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ARTICLE

Synthesis of Isoflavone Aglycones and Equol in Soy Milks Fermented by Food-Related Lactic Acid Bacteria and Their Effect on Human Intestinal Caco-2 Cells

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One hundred and three strains of lactic acid bacteria, isolated from various food ecosystems, were assayed for β-glucosidase activity toward *p*-nitrophenyl-β-D-glucopyranoside substrate. *Lactobacillus plantarum* DPPMA24W and DPPMASL33, *Lactobacillus fermentum* DPPMA114, and *Lactobacillus rhamnosus* DPPMAAZ1 showed the highest activities and were selected as the mixed starter to ferment various soy milk preparations, which mainly differed for chemical composition, protein dispersibility index, and size dimension. The soy milk made with organically farmed soybeans (OFS) was selected as the best preparation. All selected strains grew well in OFS soy milk, reaching almost the same values of cell density (ca. 8.5 log cfu/mL). After 96 h of fermentation with the selected mixed starter, OFS soy milk contained 57.0 μM daidzein, 140.3 μM genistein, 20.4 μM glycitein, and 37.3 μM equol. Fermented and nonfermented OFS soy milks were used for the in vitro assays on intestinal human Caco-2/TC7 cells. Fermented OFS soy milk markedly inhibited the inflammatory status of Caco-2/TC7 cells as induced by treatment with interferon-γ (IFN-γ) (1000 U/mL) and lipopolysaccharide (LPS) (100 ng/mL), maintained the integrity of the tight junctions, even if subjected to negative stimulation by IFN-γ, and markedly inhibited the synthesis of IL-8, after treatment with interleukin-1β (2 ng/mL). As shown by using chemical standards, these effects were due to the concomitant activities of isoflavone aglycones and, especially, equol, which were synthesized in the fermented OFS soy milk preparation.

KEYWORDS: Soy; isoflavone aglycones; equol; lactic acid bacteria; Caco-2/TC7 cells

INTRODUCTION

Isoflavones are diphenol compounds that occur naturally in plants, mainly in soybean (*1*). Isoflavones of soybean and soy foods belong to four chemical classes: aglycones and malonyl, acetyl- and β-glucoside conjugates. Of the total concentration of isoflavones in soybean, $>90\%$ exist in the glucoside forms (*1*). The β-glucoside forms are not absorbed in humans due to their high hydrophilic feature and large molecular mass, and they require hydrolysis to aglycones for bioavailability and subsequent metabolism. Hydrolysis to aglycones such as daidzein, genistein, and glycitein occurs along the entire length of the intestinal tract by the activity of both the brush border membrane and bacterial β-glucosidases (*2*). Intestinal biotransformation includes dehydroxylation, reduction, C-ring cleavage, and demethylation. Once released, aglycones are structurally similar to estrogens and mimic the function of estradiol in the human body (*3*). Consumption of isoflavone aglycones was associated with potential reduced risk of most hormone-associated health disorders (*4*, *5*) and lowest incidence of osteoporosis, menopausal symptoms, and mortality from cardiovascular disease and cancer (*6*). Contrarily to these potential health benefits, some concerns were raised about the possible detrimental effects of soy consumption due to the estrogenicity of the isoflavone constituents (*7*).

Equol [7-hydroxy-3-(4′-hydroxyphenyl)chroman] is a nonsteroidal estrogen of the isoflavone class. The main dietary origin of equol in humans is soy foods because these are the most abundant sources of the isoflavone daidzin and aglycone daidzein, its precursor (*8*). The formation of equol from daidzein occurs via a pathway that involves the synthesis of the intermediate dihydrodaidzein. Unlike isoflavone aglycones, equol is unique in having a chiral center due to the lack of a double bond in the heterocyclic ring (*2*). Overall, equol is easily absorbed through the colon wall, has a slower plasma clearance rate than daidzein, and is metabolically inert (*2*). Equol deserves increasing interest because its biological activities differ from those of its precursor: it has higher extrogenicity (*9*) and antioxidant activity (*10*), and it demonstrates antiandrogenic properties (*11*). Consumption or

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intestinal biosynthesis of equol markedly lowered plasma total cholesterol, LDL cholesterol, LDL/HDL cholesterol ratio, triglycerides, and lipoprotein (*2*).

The metabolism of soy isoflavones in humans is well documented (*8*, *12*). The capacity to metabolize isoflavone glucosides to aglycones and aglycones to equol during intestinal transit is restricted to only 30−50% of individuals in Western populations (*13*). Two main routes might be pursued for increasing the bioavailability of isoflavones from soy foods: enrichment of aglycones and equol prior to consumption and/or modulation of the intestinal microbiota via the ingestion of viable bacteria capable of in situ generation of these compounds (*14*). Several studies (*1*, *14*−*18*) considered the use of bifidobacteria and lactic acid bacteria to convert isoflavone glucosides into aglycones and/or equol during soy milk fermentation. All of these studies used a limited number of bacterial strains, which were from human fecal origin only. Besides, none of these studies assayed the biological activity of the resulting preparations. The enrichment of soy foods in aglycones and equol should also include the careful and large selection of the substrate of fermentation. Traditionally, soy milk is the aqueous extract of whole soybeans. In modern soy milk preparation, methods evolved to utilize defatted soy materials (essentially soy protein isolate) in an attempt to simplify the manufacture and to enhance the nutritional and flavor profile of the resulting soy milk.

This study screened a large number of strains of lactic acid bacteria, isolated from various food ecosystems, on the basis of their β-glucosidase activity. A pool of strains was selected and used for the synthesis of isoflavone aglycones and equol in the presence of several soy milk preparations. The preparation that contained the highest concentration of aglycones and equol was in vitro assayed for biological activities toward human intestinal Caco-2 cells.

MATERIALS AND METHODS

Bacterial Strains, Culture Condition, and Preparation of the Cell Suspension. One hundred and three strains belonging to the species *Lactobacillus alimentarius* (10N, 2B, 5A), *Lactobacillus brevis* (5Z, DPPMA869), *Lactobacillus casei* (LC10), *Lactobacillus casei* subsp. *casei* (2047, 2756, 2763, 2766), *Lactobacillus casei* subsp. *pseudoplantarum* (2742, 2745, 2749, 2750), *Lactobacillus curvatus* (13H5, 14H10, 1Hd, 2042, 2081, 2768, 2770, 2771, 2775, SAL23, SAL35), *Lactobacillus delbrueckii* subsp. *bulgaricus* (11842, $B_{15}Z$), *Lactobacillus fermentum* (DPPMA114, D13), *Lactobacillus gasseri* ($B_{30}W$), *Lactobacillus helveticus* (15009, $B_{26}W$, PR4), *Lactobacillus hilgardii* (51B), *Lactobacillus paralimentarius* (15α, 15β, 16R, 8D, DPPMA238), *Lactobacillus paracasei* (12H8, 1Hb, $B_{14}F_5$, $B_{18}S$, $B_{25}F_3$, PF6, $B_{61}F_5$), *Lactobacillus pacarbuckneri* ($B_{10}F_5$, $B_{48}F_3$, $B_{48}F_5$, B_9F_{5t}, BF_1, BF_2), *Lactobacillus paraplantarum* (4DE, DPPMA667), *Lactobacillus pentosus* (8CF, 12H5, 12H6), *Lactobacillus plantarum* (14H4, 17N, 19A, 1A7, 2A, 30, 3DM, 4H1, 4H10, DB200, DPPMASL33, DPPMA24W), *Lactobacillus rhamnosus* (11, 19, DPPMAAZ1, DPPMAAZ21), *Lactobacillus sakei* (9I, SAL1, SAL18), *Lactobacillus rossiae* (10A, 15R, 3D, 5C1, 5α, CF51, CI35, CR20, E18), *Lactobacillus sanfranciscensis* (16α, A17, BB12, DE9, E19, H10), *Lactococcus lactis* subsp. *lactis* (10γ), *Pediococcus pentosaceus* ($C_{16}F_5$, $C_{25}F_3$, $C_{30}F_{5t}$, C_6F_5, C_7F_3, C_9F_{5t}, $C_{29}F_5$), and *Weissella cibaria* (10XA16, 3XA4, 5S, 5XF12) were used in this study. All strains belong to the Culture Collection of the Department of Plant Protection and Applied Microbiology of Bari University, Italy. All of the above strains were isolated from various food ecosystems (cheeses, sourdoughs, fermented pineapple, fermented tomato, marrows, and carrots). Strains were routinely propagated in MRS broth (Oxoid, Basingstoke, Hampshire, U.K.) at 30 or 37 °C for 24 h. Twenty-fours hour cultures were harvested by centrifugation (10000*g*, 10 min at 4 °C), washed twice with sterile 50 mM potassium phosphate buffer, pH 7.0, and resuspended in sterile distilled water at a 620 nm absorbance of 2.5, corresponding to ca. log 9.5 CFU/mL. These cell suspensions were used to assay β-glucosidase activity.

