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Multifunctional Properties of Soy Milk Fermented by *Enterococcus faecium* Strains Isolated from Raw Soy Milk

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ABSTRACT: Lactic acid bacteria (LAB) isolated from soy milk were used to produce a multifunctional fermented food. Seven isolates were screened for their ability to produce peptides and free isoflavones in soy milk. The antihypertensive, antioxidant, and anti-inflammatory properties of the resulting fermented soy milks were evaluated in vitro using biochemical assays. Isolates 1−5 were found to be producers of fermented soy milk with angiotensin I converting enzyme inhibitory activity (ACEI). Isolate 3 was found to be a producer of free isoflavones that increased the antioxidant and anti-inflammatory potential of fermented soy milk. LAB isolates 2−5 were submitted to genetic profiling and a characterization scheme. These isolates were identified as *Enterococcus faecium*, and none of them contained virulence determinants or resistance to antibiotics. In conclusion, this study shows that the application of *E. faecium* isolate 3 for multifunctional food production from soy milk could be a promising strategy in the prevention therapy against cardiovascular disease.

KEYWORDS: *soy milk, Enterococcus faecium, isoflavones, peptides, hypertension, oxidative stress, inflammation*

INTRODUCTION

Cardiovascular diseases (CVD) remain the highest cause of deaths worldwide. According to the World Health Organization,[1] more than 17 million people died from CVD in 2008. Although the current economic burden of CVD is enormous, the cost is projected to increase in the future. Hypertension and atherosclerosis are two closely related pathological conditions, both of which are major risk factors of CVD.[2] Blood pressure is regulated partially by the rennin−angiotensin−aldosterone system. Angiotensin I converting enzyme (ACE) (EC 3.4.14.1) is the main component of this system.[3] ACE modulates arterial blood pressure, converting angiotensin I, an inactive decapeptide, into angiotensin II, an octapeptide with potent vasoconstrictor action.[3] Moreover, ACE degrades bradykinin, which exerts an important vasodilation activity. Inhibition of ACE by natural or synthetic inhibitors has been shown to reduce blood pressure in experimental animals and humans.[4]

Scientific evidence links oxidative stress to the development of hypertension[5] and atherosclerosis.[6] Enhanced production of reactive oxygen species (ROS) may give rise to hypertension by decreasing nitric oxide availability for smooth muscle relaxation. In addition, oxidative stress promotes inflammatory processes that result in the formation of atherosclerotic lesions.[7] Therefore, increasing cellular antioxidant capacity and reducing oxidative stress and associated inflammation can provide a beneficial effect to improve vascular health and prevent and/or inhibit the development of CVD.[8]

Nutritional interventions including either dietary changes (increasing intake of fruits, vegetables, and whole grains, reducing foods containing sugars and high sodium levels, etc.) or consumption of functional foods containing cardioprotective compounds (polyphenols, vitamins, fatty acids, carotenoids, soluble dietary fibers, sterols, organosulfur compounds, monoterpenes, etc.) have been suggested as an approach to fight against CVD.[9] A promising strategy in the prevention of CVD could be the development of multifunctional foods containing antioxidant, anti-inflammatory, and angiotensin-converting enzyme inhibitory (ACEI) compounds to target the multiple pathological conditions of CVD. In particular, soy milk contains bioactive compounds that may have beneficial roles in cardiovascular health promotion. Soy milk protein supplemented to a high-fat diet lowered concentrations of plasma lipids, total cholesterol, triglycerides, and free fatty acids in C57BL/6N mice.[10] Fermented soy milk with lactic acid bacteria (LAB) has been proven as a source of ACEI peptides. ACEI peptides are released from precursor inactive soybean proteins by the action of microbial proteases during the fermentation of soy milk.[11] On the other hand, fermentation with β-glucosidase-producing LAB has shown a great potential for the enrichment of bioactive aglycone isoflavones in soy milk.[12] The most abundant isoflavones in soybean are malonyl-, acetyl-, and β-glucoside conjugates of genistein and daidzein,[13] which are poorly absorbed in the small intestine and less bioactive compared with their aglycone isomers.[14] β-Glucosidases hydrolyze the β-glycosyl bond of the β-glucoside isoflavone to form the aglycone isomer. Aglycone isoflavones have been reported as dietary modulators of cardiovascular function by regulation

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of vascular tone, oxidative stress, and antioxidant gene transcription[9,15] as well as inflammatory processes.[16]

In this study, we explored for the first time the potential application of LAB isolated from soy milk as functional cultures able to form bioactive compounds with ACEI, antioxidant, and anti-inflammatory properties.

■ MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (Barcelona, Spain). MRS agar, M17 broth, and growth media supplements were obtained from Pronadisa (Madrid, Spain). Baird-Parker agar, Columbia agar, and API 50 CHL galleries were purchased from Biomerieux (Marcy L'etoile, France). Kanamycin Aesculin Azide Agar (KAA) was obtained from Oxoid (Basingstoke, UK). Todd−Hewitt broth was provided by Difco (Detroit, MI, USA). Genistein, daidzein, and glycitin standards were purchased from Extrasynthese SAS (Genay, France).

Materials. Soybeans were provided by MangFong S.A. (Madrid, Spain). Reference LAB strains *Lactobacillus delbrueckii* subsp. *lactis* CECT 372, *Streptococcus thermophilus* CECT 986, and *Lactobacillus plantarum* CECT 784 were provided from the Spanish Type Culture collection (Valencia, Spain). The reference strains were subcultured in appropriate media and stored at −80 °C in the presence of glycerol (20%, v/v). Commercial fermented milk containing ACEI peptides was purchased in a local supermarket.

Soy Milk Preparation. Briefly, soybeans were soaked in distilled water at 1:4 ratio (w/v) for 16 h at 20 °C. Subsequently, 190 g of soaked beans in 500 mL of distilled water was ground in a Thermomix blender (Vorwerk, Germany) at 50 °C and maximum speed for 3 min. The slurry was vacuum-filtered on Whatman no. 1 paper and autoclaved at 115 °C for 15 min.

Isolation and Selection of LAB. Raw soy milk supplemented with 5% glucose (w/v) and 3% NaCl (w/v) was incubated at 30 °C for 24, 48, and 72 h. Serial decimal dilutions ($10-10^8$) of fermented soy milk in peptone water at each incubation time were plated in duplicate onto MRS agar, a medium for the isolation of LAB and bifidobacteria, and M17 containing 0.5% (w/v) lactose, a medium for the isolation of lactococci and *S. thermophilus*. MRS and M17 plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at two different temperatures (30 and 37 °C) for 48 h.

Ten isolates from each culture on which growth was observed (~50 isolates per sample) were randomly selected, grown in LAPTg broth (1% meat peptone, 1.5% tryptone, 1% yeast extract, 1% glucose, and 0.1% Tween 80), and stored at −80 °C in the presence of glycerol (20%, v/v). The isolates were observed by optical microscopy to determine the morphology and Gram staining and, additionally, catalase and oxidase tests were performed to select lactic acid bacteria. A total of seven Gram-positive, catalase-negative, and oxidase-negative isolates were randomly selected for further assays as presumptive lactic acid bacteria. After this initial selection, LAB were first characterized for their growth, acidification rate, proteolytic activity, and isoflavone bioconversion in sterile soy milk incubated at 37 °C for 16 h. Fermented soy milk by isolated strains was further screened for ACEI activity, oxygen radical absorbance capacity (ORAC), and nitric oxide inhibitory (NOI) activity.

Culture Preparation and Fermentation of Soy Milk. Stock cultures of reference strains and isolated LAB were thawed, inoculated at 2% (v/v) in their corresponding growth medium (LAPTg for isolated LAB, MRS for *Lactobacillus* CECT strains, and M17 supplemented with 0.5% lactose for *S. thermophilus* CECT 986) and incubated at 37 °C for 20 h. Furthermore, cultures were transferred at 2% (v/v) to sterile soy milk and incubated at 37 °C for 8 h (*S. thermophilus* CETC 986), 10 h (*L. lactis* CETC 372), and 16 h (*L. plantarum* CECT 784 and isolated LAB). Finally, soy milk was inoculated with the bacterial cultures at 1×10^6 CFU/mL to initiate the respective fermentations, which were carried out at 37 °C for 16 h.

Bacterial Growth, Acidification, and Proteolytic Activity. At the end of fermentation, bacterial growth was determined by plating decimal peptone water dilutions (10^6-10^8) of the fermented samples in triplicate onto appropriate medium for each culture. The pH value of the fermented soy milk was measured with a pH-meter (Crison Instruments S.A., Barcelona, Spain).

Proteolytic activity of LAB was assessed by measuring the free amino groups in the whey fraction of fermented soy milk following the method reported by Adler-Nissen.[17] The absorbance of the samples was measured at 340 nm in a microplate reader (Biotek, Winooski, VT, USA). An external calibration curve was prepared with L-leucine from 0.2 to 4 mM. Unfermented soy milk was used as blank and was subtracted from each sample value to calculate the concentration of peptides produced by fermentation, and results were expressed as milligrams of peptides per milliliter of sample.

