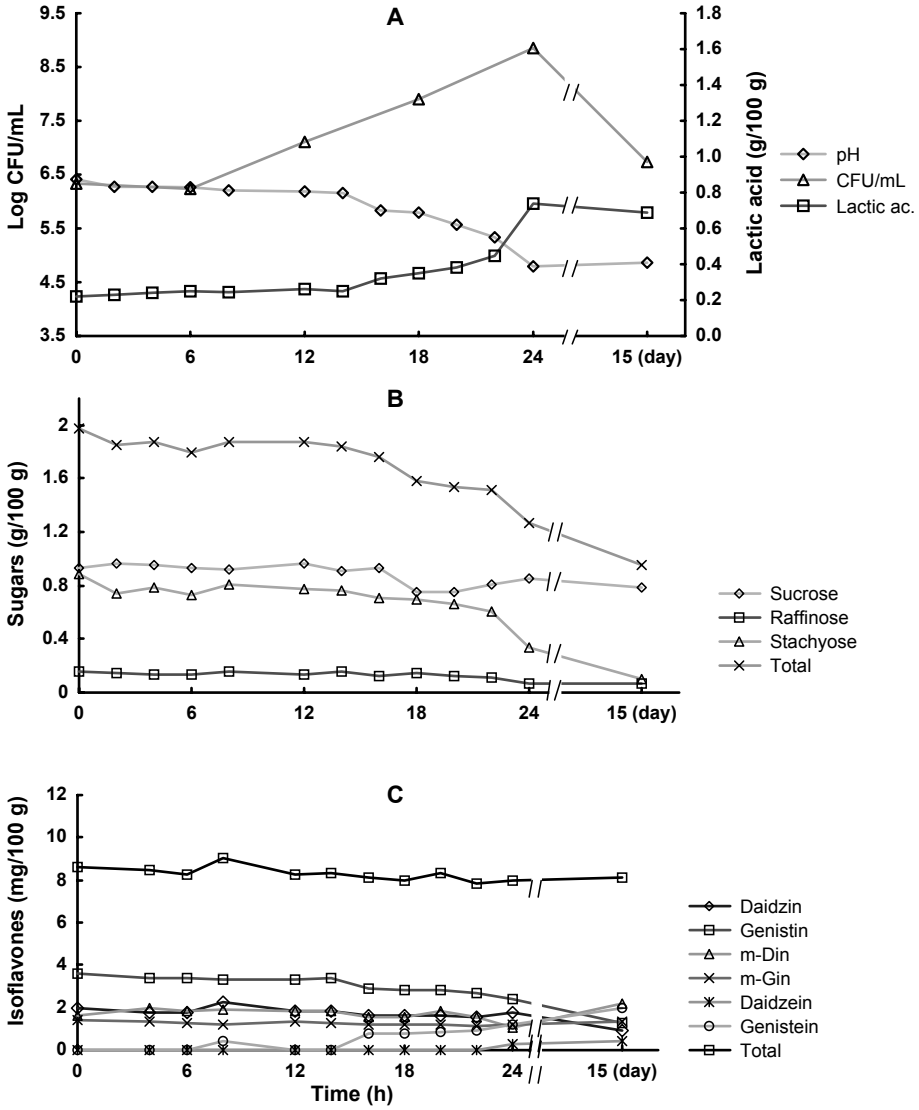

Fig. 2. Fermentation of soybean milk with *Bifidobacterium adolescentis* JCM 1275
A: CFU/mL, pH and lactic acid (titratable acidity).
B: Sugar concentration.
C: Isoflavone concentration.

It has been reported that many bifidobacteria[17] and some other lactic acid bacteria[3,14] hydrolyze isoflavone glycosides into corresponding aglycones. In general, isoflavones express higher biological activity such as estrogen-like activity in the aglycone form, since aglycone isomers are able to bind to hydrophobic sites of receptors or enzyme proteins[16]. Thus, it is expected that the biological activity of soybean milk increases after fermentation using the strains which could hydrolyze isoflavone glycosides.