Chemicals. Daizdein, glycitein, genistein, and equol were purchased from Sigma (Sigma-Aldrich Chemical Corp., St. Louis, MO). Dulbecco's Modified Medium (DMEM), nonessential amino acids, penicillin−streptomycin, and Hepes were purchased from EuroClone (EuroClone S.p.A., Pero, Milano, Italy). Fetal bovine serum (FBS) was from Hyclone (Thermo Fisher Scientific Inc., Waltham, MA).

β-Glucosidase Activity. The β-glucosidase activity was measured in terms of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) substrate (Sigma) (*19*). The assay mixture contained 900 μL of 2.5 mM *p*NPG (final concentration) in 0.5 M potassium phosphate buffer, pH 7.5, and 100 μL of cell suspension. The mixture was incubated at 40 °C, and the reaction was stopped by heating the mixture at 95 °C for 5 min. The absorbance was measured at 410 nm. One unit (U) of enzyme activity was defined as the amount of β-glucosidase that released 1 μmol of *p*-nitrophenol from the substrate *p*NPG per milliliter per minute under the assay conditions (*16*).

Soy Milks. Organically farmed soybean variety PR9M10 (OFS) (EcorNaturaSi, Verona, Italy), soy protein isolate (SPI) (Supro Soja 80 Aptonia, Villeneuve d'Ascq, France), and various commercial soy flours (Cargill Texturizing Solutions Soy Protein, Gent, Belgium) were used for the manufacture of soy milk. OFS was washed overnight and soaked in distilled water (ratio of 5:1, w/v). Swollen soybeans (moisture content ca. 46.5%) were manually dehulled, and the resulting grind was diluted with distilled hot water (ca. 90 °C) (1:10, w/v) and homogenized by using a mixer blender (PBI International, Milan, Italy). Homogenization was at 10000 rpm for 2 min, followed by 1 min of pause and further treatment at 10000 rpm for 2 min. The slurry was centrifuged (7000*g*, 10 min at 4 °C), and soy milk was sterilized by filtration through 0.22 μm membrane filters (Millipore Corp., Bedford, MA). The pH was 6.2. SPI was diluted with distilled water (40 °C) (0.4:10, w/v) and heated at ca. 55 °C for 30 min under stirring conditions (120 rpm). After cooling at room temperature, the pH was adjusted to 6.7 using 5 M NaOH (*1*). Sterilization was at 121 °C for 15 min. Commercial soy flours mainly differed for chemical composition (protein, fat, crude fiber, and soluble carbohydrates), protein dispersibility index, and size dimension (**Table 1**). ProLia 68237 and 68238, Provasoy 68288, 68290, 68282, and 68280, Provafull 68147, soy flour, soy semolina, soy grits, full-fat soy flour, and low-fat soy flour were diluted with distilled water (40 °C) (1:10, w/v) according to the method described by Chun et al. (*15*). The pH was ca. 6.3. Sterilization was at 121 °C for 15 min. During sterilization, a slight precipitation of proteins could not be excluded due to the suspension of soy flour in distilled water.

Fermentation of Soy Milks. Soy milks were inoculated with 4% (v/v) of the pooled cell suspension of selected lactic acid bacteria. The proportion of the four strains in the selected starter was approximately 1:1:1:1. The initial cell density of individual strains was ca. log 7.0 CFU/mL. Fermentation was at 30 °C for 96 h under stirring conditions (120 rpm). Soy milks without bacterial inoculum were incubated under the same conditions and used as the control. For assays on human intestinal cells, soy milk was previously freeze-dried, resuspended in DMEM, and sterilized through filtration (0.22 μm).

Randomly Amplified Polymorphic DNA−Polymerase Chain Reaction (RAPD-PCR) Analysis of Lactic Acid Bacteria. The monitoring of *L. plantarum* DPPMASL33 and DPPMA24W, *L. fermentum* DPPMA114, and *L. rhamnosus* DPPMAAZ1 during fermentation of soy milks was carried out by RAPD-PCR after isolation and restreaking of ca. 10 colonies from the highest MRS plate dilutions. Two primers (Invitrogen, Milan, Italy), with arbitrarily chosen sequences (P7 5′-AGCAGCGTGG-3′, and M13, 5′-GAGGGTGGCGGTTCT-3′) (*20*, *21*), were used singly in two series of amplification. The reaction mixture contained 200 μM of each dNTP, 1−2 μM primer, 1.5−3 μM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Invitrogen), 2.5 μL of PCR buffer, 25 ng of DNA, and sterile double-distilled water to 25 μL. For amplifications with primer P7, the PCR program comprised 45 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 35 °C, and elongation for 2 min at 72 °C; the cycles were preceded by denaturation at 94 °C for 4 min and followed by elongation at 72 °C for 5 min. For primer M13, amplification reactions were carried out according to the protocol described by Rossetti and Giraffa (*21*): one cycle at 94 °C for 60 s (denaturing), 42 °C for 20 s (annealing), and 72 °C for 2 min (elongation). PCR products were separated by electrophoresis (2 h at 130 V) on 1.5% (w/v) agarose gel (Invitrogen), and the DNA was detected by UV transillumination after staining with ethidium bromide (0.5 μg/mL). The molecular weight of the amplified DNA fragments was estimated by comparison with 1 kb Plus DNA Ladder (Invitrogen). For RAPD analysis,

Table 1. Chemical Composition, Protein Dispersibility Index, and Size Dimension of Commercial Soy Flours

soy flour	protein (%)	fat (%)	crude fiber (%)	soluble carbohydrate (%)	protein dispersibility index	particle size (mesh)[a]	description
OFS[b]	13.1	6.7	1.1	7.5	65.3	160	raw soybeans manually dehulled
SPI[c]	83	4.4	1.5	1.7	20.1	72	soy protein isolate, flavored with vanilla and sweetened with aspartame
Prolia 68237	54	0.95	3.5	13.5	70	200	defatted, slightly heat treated
Prolia 68238	55	1.1	3.5	14	77.5	200	defatted, nontoasted
Provasoy 68288	54	1.25	3.5	14	22.5	100	defatted, enzymatically debittered
Provasoy 68290	54	1.25	3.5	13.5	22.5	200	defatted, enzymatically debittered
Provasoy 68282	54.2	1.0	3.5	14	22.5	100	defatted, enzymatically debittered
Provasoy 68280	55.6	1.7	3.5	13.8	22.5	80	defatted, enzymatically debittered
Provafull 68147	39.0	21.0	3.5	12	15	72	full-fat toasted
soy flour	40	22	20	7.0	17	120	toasted
soy semolina	38	22	12.4	6.5	21	120	dehulled and toasted
soy grits	38	22	20	6.5	19	11	dehulled and toasted
full-fat soy flour	38.2	23	16.7	12.5	20	100	cleaned, mechanically processed
low-fat soy flour	45.6	11.7	18.2	13	22	100	extruded, mechanically expelled and milled

[a] Mesh, number of openings per inch. [b] OFS, organic farming soybean. [c] SPI, soy protein isolate.

the presence or absence of fragments was recorded as 1 or 0, respectively. Only reproducible well-marked amplified fragments were scored, faint bands being ignored.