Isoflavone Extraction and Analysis. Isoflavones from freeze-dried samples were analyzed by RP-HPLC and HPLC-MS according to the method of Dueñas et al.[18] Chromatographic peaks were identified by comparison of retention times and UV and mass spectra with those of standards. Maximum UV wavelength and m/z ratios of molecular and fragment ions were used for identification of each compound as shown in Table 1. Quantification was made using external calibration curves (linearity of calibration was >0.999; standard concentration range was 0−25 μg/mL), with genistein, daidzein, and glycitin standards. The concentrations of malonyl, acetyl, glucoside, and aglycone forms were calculated using the calibration conversion factors shown in Table 2 and reported by Collison.[19]

Table 1. Identification of Major Isoflavones in Non-fermented and Fermented Soy Milk Samples by HPLC-MS

peak	UV wavelength (nm)	$[H]^-$ (m/z)	fragment ions (m/z)	isoflavones
1	249, 313	415	253	daidzein 7-*O*-glucoside (daidzin)
2	256, 320	445	283	glycitein 7-*O*-glucoside (glycitin)
3	260, 327	431	269	genistein 7-*O*-glucoside (genistin)
4	250, 301	501	253, 457	daidzein malonylglucoside
5	252, 301	457	253	daidzein acetylglucoside
6	258, 320	518	269, 473	genistein malonylglucoside
7	250, 298	253		daidzein
8	258, 327	283		glycitein
9	260, 315	473	431, 269	genistein acetylglucoside
10	260	269		genistein

Table 2. Calibration Conversion Factors for Soy Isoflavones

	aglycone	glucoside	acetyl glucoside	malonyl glucoside
daidzein	1.000	0.611	0.555	0.506
glycitin	1.570	1.000	1.094	1.193
genistein	1.000	0.625	0.570	0.521

ACEI Activity. ACEI activity was determined in whey fractions of fermented soy milk. The whey fraction was obtained by sample stirring and centrifugation at 20000*g* at 4 °C for 10 min. ACEI activity was measured following the fluorescence-based protocol of Santandreu and Toldrá.[20] The generated fluorescence was read every minute for 30 min at emission and excitation wavelengths of 355 and 405 nm, respectively, in a microplate fluorometer (Biotek, USA). ACEI activity was expressed as the protein concentration (μg/mL) needed to inhibit 50% of ACE activity (IC_{50}). IC_{50} values were determined by dose−response curves in which the range of concentrations (0−160 μg protein/mL) was distributed in a logarithmic scale and using the nonlinear regression sigmoidal curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA). Protein

concentration was measured by Bior-Rad DC protein assay (Bio-Rad, Spain) using bovine serum albumin as standard.

Oxygen Radical Absorbance Capacity (ORAC). ORAC was measured by fluorescence as described previously.[21] Results were expressed as milligram Trolox equivalents (TE) per gram of dry matter (mg TE/g dm).

Nitric Oxide Inhibitory (NOI) Activity. Murine macrophages RAW 264.7 from American Type Culture Collection (ATCC, Manassas, VA, USA) were used to measure the potential anti-inflammatory activity of fermented soy milk. Macrophages were cultured in DMEM containing 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO_2/95% air as described elsewhere.[21] The cells (5×10^4/well) were treated with 1 μg/mL of lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 and the 10-fold diluted whey fraction of fermented samples (50 μg of protein/mL), for 24 h. Medium was collected after treatment, and nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction. Cell viability was conducted using the CellTiter 96 Aqueous One Solution Proliferation assay kit from Promega (Barcelona, Spain) following the manufacturer's instructions.

Identification of the Bacterial Isolates. LAB isolates 2−5 were tested for coagulase activity and for growth on plates of Baird−Parker, a selective medium for the isolation of staphylococci, and kanamycin aesculin azide agar, a selective medium for the isolation of enterococci. The fermentation of carbohydrates was also assessed using API 50 CHL galleries. Phenotyping of selected strains indicated that they belonged to the genus *Enterococcus*; therefore, identification was performed by PCR species-specific detection of enterococcal *ddl* genes, which encode D-alanine:D-alanine ligases, following the protocol described by Dutka-Malen et al.[22]

Confirmation of enterococci identification was performed by PCR sequencing of a 470 pb fragment of the 16S rRNA gene as described by Kullen et al.[23] The amplicons were purified using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced at the Genomics Unit of the Universidad Complutense de Madrid, Spain. The resulting sequences were used to search sequences deposited in the EMBL database using BLAST algorithm, and the identity of the isolates was determined on the basis of the highest scores (>98%).

Phenotypic Assays of Enterococcal Isolates. The hemolytic activity of the isolates was determined on Columbia agar supplemented with 5% horse blood. After an incubation of 72 h at 37 °C, the plates were analyzed, and the isolates were classified as nonhemolytic (no halo), moderately hemolytic (halo <1.5 mm), or strongly hemolytic (halo >1.5 mm).

Production of gelatinase was determined on Todd−Hewitt agar containing 30 g of gelatin/L. Single colonies were streaked onto plates, grown overnight at 37 °C, and placed at 4 °C for 5 h before examination for zones of turbidity around the colonies, indicating hydrolysis.