From these results, it is concluded that the most suitable strains to produce the yogurt-like fermented soybean milk containing high concentrations of isoflavone aglycones are *B. breve*, *B. bifidum* and *Lb. casei* subsp. *rhamnosus*. Meanwhile, two strains, *Lb. casei* subsp. *casei* and *Lc. lactis* subsp. *lactis* exhibited isoflavone hydrolyzing capacity, even though they lacked lactic acid fermentation capacity. These strains are expected to be useful for soybean beverage production or for use in mixed culture fermentation. In contrast, *Lb. delbrueckii* subsp. *bulgaricus* IFO 13953, CYLa, CYLb, *S. thermophilus* MAFF 400301, CYSa and CYSb did not hydrolyze isoflavone glycosides, however they efficiently produced lactic acid in soybean milk. These strains are also expected to be used in mixed cultures with other microorganisms.

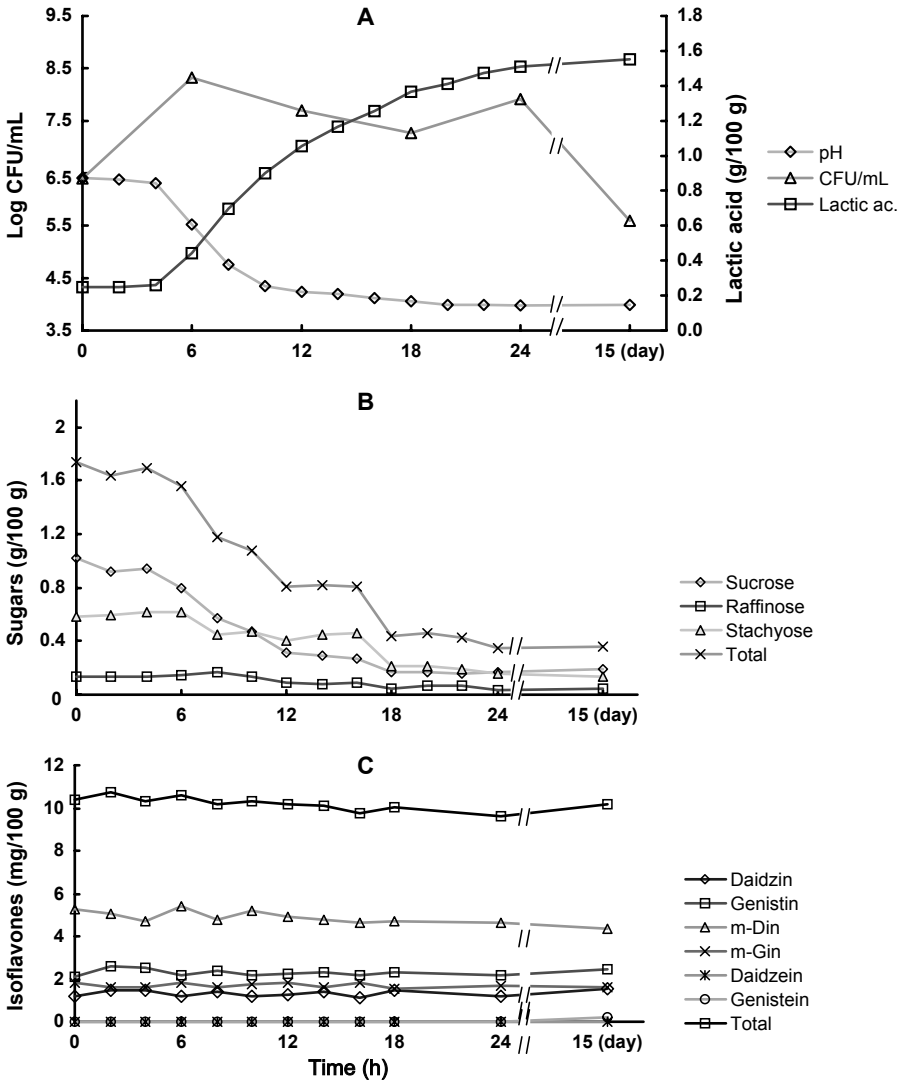

Fig. 3. Fermentation of soybean milk with *Lactobacillus delbrukii* subsp. *bulgaricus* IFO 13953

A: CFU/mL, pH and lactic acid (titratable acidity).
B: Sugar concentration.
C: Isoflavone concentration.

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