Extraction of Isoflavones−Aglycones and Equol from Soy Milks and HPLC Analysis. The extraction of isoflavones from fermented and nonfermented soy milks was carried out as described by Otieno and Shah (*22*). After incubation, samples of soy milk were freeze-dried, and an aliquot of 0.5 g was added to 25 mL of methanol and refluxed on a heating mantle for 1 h. The mixture was filtered through Whatman no. 1 filter paper (Carlo Erba Reagenti SpA, Rodano, Milano, Italy). Determination of isoflavones was carried out using an Äkta purifier HPLC equipped with a detector UV900 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The reversed phase silica column was an XTerraTM MS C18 5 μm, 4.6 × 250 mm (Waters, Brussels, Belgium). Analyses were carried out under isocratic conditions with an eluent mixture consisting of 40% solvent B (acetonitrile/methanol 20:80, v/v) in solvent A (0.05% formic acid in water, v/v). The flow rate was 0.8 mL/min, the temperature of the column was 40 °C, and the run time was set to 30 min (*23*). Peak identification was carried out by comparing retention times with those of standards (Sigma Chemical Co.). Calibration curves for daizdein, glycitein, genistein, and equol were obtained using concentrations in the range of 0.2−200 μM.

Cell Cultures. Human intestinal Caco-2 cells (TC7 clone) (*24*) supplied by the Istituto Superiore di Sanità (Rome, Italy) were routinely cultured in DMEM, supplemented with 10% (w/v) heat-inactivated FBS, 1% (w/v) nonessential amino acids, 50 μg/mL penicillin/streptomycin, and 1 mM Hepes. Cells were maintained in 25 cm^3 culture flasks at 37 °C under 97% humidified atmosphere and 5% CO_2. The culture medium was replaced three times a week. Passage was performed at 80% of confluence. Experiments were carried out on passages from 55 to 68.

Human Cell Proliferation. Cell viability was measured using the Neutral Red (NR) uptake assay (*25*). Caco-2/TC7 cells were seeded in 96-well plates at the density of 1×10^4 cells/well and cultivated for 24 h at 37 °C. Incubation was carried out with chemical standards of daizdein and glycitein dissolved in dimethyl sulfoxide (DMSO), and equol was dissolved in methanol at the final concentrations of 5, 50, and 100 μM. After 24, 48, and 72 h of incubation, the culture media were removed from the well plates, and the cells were washed with 200 μL of PBS and incubated for 4 h at 37 °C with 150 μL of freshly prepared NR solution (33 mg/L). Furthermore, cells were washed twice with PBS, and 150 μL of lysing solution (50%, v/v, ethanol in Milli-Q water with 1%, v/v, acetic acid) was added to wells. The 96-well plates were shaken for 10 min, and the absorbance was measured at 540 nm using a Novapath microplate reader (Bio-Rad, Hercules, CA). DMEM containing methanol (0.5%, v/v) and DMSO (1%, v/v) was used as the negative control for equol and genistein, daizdein, or glycitein, respectively.

Nitric Oxide (NO) Determination. NO was determined by measuring the stable oxidation products nitrite and nitrate (*26*). Caco-2/TC7 cells were seeded in 24-well plates by resuspending on DMEM medium (Gibco, BRL, Gaithersburg, MD) without phenol red at the density of 2.5×10^4 cells/well and cultivated for 7 days at 37 °C. After cultivation, chemical standards of daizdein, glycitein, or equol (10 μM) or fermented or nonfermented OFS soy milks, diluted at the final concentration of 10 μM of equol, were added to DMEM, and cells were further treated for 24 h. Diluted OFS soy milks were sterilized through 0.22 μm filter membrane (Millipore) to remove lactic acid bacteria cells. After the addition of chemical standards and fermented and nonfermented OFS soy milks, interferon-γ (IFN-γ) was added to cells. All experiments were carried out also using 1000 U/mL of IFN-γ (Peprotech, Rocky Hill, NJ) and 100 ng/mL of lipopolysaccharide (LPS) (Sigma, St. Louis, MO). After 48 h of incubation, cell culture supernatants were mixed with an equal volume of Griess reagent (Sigma) (1%, w/v, sulfanilic acid in 0.5 M HCl and 0.1%, w/v, N^1-1-naphthylethylendiamine hydrochloride), and the absorbance at 540 nm was measured after 30 min. The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

Measurement of the Transepithelial Electric Resistance (TEER). To allow differentiation, Caco-2/TC7 cells were seeded (1×10^4 cells/mL) onto 12-well insert plates with polyethylene terephthlate (PET) membrane (pore size of 0.4 μm) (BD Falcon, Franklin Lakes, NJ) and cultivated for 18 days at 37 °C. After cultivation, cells were treated for 24, 48, and 72 h with chemical standards of daizdein, glycitein, genistein, or equol at concentrations of 5, 50, and 100 μM. DMEM containing methanol (0.5%, v/v) and DMSO (1%, v/v) were used as the negative controls. The integrity of the monolayer was monitored by measuring the TEER through the Millicell-ERS Voltohmmeter (Millipore, Billerica, MA). Fermented and nonfermented OFS soy milks were diluted at the final concentration of 10 μM equol and sterilized through filtration. All experiments were carried out also using 1000 U/mL IFN-γ. Measurements were expressed in ohms × cm^2, after subtracting the mean values of the resistance from cell-free inserts. TEER data were recorded at room temperature.

Interleukin-8 (IL-8). Caco-2/TC7 cells were incubated for 24 h at 37 °C with interleukin-1β (2 ng/mL) (Peprotech) and then stimulated for another 24 h with chemical standards of daizdein, glycitein, or equol at the concentration of 10 μM or with fermented and nonfermented OFS soy milks diluted and filtered at the final concentration of 10 μM equol. DMEM containing methanol (0.5%, v/v) and DMSO (1%, v/v) was used as the negative controls. Synthesis of the pro-inflammatory IL-8 was measured using the enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems, Vienna, Austria). Quantification was carried out using a reference standard curve as provided by the manufacturer.

Statistical Analysis. All data were obtained at least in triplicates. Analysis of variance was carried out on transformed data followed by the separation of means with Tukey's HSD using a statistical software Statistica for Windows (Statistica 6.0 for Windows 1998, StatSoft, Vigonza, Italy). Letters indicate significantly different groups ($P < 0.05$) by Tukey's test. For NO, TEER, and IL-8 determinations, DMEM containing methanol (0.5%, v/v) and DMSO (1%, v/v) was used as the control for statistical Student's *t* test.