Screening for Potential Virulence Determinants, *van* Genes, and Antibiotic Susceptibility among the Enterococcal Isolates. Screening for potential virulence determinants, *van* genes, and antibiotic susceptibility among the enterococcal isolates was performed. A multiplex PCR method was used to detect the presence of virulence determinants encoding sex pheromones (*ccf, cpd, cad, cob*), adhesins ($efaA_{fs}$, $efaA_{fm}$), and products involved in aggregation (agg_2), biosynthesis of an extracellular metalloendopeptidase (*gelE*), biosynthesis of cytolysin (*cylA*), and immune evasion (eps_{fs}).[24] The primer couples and PCR conditions used to detect all of the genes cited above were those proposed by Eaton and Gasson.[25] Control strains used in PCR experiments were *E. faecalis* strains F4 ($efaA_{fs}^+$ $gelE^+$ agg^+ $cylMBA^+$ esp^+ cpd^+ cob^+ ccf^+ cad^+), P36 ($efaA_{fs}^+$ $gelE^+$ agg^+ $cylA^+$ esp^+ cpd^+ cob^+ ccf^+ cad^+), and P4 ($efaA_{fs}^+$ $gelE^+$ agg^+ $cylA^+$ cpd^+ cob^+ ccf^+ cad^+) as well as *E. faecium* P61 ($efaA_{fm}^+$ esp^+).[25]

PCR reactions for *vanA* and *vanB* genes were prepared as described by Dutka-Malen et al.[22] and Ramos-Trujillo et al.,[26] respectively. *E. faecium* BM4147 (resistant to vancomycin, VanA⁺) and *E. faecalis* V583 (resistant to vancomycin, VanB⁺) were used as positive controls. Detection of *vanD*, *vanE*, and *vanG* genes in the *E. faecalis* isolates was performed as previously described.[27−29]

The determination of the minimal inhibitory concentration (MIC) to several antibiotics was evaluated by a microdilution method using the Sensititer plates Staenc1F (Trek Diagnostic Systems, Cleveland, OH, USA) as described by Jiménez et al.[24]

Data Aalysis. Experiments were performed in duplicate; each replicate was analyzed at least in duplicate. Data were expressed as the mean ± standard deviation (SD) of two independent experiments. The statistical method used was one-way analysis of variance (ANOVA), to determine whether there were significant ($P \leq 0.05$) differences between samples using Statgraphics 5.0 (Statistical Graphics Corp., Rockville, MD, USA) software. Pattern recognition methods such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to the data collected from each sample using The Unscrambler X (CAMO, Oslo, Norway) software package. PCA was applied to the data set after standardization (the mean of the values for each variable is subtracted from each variable value, and the result is divided by the standard deviation of the values for each variable). HCA was applied to the standardized data to investigate similarities and sample types.

RESULTS

Growth, Acidification, and Proteolytic Activity of Bacterial Isolates in Soy Milk. Table 3 shows the bacterial growth, pH, and proteolytic activity of LAB isolates and reference strains. Bacterial isolates grew well in soy milk, attaining values of Δlog CFU/mL that ranged from 1.5 to 2.46. The cell density values for all isolates (8.09−8.96 log CFU/mL) were similar or even higher ($P < 0.05$) compared to the reference strains *L. plantarum* CECT 784 (LP), *L. delbrueckii* subsp. *lactis* CECT 372 (LL), and *S. thermophilus* CECT 986 (ST).

Table 3. Cell Growth, Acidification, and Proteolytic Activity (Expressed as the Content of Peptides Released during Soy Milk Fermentation) of LAB Isolates and Reference Strains in Soy Milk Incubated at 37 °C for 16 h[a]

	$\log_{10}$ (CFU/mL)	pH	peptides (mg/mL)
NFS	6.32 ± 0.24a	6.50 ± 0.12g	
isolate 1	8.32 ± 0.1cd	4.82 ± 0.08f	0.71 ± 0.03cd
isolate 2	8.09 ± 0.3c	4.44 ± 0.23bcd	0.87 ± 0.04ef
isolate 3	8.27 ± 0.10cd	4.54 ± 0.10cde	0.61 ± 0.01bc
isolate 4	8.24 ± 0.14cd	4.75 ± 0.01ef	0.72 ± 0.09bc
isolate 5	8.42 ± 0.12d	4.27 ± 0.01b	0.63 ± 0.06bc
isolate 6	8.88 ± 0.03e	4.78 ± 0.09ef	0.63 ± 0.04bc
isolate 7	8.96 ± 0.09e	4.33 ± 0.01bc	0.49 ± 0.06a
ST	7.48 ± 0.03b	4.39 ± 0.22bc	0.67 ± 0.09bcd
LL	8.43 ± 0.09d	3.98 ± 0.01a	0.94 ± 0.08f
LP	8.37 ± 0.15d	3.99 ± 0.01a	0.58 ± 0.05b

[a]Data indicate the mean ± standard deviation of two independent experiments. Means with different letters in the same column are significantly different ($P < 0.05$ in one-way ANOVA). NFS, inoculated nonfermented soy milk; ST, *S. thermophilus* CETC 986; LL, *L. delbrueckii* subsp. *lactis* CETC 372; LP, *L. plantarum* CETC 784.