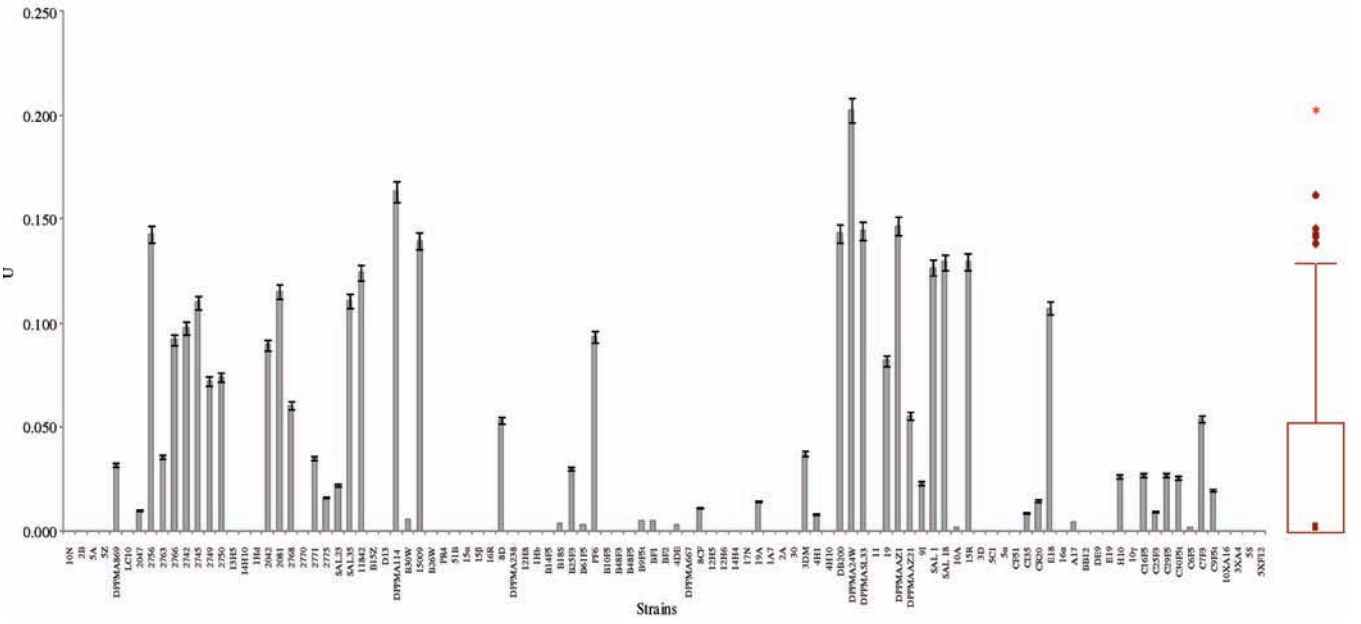

Figure 1. β-Glucosidase activity of strains of lactic acid bacteria on *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) substrate. One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute under the assay conditions. Strains are indicated by code; for correspondence to species, see Materials and Methods. Box plot is shown also. The center line of the box represents the median (■), and the top and bottom of the box represent the 75th and 25th percentiles of the data, respectively. The top and bottom of the error bars represent the 5th and 95th percentiles of the data, respectively. The circles in the box plot extend to the outliers of the data (●), and very extreme points are represented as individual data points (∗).

RESULTS

***β*-Glucosidase Activity.** Preliminarily, the β-glucosidases of 103 strains of lactic acid bacteria, isolated from various food ecosystems, was assayed using the synthetic *p*NPG substrate. The activity ranged from 0 to 0.202 U (**Figure 1**). Forty-eight strains mainly belonging to the species *L. alimentarius*, *L. brevis*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*, *L. hilgardii*, *L. paralimentarius*, *L. paraplantarum*, *L. pentosus*, *L. sanfranciscensis*, *Lc. lactis* subsp. *lactis*, *L. parabuckneri*, and *W. cibaria* did not exhibit β-glucosidase activity. The median value was found to be 0.003 U, and the values corresponding to the 25th and 75th percentile of the data were 0 and 0.045 U, respectively. Twenty-five strains belonging to various species of lactic acid bacteria showed β-glucosidase activity higher than 0.055 U. *L. plantarum* DPPMA24W isolated from cheese, *L. fermentum* DPPMA114 from wheat flour, *L. rhamnosus* DPPMAAZ1 from cheese, and *L. plantarum* DPPMASL33 from Italian sausages showed the highest activities (0.202 ± 0.07, 0.163 ± 0.06, 0.147 ± 0.06, and 0.144 ± 0.07 U, respectively), and the values were located out of the error bars of the box plot. On the basis of these results, these four strains were used as a mixed starter to ferment various soy milk preparations.

Fermentation of Soy Milks. Fourteen soy milks (**Table 1**) were subjected to fermentation by using the mixed starter consisting of selected *L. plantarum* DPPMA24W and DPPMASL33, *L. fermentum* DPPMA114, and *L. rhamnosus* DPPMAAZ1. All substrates underwent a process of lactic acidification (**Figure 2A**). After 96 h of incubation at 37 °C, the values of ΔpH ranged from 0.59 ± 0.06 to 1.19 ± 0.09 for Provasoy 68288 and low-fat soy flour, respectively. The median value was found to be 0.93, and the values corresponding to the 25th and 75th percentiles of the data were 0.79 and 1.01, respectively. After fermentation, the values of pH for all soy milks ranged from 5.1 to 5.3.

Lactic acid bacteria grew in all soy milks (**Figure 2B**). The values of ΔLog CFU/mL ranged from 0.99 ± 0.29 to from 1.61 ± 0.30 for full-fat soy flour and low-fat soy flour, respectively. The median value was found to be 1.31 ΔLog CFU/mL corresponding to the final cell density of 8.31 Log CFU/mL. The values corresponding to the 25th and 75th percentiles of the data were 1.21 and 1.43 ΔLog CFU/mL, respectively. Growth of lactic acid bacteria was completed during 24−36 h of incubation. As shown by RAPD-PCR typing, all four species of lactic acid bacteria used in the mixed starter grew in soy milks, reaching almost the same cell density.

The initial concentration of isoflavones in soy milks ranged from 142.3 ± 12.5 to 171.5 ± 10.8, from 102.2 ± 8.6 to 123.0 ± 11.3, and from 10.5 ± 1.1 to 18.0 ± 0.9 μM for daidzin, genistin, and glycitin, respectively (data not shown). Low concentrations (0−7.8 ± 0.5 μM) of aglycones were also found (**Table 2**). Except for soy milk made with full-fat soy flour, the concentration of aglycones increased during incubation of all soy milks. After 96 h, the highest concentration of daidzein was found in OFS (57.0 ± 4.0 μM) followed by Prolia 68238 (50.7 ± 2.1 μM) and Prolia 68237 (46.4 ± 1.7 μM). Also, the final concentration of genistein was the highest in the above three soy milks (140.3 ± 9.4, 102.9 ± 6.4, and 94.0 ± 5.3 μM for OFS and Prolia 68238 and 68237, respectively). Compared to the other aglycones, the concentration of glycitein was lower in all soy milks. The highest concentrations of glycitein were 23.9 ± 2.4, 22.5 ± 1.3, and 20.4 ± 1.0 μM for Prolia 68237 and 68238, and OFS, respectively. After 96 h of fermentation, the ratios of conversion (μM product/μM substrate) for OFS soy milks were 0.72, 0.85, and 0.98 for daidzin into daidzein, genistin into genistein, and glycitin into glycitein, respectively. Although the concentration of aglycones increased during incubation, all of the ratios of conversion after 24 h ranged from 1.0 to 0.95. Malonyl- and acetyl-glucosides were not considered from the assays of isoflavones because their concentrations were very low in all soy milk preparations (< 5% of total isoflavone concentrations) (data not shown). Nevertheless, the synthesis of aglycones also from this source might not be excluded.

Before incubation, equol was not found in the soy milk preparations (**Table 2**). Several soy milk preparations (e.g., SPI,

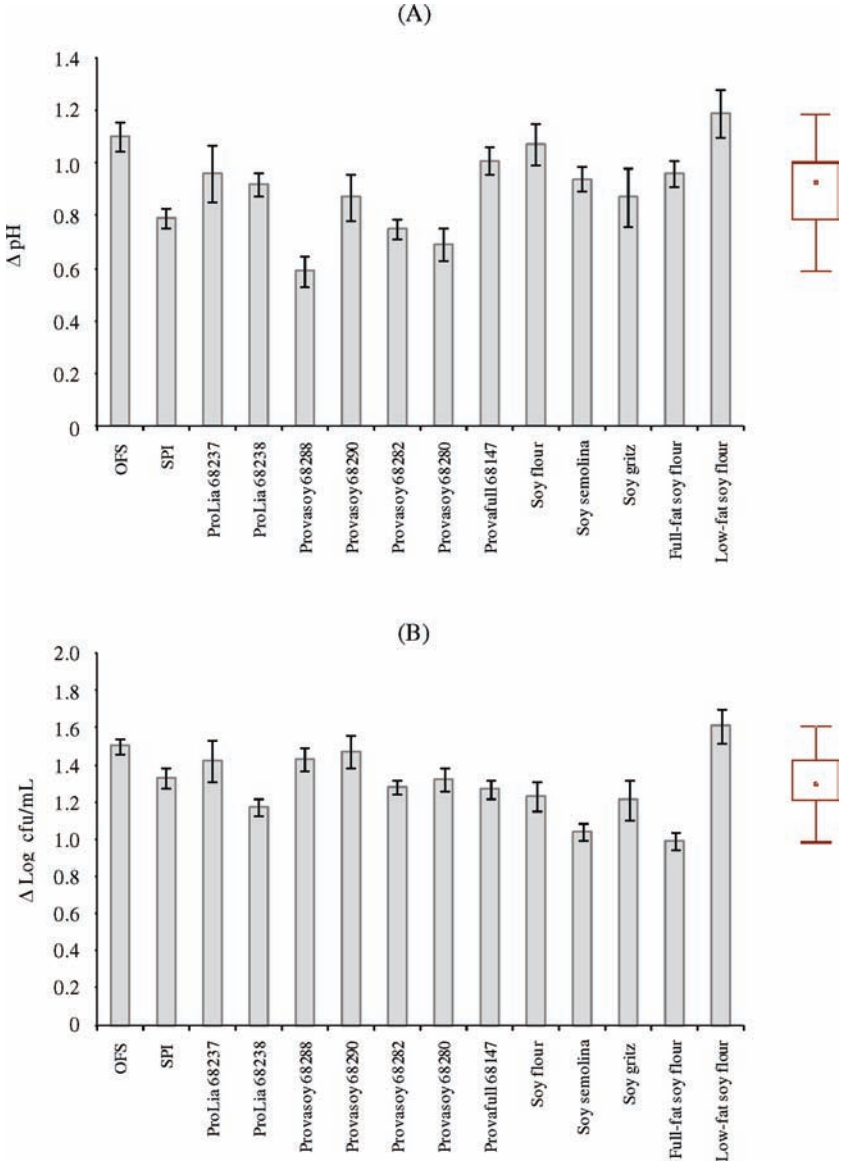

Figure 2. Lactic acidification, expressed as ΔpH (difference of pH before and after 96 h of fermentation) (**A**), and cell density, expressed as Δlog CFU/mL (difference of log CFU/mL before and after 96 h of fermentation) (**B**), of various soy milk preparations. For the manufacture and composition of soy milks see Materials and Methods and **Table 1**. OFS, organic farming soybean; SPI, soy proteins isolate. Box plot is shown also. The center line of the box represents the median (■), the top and bottom of the box represent the 75th and 25th percentiles of the data, respectively. The top and bottom of the error bars represent the 5th and 95th percentiles of the data, respectively.

Provasoy 68290 and 68282, soy flour) were not suitable for the synthesis of equol. For the other soy milks the concentration of equol increased during fermentation. After 96 h of incubation, the highest concentration of equol was found in soy milks such as Prolia 68238 and 68237 (20.0 ± 1.1 and 18.5 ± 0.9 μM, respectively) and, especially, in OFS (37.3 ± 1.5 μM). Due to the simultaneous synthesis of daidzein, the ratio of its conversion into equol was unpredictable.

On the basis of the above results, fermented and nonfermented OFS soy milks were chosen for the in vitro assays on intestinal human Caco-2/TC7 cells.

Viability of Caco-2/TC7 Cells. To assay the potential immunomodulatory properties of isoflavone aglycones contained in OFS soy milk, first, the cytotoxicity of the chemical standards (5−100 μM) was checked using the Neutral Red (NR) uptake assay. Genistein, glycitein, and equol behaved similarly to methanol and DMSO (negative controls) and did not show significant effect on the Caco-2/TC7 cell proliferation. After 72 h of treatment, daizdein significantly ($P < 0.03$) inhibited the cell proliferation (30%) at the highest concentration of 100 μM.

Nitric Oxide Release. Preliminarily, Caco-2/TC7 cells were treated for 24 h with chemical standards (10 μM), fermented (diluted to the concentration of 10 μM equol) OFS, or nonfermented OFS. These compounds or preparations did not induce the release of NO and behaved as the negative controls DMSO and methanol (**Figure 3**). Furthermore, Caco-2/TC7 cells were stimulated with IFN-γ (1000 U/mL) and LPS (100 ng/mL) for 24 h. This treatment significantly ($P < 0.01$) increased the release of NO and mimicked the inflammatory status of Caco-2/TC7 cells previously treated with negative control (DMSO or methanol), daidzein, or nonfermented OFS soy milk. On the contrary, treatments with equol or fermented OFS soy milk markedly ($P < 0.01$) inhibited the release of NO. Considerable inhibition of the release of NO was also found by treatments with genistein and glycitein ($P < 0.01$). Because the concentration (10 μM) of the isoflavone aglycones used in the assay was previously shown to be nontoxic,

Table 2. Concentration (Micromolar) of Isoflavone Aglycones and Equol Synthesized during Fermentation of Soy Milks Prepared by Using Various Soy Flours[a]