With regard to acidification activity, all isolates underwent a process of lactic acidification (Table 3). After 16 h of incubation at 37 °C, the ΔpH values ranged from 1.68 to 2.23 units. The pH values of soy milk fermented with the bacterial isolates (pH range from 4.27 to 4.82) were slightly higher ($P < 0.05$) compared to LP and LL. In addition, soy milk fermentation with isolates 2, 3, 5, and 7 showed pH values similar to those observed in fermented soy milk with ST; however, isolates 1, 4, and 6 showed the lowest ($P < 0.05$) acidification activity.

Proteolytic activity was expressed as the peptide content released during fermentation (Table 3). Isolate 7 showed the lowest proteolytic activity ($P < 0.05$). In contrast, isolate 2 exhibited the highest proteolytic activity, which was similar to LL. The other isolates, 1, 3, 4, 5, and 6, exhibited an intermediate proteolytic activity that was similar to those of ST and LP.

Biotransformation of Isoflavones in Fermented Soy Milk with Bacterial Isolates. The concentration of isoflavone glucosides in nonfermented soy milk (NFS) was 85.48 μg/g fresh weight (fw) (Table 4). In addition, daidzin and genistin accounted for 32 and 46% to the total isoflavone glucoside concentration, respectively. Low concentrations of isoflavone aglycones were also observed (18.80 μg/g fw), genistein being the most abundant aglycone found in NFS (11.12 μg/g fw). Biotransformation of isoflavones in soy milk during fermentation was strain specific. Isoflavone glucosides (glycitin, genistin, and daidzin) markedly decreased ($P < 0.05$) in soy milk fermented with isolate 3 and LP and, to a lesser extent, in LL. In general, malonylglucoside content of soy milk was stable after fermentation, with the exception of isolates 3 and 5 and LL. In contrast, lactic acid fermentation decreased ($P < 0.05$) acetylglucoside isoflavone concentration of soy milk. With regard to aglycones, fermentation with isolate 3 and LP increased 3- and 4-fold the aglycone content, respectively. As an example, Figure 1 compares the isoflavones chromatographic profile of NFS and soy milk fermented with isolate 3. In soy milk fermented by isolate 3 and LP, the concentrations of aglycones reached 71 and 82% of the total isoflavone concentration. The highest concentrations of daidzein and genistein were found in soy milk fermented with isolate 3 (34.32 and 28.07 μg/g fw, respectively) and LP (43.06 and 31.99 μg/g fw, respectively). Compared to other aglycones, the concentration of glycitein was much lower in all fermented soy milks. Higher glycitein concentrations were found only in soy milk fermented with LL (2.58 μg/g fw), isolate 3 (2.92 μg/g fw), and LP (5.6 μg/g fw).

ACEI, Antioxidant, and NOI Activities of Fermented Soy Milk with Bacterial Isolates. Table 5 shows the ACEI activity, expressed as IC_{50}, of NFS soybean milk (101.6 μg soluble protein/mL) and fermented soy milks (24.26−79.78 μg soluble protein/mL) compared to NFS. These results indicate that ACEI activity is enhanced ($P < 0.05$) by fermentation with all LAB tested. However, differences among isolates were observed, as shown in the dose−response curves of some LAB isolates illustrated in Figure 2. With the exception of isolates 6 and 7, ACEI activity of fermented soy milk was higher ($P < 0.05$) compared to ST and LP and similar to that of LL. Soy milk fermented with isolate 2 showed the highest ($P < 0.05$) ACEI activity (IC_{50} = 24.26 μg protein/mL) followed by isolates 1 and 3−5 (IC_{50} = 34.26−39.5 μg protein/mL). The ACEI activity of fermented soy milks containing isolates 1−5 was up to 2-fold higher ($P < 0.05$) compared to a commercial fermented bovine milk containing ACE-inhibitory peptides (IC_{50} = 49.62 μg protein/mL) (data not shown).

Soy milk fermentation with LAB isolates 1 and 4−7 showed ORAC values (1.15−1.38 mg Trolox/g fw) similar to that of NFS (1.31 mg Trolox/g fw). In contrast, soy milk fermented with isolates 2 and 3 and LP showed higher ($P < 0.05$) ORAC values (1.52, 1.75, and 2.02 mg Trolox/g fw) compared to NFS.