	daidzein						genistein						glycitein						equol					
soy milk	0 h	24 h	48 h	72 h	96 h		0 h	24 h	48 h	72 h	96 h		0 h	24 h	48 h	72 h	96 h		0 h	24 h	48 h	72 h	96 h	
Organically Farmed Soybean (OFS)																								
OFS	6.1 ± 0.7e	40.9 ± 4.8h	48.7 ± 3.5k	53.8 ± 5.3i	57.0 ± 4.0m	3.6 ± 1.4b	98.8 ± 8.9i	125.4 ± 12.1j	136.9 ± 10.3l	140.3 ± 9.4k	11.3 ± 0.5g	13.4 ± 1.2b	17.2 ± 0.9i	19.3 ± 1.6h	20.4 ± 1.0h	–	7.4 ± 1.1f	12.3 ± 1.8g	22.3 ± 1.3f	37.3 ± 1.5g				
Soy Protein Isolate (SPI)																								
SPI	4.9 ± 1.1c	23.2 ± 2.3g	31.1 ± 3.5j	35.4 ± 2.6g	36.6 ± 2.2j	7.4 ± 1.8c	35.2 ± 2.1h	64.0 ± 3.6i	75.1 ± 3.1i	75.9 ± 2.9h	–	12.3 ± 0.7a	19.7 ± 1.3j	19.8 ± 0.6h	20.1 ± 0.8h	–	–	–	–	–				
Commercial Soy Flours																								
Prolia 68237	4.7 ± 0.3c	8.70 ± 0.6f	26.3 ± 1.0h	40.1 ± 2.4h	46.4 ± 1.7k	7.8 ± 0.5d	21.1 ± 1.3f	62.2 ± 3.2i	88.1 ± 6.5j	94.0 ± 5.3i	6.7 ± 1.0d	10.9 ± 1.5d	17.6 ± 2.1i	21.5 ± 3.2i	23.9 ± 2.4i	–	6.1 ± 0.8e	9.3 ± 1.2e	11.2 ± 1.4d	18.5 ± 0.9e				
Prolia 68238	5.9 ± 0.8e	9.8 ± 1.7	29.5 ± 2.0i	43.3 ± 2.7h	50.7 ± 2.1l	7.0 ± 0.6c	24.4 ± 2.0g	58.2 ± 3.7h	93.6 ± 6.3k	102.9 ± 6.4j	6.3 ± 0.4d	10.5 ± 1.2d	14.8 ± 3.2h	21.1 ± 1.8i	22.5 ± 1.3i	–	5.5 ± 1.3d	10.9 ± 1.0f	18.7 ± 1.4e	20.0 ± 1.1f				
Provasoy 68288	3.1 ± 0.6b	6.3 ± 0.4d	14.9 ± 0.7d	19.3 ± 1.8e	21.6 ± 1.2g	–	9.6 ± 0.4d	25.9 ± 1.4f	35.1 ± 0.7h	38.1 ± 0.8g	–	4.2 ± 0.5c	7.0 ± 0.8c	9.8 ± 0.6b	10.5 ± 0.4c	–	3.2 ± 0.2b	5.3 ± 0.6b	10.4 ± 0.5d	13.6 ± 0.8c				
Provasoy 68290	–	3.5 ± 0.3b	7.5 ± 0.6b	11.0 ± 1.1c	12.6 ± 0.7c	–	4.8 ± 0.5a	16.3 ± 0.5e	22.6 ± 0.8f	24.8 ± 1.0e	–	5.6 ± 0.5d	8.1 ± 0.7d	9.8 ± 0.6b	10.2 ± 0.5c	–	–	–	–	–				
Provasoy 68282	2.0 ± 0.5a	4.3 ± 0.8c	7.9 ± 1.3b	10.2 ± 0.7b	11.4 ± 1.0b	–	4.8 ± 0.3a	7.4 ± 0.5b	11.5 ± 0.3c	14.4 ± 0.6c	7.4 ± 0.5f	9.8 ± 0.7f	12.3 ± 0.6f	14.4 ± 1.3e	15.5 ± 1.1f	–	–	–	–	–				
Provasoy 68280	3.1 ± 0.2b	7.1 ± 0.6e	12.6 ± 1.0c	15.7 ± 0.5d	18.5 ± 0.8e	2.2 ± 0.1a	6.3 ± 0.6c	9.6 ± 0.7d	17.0 ± 0.4e	18.9 ± 0.7e	6.0 ± 0.5d	8.8 ± 0.4e	13.7 ± 0.4h	16.9 ± 1.1g	17.6 ± 0.8g	–	4.4 ± 0.5c	7.4 ± 0.1d	10.6 ± 0.7d	14.5 ± 0.3d				
Provafull 68147	–	9.0 ± 0.6f	15.7 ± 0.9e	22.8 ± 1.2e	26.3 ± 0.5h	–	6.7 ± 0.3c	10.0 ± 0.6d	14.4 ± 0.2d	16.6 ± 0.4d	–	3.2 ± 0.2b	4.9 ± 0.5a	6.0 ± 0.7a	6.7 ± 0.5a	–	3.4 ± 0.3b	5.6 ± 0.4b	8.5 ± 0.2b	10.3 ± 0.5b				
soy flour	–	0.8 ± 0.4a	7.1 ± 0.8b	11.0 ± 0.7c	13.4 ± 0.5d	–	4.1 ± 0.3a	10.0 ± 0.4d	11.5 ± 0.2c	11.8 ± 0.5b	3.9 ± 0.2b	12.0 ± 0.7h	14.1 ± 1.0h	15.1 ± 0.8f	17.2 ± 0.6g	–	–	–	–	–				
soy semolina	–	7.5 ± 0.3e	17.3 ± 1.2f	23.2 ± 1.4e	26.3 ± 0.9h	–	5.9 ± 0.2b	9.2 ± 0.5c	10.7 ± 0.6b	11.1 ± 0.8b	–	3.5 ± 0.1b	5.6 ± 0.3b	6.3 ± 0.5a	7.4 ± 0.2b	–	3.1 ± 0.9b	6.3 ± 0.6c	9.2 ± 0.7c	10.3 ± 0.2b				
soy grits	–	4.7 ± 0.3c	17.7 ± 0.5f	25.6 ± 1.3f	28.3 ± 1.0i	7.0 ± 0.3c	16.3 ± 0.8e	28.5 ± 2.1g	30.3 ± 3.2g	32.9 ± 1.7f	3.2 ± 0.3a	9.8 ± 0.2f	10.9 ± 0.7e	11.6 ± 0.6c	11.6 ± 0.3d	–	0.4 ± 0.4a	3.5 ± 0.2a	5.5 ± 0.3a	7.6 ± 0.1a				
full-fat soy flour	2.7 ± 0.6b	3.5 ± 0.5b	3.9 ± 0.2a	3.9 ± 0.5a	3.9 ± 0.8a	–	–	–	–	–	6.7 ± 0.1e	10.2 ± 0.6g	12.0 ± 0.5f	12.7 ± 0.4d	13.7 ± 0.9e	–	–	–	–	–				
low-fat soy flour	5.1 ± 0.9d	11.0 ± 0.4g	18.5 ± 1.0g	19.7 ± 1.6	20.1 ± 1.2f	–	4.4 ± 0.3a	6.3 ± 0.4a	7.4 ± 0.6a	7.8 ± 0.4a	4.6 ± 0.2c	9.5 ± 0.4f	13.0 ± 0.9g	14.1 ± 0.6e	14.8 ± 0.5f	–	–	–	–	–				

[a] For the manufacture and composition of soy milks, see Materials and Methods. Fermentation was carried out with the mixed starter consisting of *Lactobacillus plantarum* DPPMA24W and DPPMASL33, *Lactobacillus fermentum* DPPMA114, and *Lactobacillus rhamnosus* DPPMAAZ1. –, not found.

the death of Caco-2/TC7 cells did not interfere with the release of NO.

Transepithelial Electric Resistance. Under culture conditions, Caco-2/TC7 cells develop the morphological and functional characteristics of enterocytes including intercellular tight junctions, the integrity of which is measured by TEER. Preliminarily, TEER was measured in the presence of chemical compounds (10−100 μM) or fermented (diluted to the concentration of 10 μM equol) or nonfermented OFS soy milk. Except for equol at the concentration of 100 μM, no effects on TEER were found during 72 h of incubation (data not shown). Treatment of Caco-2/TC7 cells with IFN-γ (1000 U/mL) markedly ($P < 0.01$) decreased the value of TEER (**Figure 4**). When Caco-2/TC7 cells stimulated with IFN-γ were also subjected to treatment with fermented OFS soy milk, the negative effect of IFN-γ was markedly attenuated ($P < 0.01$). A negligible effect was found for treatment with nonfermented OFS soy milk. Genistein, glycitein, and, especially equol, behaved similarly to fermented OFS soy milk. Daidzein did not interfere with the negative effect of IFN-γ (data not shown).