Soy milk samples did not show significant effect on the macrophage RAW 264.7 cell proliferation (data not shown) at the concentrations tested (10-fold dilutions of the whey fraction corresponding to 40 ± 0.39 μg of soluble protein/mL of

Table 4. Isoflavone Content (Micrograms per Gram Fresh Weight) of Soy Milks Fermented with LAB Isolates and Reference Strains[a]

	NFS	isolate 1	isolate 2	isolate 3	isolate 4	isolate 5	isolate 6	isolate 7	ST	LL	LP
glycitin	6.47 ± 0.46d	6.13 ± 0.44cd	5.74 ± 0.26bcd	4.94 ± 0.02b	6.19 ± 0.14d	6.16 ± 0.02cd	6.24 ± 0.25d	6.31 ± 0.11d	6.58 ± 0.22d	5.25 ± 1.04bc	3.72 ± 0.22a
genistin	39.51 ± 0.30def	41.93 ± 1.19d	40.65 ± 0.09def	10.93 ± 0.09b	40.30 ± 0.67def	41.26 ± 0.89ef	40.12 ± 1.51de	39.87 ± 1.38def	41.71 ± 1.44f	34.95 ± 2.38c	2.14 ± 0.03a
daidzin	26.97 ± 1.65cd	28.42 ± 0.44d	25.81 ± 0.51bcd	2.59 ± 0.16a	25.07 ± 0.71bc	26.64 ± 0.08cd	26.24 ± 1.28cd	26.80 ± 0.55cd	26.71 ± 2.25cd	23.81 ± 1.97b	1.18 ± 0.10a
malonylgenistin	5.14 ± 0.44de	4.67 ± 0.04bcd	4.74 ± 0.12bcd	4.22 ± 0.08ab	5.15 ± 0.12de	4.56 ± 0.17abc	4.58 ± 0.11bcd	5.43 ± 0.34e	5.08 ± 0.22cde	4.03 ± 0.10a	5.36 ± 0.49e
malonyldaidzin	3.53 ± 0.30bc	3.24 ± 0.08bc	3.23 ± 0.16bc	3.18 ± 0.04abc	3.24 ± 0.06bc	3.15 ± 0.18ab	3.39 ± 0.21bc	3.59 ± 0.24c	3.36 ± 0.11bc	2.78 ± 0.21a	3.57 ± 0.35c
acetylgenistin	2.23 ± 0.04d	0.13 ± 0.01a	0.08 ± 0.01a	0.11 ± 0.01a	0.82 ± 0.06b	0.09 ± 0.01a	1.30 ± 0.19cd	1.03 ± 0.06b	0.24 ± 0.02a	1.83 ± 0.00c	1.03 ± 0.03b
acetyldaidzin	1.63 ± 0.10e	0.41 ± 0.00a	0.46 ± 0.02a	0.44 ± 0.02a	0.60 ± 0.03ab	0.42 ± 0.03a	1.24 ± 0.09d	0.78 ± 0.04bc	0.44 ± 0.02a	1.39 ± 0.16d	0.96 ± 0.09d
total glucosides	85.48 ± 1.61d	81.54 ± 0.93d	80.69 ± 0.97d	26.41 ± 0.09b	81.36 ± 1.71d	82.27 ± 0.45d	83.85 ± 3.92d	83.82 ± 2.52d	84.12 ± 0.91d	74.05 ± 5.87c	17.96 ± 1.31a
glycitein	1.86 ± 0.16ab	1.83 ± 0.16ab	2.40 ± 0.13cd	2.92 ± 0.25e	1.51 ± 0.13a	1.68 ± 0.10ab	1.78 ± 0.10ab	1.90 ± 0.16ab	2.03 ± 0.06bc	2.58 ± 0.26de	5.60 ± 0.26f
genistein	11.12 ± 0.18a	10.23 ± 0.50a	10.90 ± 0.58a	28.07 ± 0.26c	11.68 ± 0.27a	10.93 ± 0.97a	10.04 ± 0.54a	10.56 ± 0.85a	13.94 ± 0.28b	13.54 ± 0.28b	31.99 ± 1.84d
daidzein	5.82 ± 0.37a	6.44 ± 0.32ab	6.71 ± 0.30ab	34.32 ± 0.41d	6.51 ± 0.05ab	6.85 ± 0.58ab	6.07 ± 0.21a	7.45 ± 0.13ab	6.87 ± 0.16ab	10.13 ± 0.16c	43.06 ± 2.41e
total aglycones	18.80 ± 0.39a	18.54 ± 0.98a	21.11 ± 0.40ab	65.58 ± 0.10d	19.70 ± 0.44ab	18.76 ± 0.29a	17.89 ± 0.43a	19.91 ± 0.56ab	22.47 ± 1.10b	26.25 ± 0.17c	80.65 ± 4.51e

[a]Data indicate the mean ± standard deviation of two independent experiments. Means with different letters in the same row are significantly different ($P < 0.05$ in one-way ANOVA). NFS, inoculated nonfermented soy milk; ST, *S. thermophilus* CETC 986; LL, *L. delbrueckii* subsp. *lactis* CETC 372; LP, *L. plantarum* CETC 784.