Interleukin-8. IL-8 is a member of the C-X-C chemokine family and plays an essential role in the recruitment and activation of neutrophils, thereby initiating the inflammatory response. When Caco-2/TC7 cells were subjected to treatment with interleukin-1β (2 ng/mL), a significant ($P < 0.01$) increase of the synthesis of IL-8 was found (**Figure 5**). When Caco-2/TC7 cells stimulated with interleukin-1β were also subjected to treatment with equol and daidzein, a significant ($P < 0.01$) decrease of the synthesis of IL-8 was found. The inhibition of the synthesis of IL-8 was the highest ($P < 0.01$) by treatment with fermented OFS soy milk. On the contrary, treatments with genistein, glycitein, or nonfermented OFS soy milk did not significantly ($P < 0.01$) affect the concentration of IL-8 compared to Caco-2/TC7 cells subjected to treatment with interleukin-1β.

DISCUSSION

Several studies considered the use of presumptive probiotic strains, isolated from human fecal source, to enrich various soy milk preparations with isoflavone aglycones (*1*, *14*−*18*). Strains mainly belonged to bifidobacteria and *L. acidophilus*, *L. casei*, *L. paraplantarum*, *E. durans*, *S. salivarius*, *W. confusa*, and *L. paracasei* (*1*, *14*−*18*). This study considered a very large number of strains of lactic acid bacteria, isolated only from food ecosystems. Most of the species used were not previously assayed. Preliminarily, strains were selected on the basis of β-glucosidase activity toward the synthetic *p*NPG substrate. Although β-glucosidase activity was confirmed to be extremely strain dependent (*16*), ca. 25% of the assayed strains showed rather elevated enzyme activity. Four new strains, corresponding to *L. plantarum* DPPMA24W and DPPMASL33, *L. fermentum* DPPMA114, and *L. rhamnosus* DPPMAAZ1, showed the highest enzyme activity and were selected as the mixed starter to ferment various soy milk preparations. Variability was also found regarding the suitability of soy milk preparations to be enriched in isoflavone aglycones. Apart from the chemical composition, the main features shared by soy milks containing the highest levels of aglyycones were high indices of protein dispersibility (65.3−77.5) and small values of particle size (160−200 mesh). Other studies considered soy protein isolate (*1*, *16*), mixture of soy protein isolate and soy germ (*16*), or soy protein isolate supplemented with skim milk powder (*17*) or sucrose, fructose, and lactose (*18*). The soy milk prepared from OFS manually dehulled was selected as the best substrate for the conversion. The same protocol was successfully applied for the manufacture of the aglycones-enriched soy milk from the Tae-Kwang variety of soybean (*15*).

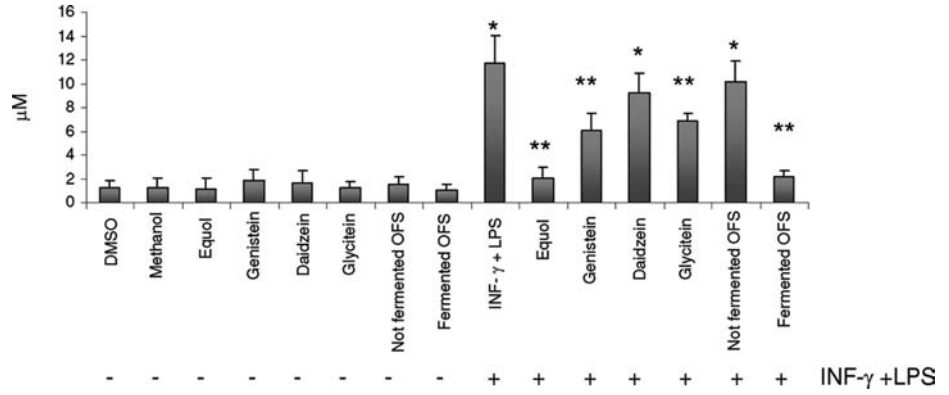

Figure 3. Nitric oxide (NO) release (μM) by Caco-2/TC7 cells. Cells were pretreated for 24 h with chemical standards (equol, daidzein, genistein, or glycitein) (10 μM), fermented (diluted to the concentration of 10 μM equol) or nonfermented OFS. Furthermore, cells were stimulated with interferon-γ (IFN-γ) (1000 U/mL) and lipopolysaccharide (LPS) (100 ng/mL) for 24 h. DMEM containing DMSO (1%,v/v) or methanol (0.5%, v/v) was the negative control. Data are the mean $\pm$ SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t* test. One asterisk indicates a significant difference ($P < 0.01$) with respect to the negative controls DMEM with added DMSO or methanol. Two asterisks indicate a significant difference ($P < 0.01$) with respect to IFN-γ and LPS.

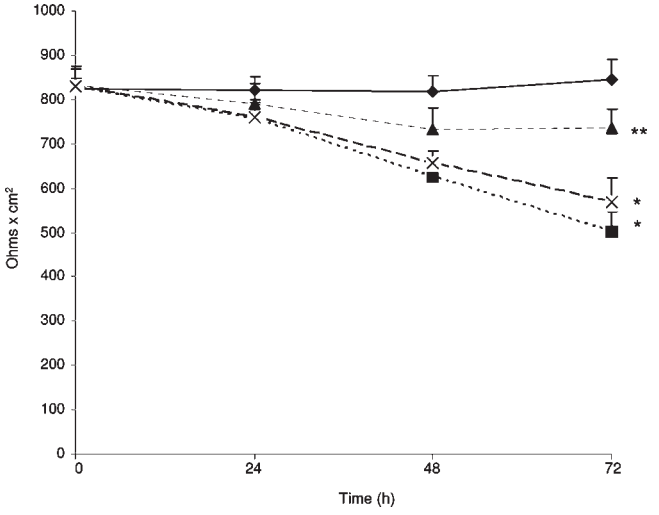

Figure 4. Transepithelial electric resistance (TEER) (ohms $\times$ cm^2) of Caco-2/TC7 cells after 24, 48, and 72 h. Incubation was with interferon-γ (IFN-γ, 1000 U/mL) (■), IFN-γ and fermented (▲) (diluted to the concentration of 10 μM equol), or not fermented ($\times$) OFS soy milks. DMEM medium was used as the negative control (◆). Data are the mean $\pm$ SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t* test. One asterisk indicates a significant difference ($P < 0.01$) with respect to the negative control DMEM. Two asterisks indicate a significant difference ($P < 0.01$) with respect to IFN-γ.

All selected strains grew well in OFS soy milk, attaining almost the same values of cell density (ca. 8.5 Log CFU/mL) found for other strains of lactic acid bacteria and bifidobacteria (*1*, *15*−*17*). Also, the moderate acidification of the soy milk seemed to be a common feature between OFS soy milk and other preparations (*1*, *15*−*17*). The time of incubation to get the highest synthesis of aglycones distinguished this study. Contrarily to other studies, which considered 12, 24, or 48 h of incubation (*1*, *15*−*17*), the highest concentration of aglycones was found after 96 of fermentation of OFS soy milk, even though partial degradation of these and other compounds could not be excluded. Under these conditions, the mixed starter synthesized 57.0 μM daidzein (corresponding to 1.45 mg/100 mL), 140.3 μM genistein (3.9 mg/100 mL), and 20.4 μM glycitein (0.58 mg/100 mL). The OFS soy milk had ca. 90% moisture. The concentrations of daidzein, genistein, and glycitein synthesized by *B. animalis* strains ranged from 19.4 to 21.4 mg/100 g, from 35.2 to 39.2 mg/100 g, and from 3.7 to 3.8 mg/100 g of freeze-dried sample, respectively (*17*). Those synthesized by *B. pseudolongum* and *B. longum* ranged from ca. 0.48 to 0.59 mg/100 mL, from 1.57 to 1.98 mg/100 mL, and from 0.17 to 0.35 mg/100 mL, respectively (*1*). After 12 h of incubation, *L. paraplantarum* synthesized ca. 131.4, 212.1, and 38.4 mg/100 g of dry soy milk of daidzein, genistein, and glycitein, respectively (*15*). The concentrations of aglycones produced by *E. durans*, *S. salivarius*, and *W. confusa* were markedly lower. The synthesis of daidzein and genistein by other strains of lactic acid bacteria and bifidobacteria was in the range of 26.3−30.6 mg/L (*18*). Except for *L. paraplantarum* (*15*), the mixed starter selected in this study had the capacity to synthesize aglycones as the most promising probiotic strains. Overall, the rates of hydrolysis of isoflavones into glucosides markedly varied depending on the strain. For instance, *L. paraplantarum* showed rates of conversion of 90, 100, and 61% for daizein, genistein, and glycitein, respectively (*15*). In the same study, *S. salivarius* and *W. confusa* preferentially metabolized daidzin and genistin, the rate of conversion of glycitin into glycitein being $<20\%$. The biotransformation of isoflavone into algycones by two strains of *B. animalis* varied between 75 and 85% depending on the soy milk preparation (*17*). After 96 h of fermentation, the mixed starter selected in this study showed ratios of conversion of 0.72, 0.85, and 0.98 for daidzein, genistein, and glycitein, respectively. Overall, it was well established that the synthesis of isoflavone aglycones improves the biological functionality of soy milk (*16*). Isoflavones contained in fermented foods such as tempe, natto, and soy sauce are more available to humans than those from unfermented products (*22*, *27*).

Only a few studies also considered the synthesis of equol during soy milk fermentation. Equol is mainly identified in human and animal urine (*8*, *28*) and bovine milk (*29*). The biologically potent equol metabolite was found in soy milks fermented with bifidobacteria (*1*). After 24 h of incubation, the highest concentration of equol (0.521 mg/100 mL) was synthesized by *B. animalis*, compared to 0.338 and 0.433 mg/100 mL for strains of *B. pseudolongum* and *B. longum* (*1*). The OFS soy milk fermented with the mixed starter selected in this study also contained 37.3 μM equol (corresponding to 0.9 mg/100 mL). Currently, individuals are distinguished in equol and non-equol producers on the basis of the concentration of this compound in the plasma (*2*). Typing of bacteria in these two categories led to the conclusion that certain

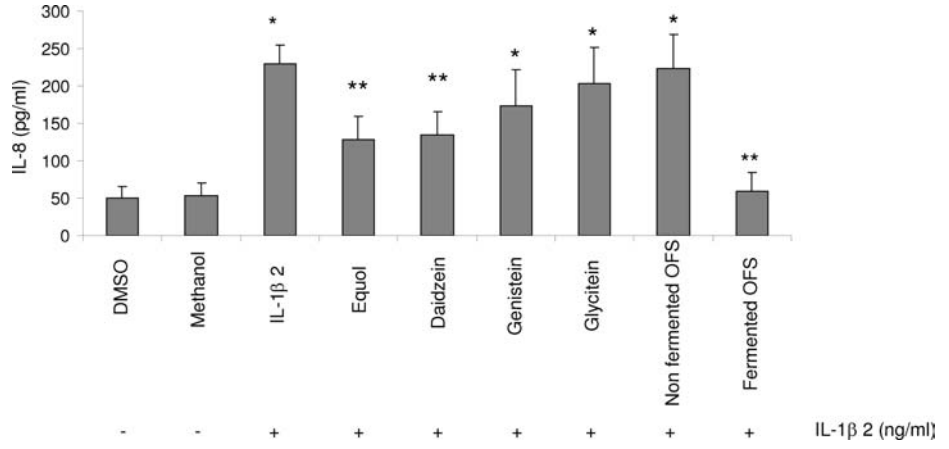

Figure 5. Interleukin-8 (IL-8) release (pg/mL) by Caco-2/TC7 cells stimulated for 24 h with intelukin-1 β (IL-1β) (2 ng/mL) and subsequently treated (24 h) with chemical standards of equol, daizdein, genistein, or glycitein at the concentration of 10 μM or with fermented (diluted to the concentration of 10 μM equol) and not fermented OFS soy milks. DMEM containing DMSO (1%, v/v) or methanol (0.5%, v/v) was the negative control. Data are the mean $\pm$ SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t* test. One asterisk indicates a significant difference ($P < 0.01$) with respect to the negative controls DMEM with added DMSO or methanol. Two asterisks indicate a significant difference ($P < 0.01$) with respect to IL-1β.

intestinal bacteria determined the equol production status of individuals (*2*). A mixed culture, consisting of intestinal strains, was administered to the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The SHIME was fed soy germ powder and inoculated with fecal samples from two non-equol-producing individuals. Administration of the mixed microbial culture seemed to convert the non-equol producer into the equol producer status (*30*). This study showed that selected strains isolated from food ecosystems also had the capacity to synthesize equol at a level comparable to or even higher than isolates from the intestinal microbiota.

Almost all of the studies that aimed at using bacteria for the synthesis of isoflavone aglycones and equol during soy milk preparation did not assay the resulting preparation for functional activities. This study used Caco-2/TC7. Caco-2 cells (human colon carcinoma) are one of the in vitro systems most widely used to mimic the intestinal mucosa. Despite their neoplastic origin, these cells have the capacity to spontaneously differentiate to mature enterocytes and to express brush border enzymes. Fermented OFS soy milk markedly inhibited the inflammatory status of Caco-2/TC7 cells as induced by treatment with IFN-γ (1000 U/mL) and LPS (100 ng/mL), contributed to maintain the integrity of the tight junctions, even if subjected to the negative stimulation of IFN-γ, and markedly inhibited the synthesis of IL-8, after treatment with intelukin-1β (2 ng/mL). As shown by using chemical standards, these effects were due to the concomitant activities of aglycones and, especially, equol, which were contained in the soy milk preparation. In agreement, it was shown that food-derived flavonoids suppressed the induced IL-8 secretion in intestinal epithelial cell lines (*31*, *32*). The immunomodulatory bioactivity of a soy beverage, fermented with mixed cultures of lactic acid bacteria and bifidobacteria, was also shown toward human intestinal epithelial cells (IEC) on the basis of the inhibition of the IL-8 synthesis (*33*). Genistein, at 25, 50, and 100 μM, and daidzein, at 25 and 50 μM, did not induce cell injury to Caco-2 cells and decreased the cell injury and DNA oxidative damage as induced by 5 μM oleic acid hydroperoxides (*34*). Natural polyphenolic extracts modulated the intestinal inflammation using Caco-2 cells, which were stimulated by cytokines and chemokines (IL-1, IL-6, IL-8, TFN-α). The inhibition of those pathways at any point of the cascade repressed the synthesis of these proteins and/or their reaction products (*31*).

Soy beverages have inherent and desirable nutritional properties compared to dairy milk (e.g., low cholesterol and saturated fat levels, absence of lactose). Fermentation of soy flours, also by nonintestinal bacteria, increased the bioavailability of isoflavone algycones and equol, suggesting their use as functional beverages and/or substrates for the synthesis of functional molecules to be used for medical purposes.

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