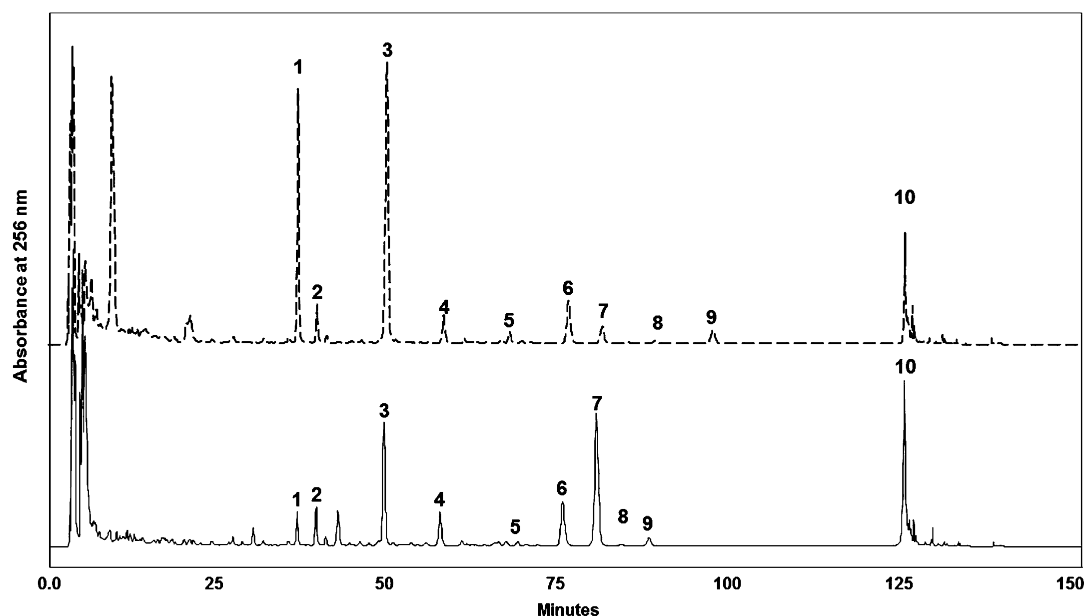

Figure 1. HPLC chromatograms comparing the isoflavone chromatographic profiles of nonfermented soy milk (solid) and fermented soy milk with isolate 3 (dashed). Peaks: 1, daidzin; 2, glycitin; 3, genistin; 4, daidzein malonylglucoside; 5, daidzein acetylglucoside; 6, genistein malonylglucoside; 7, daidzein; 8, glycitein; 9, genistein acetylglucoside; 10, genistein.

Table 5. ACEI Activity, ORAC, and NOI Activities of Soy Milk Fermented with LAB Isolates and Reference Strains[a]

	ACEI (IC_{50} in mg protein/mL)	ORAC (mg Trolox equiv/g fw)	NOI (% inhibition of NO production in macrophages)
NFS	101.6 ± 7.09f	1.31 ± 0.1bc	23.87 ± 0.92a
isolate 1	35.11 ± 1.50b	1.31 ± 0.07bc	24.44 ± 2.93a
isolate 2	24.26 ± 0.30a	1.52 ± 0.07e	25.43 ± 2.78abc
isolate 3	39.5 ± 3.45b	1.75 ± 0.27f	46.64 ± 4.28d
isolate 4	34.26 ± 4.91b	1.15 ± 0.06ab	24.00 ± 0.73a
isolate 5	34.45 ± 2.67b	1.38 ± 0.06cd	30.38 ± 3.40b
isolate 6	79.78 ± 8.87e	1.24 ± 0.02abc	23.58 ± 3.41a
isolate 7	41.98 ± 2.96bc	1.28 ± 0.12bc	30.51 ± 2.18bc
ST	66.73 ± 0.56d	1.46 ± 0.10de	26.76 ± 2.45abc
LL	39.86 ± 3.64b	1.09 ± 0.10a	26.10 ± 1.25abc
LP	51.37 ± 0.95c	2.02 ± 0.10g	48.78 ± 3.83d

[a]Data indicate the mean ± standard deviation of two independent experiments. Means with different letters in the same column are significantly different ($P < 0.05$ in one-way ANOVA). NFS, inoculated nonfermented soy milk; ST, *S. thermophilus* CETC 986; LL, *L. delbrueckii* subsp. *lactis* CETC 372; LP, *L. plantarum* CETC 784.

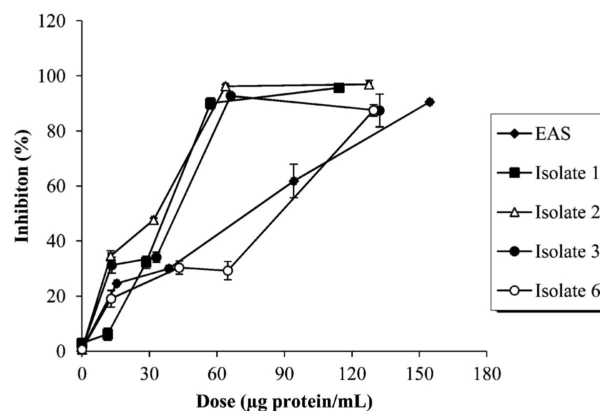

Figure 2. Dose−response curves for nonfermented (NFS) and fermented soy milk samples produced by some LAB isolated from soy milk. Values are the average of two independent experiments. Bars indicate the standard deviation.

medium). Subsequently, macrophages activated with LPS (1 μg/mL) were treated for 24 h with NFS and fermented samples (10-fold dilutions of the whey fraction corresponding to 40 ± 0.39 μg of soluble protein/mL of medium). Cells treated with LPS and sample vehicle (water) showed increased release of NO in the medium (data not shown), which mimics the inflammatory status of macrophages. NFS and soy milk fermented with isolates 1, 2, and 4−7, ST, and LL showed a weak inhibition of the NO production in LPS-activated macrophages ranging from 23 to 30% (Table 4). On the contrary, fermented soy milk with isolate 3 and LP markedly inhibited ($P < 0.05$) the NO production (46.64 and 48.78%, respectively) in LPS-stimulated macrophages.

Principal Component and Hierarchical Cluster Analyses. The score and loading plots for PC1 versus PC2 are superimposed in Figure 3. PC1 explained 68% of the total variance in the data set, whereas PC2 explained 30%. The location of soy milk fermented by isolate 3 and LP may be explained by their higher values in aglycones and ORAC and NOI activities. In contrast, soy milk fermented by isolates 1 and 4−7 and LL showed lower aglycones and ORAC and NOI activities; therefore, they are located diametrically opposite from LP and isolate 3. The location of fermented soy milk with isolate 2 may be explained by its high peptide content and ACEI activity, which is opposite from the location of soy milk fermented with ST characterized by its lower peptide content and ACEI activity. Aglycone content was found to be significantly correlated ($P < 0.05$) with ORAC and NOI activities as evidenced by their Pearson correlation coefficients ($r = 0.898$ and 0.952, respectively). In addition, ORAC and NOI activities were also positively correlated ($r = 0.871$; $P < 0.05$).

The results obtained following HCA are shown as a dendogram in which four well-defined clusters are found (Figure 4). Samples are grouped in clusters based on their relative distance. Group I included soy milk fermented with isolate 2, which

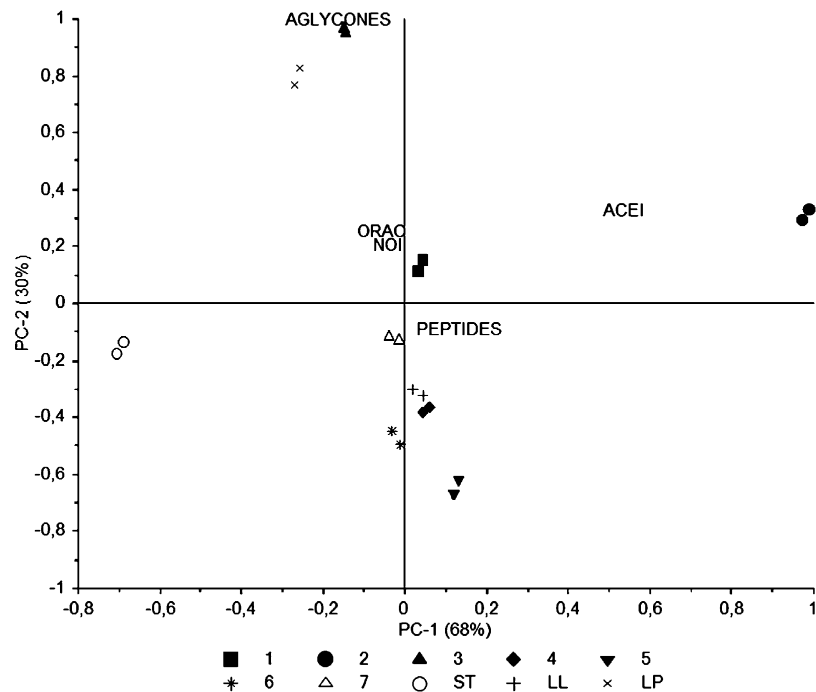

Figure 3. Principal component analysis (PCA) score and loading biplot. Samples (score plot) correspond to the soy milks fermented with different LAB strains. Variables measured (loading plot) correspond to peptides, isoflavone aglycones, oxygen radical absorbance capacity (ORAC), inhibition of angiotensin I converting enzyme (ACEI), and nitric oxide (NOI).

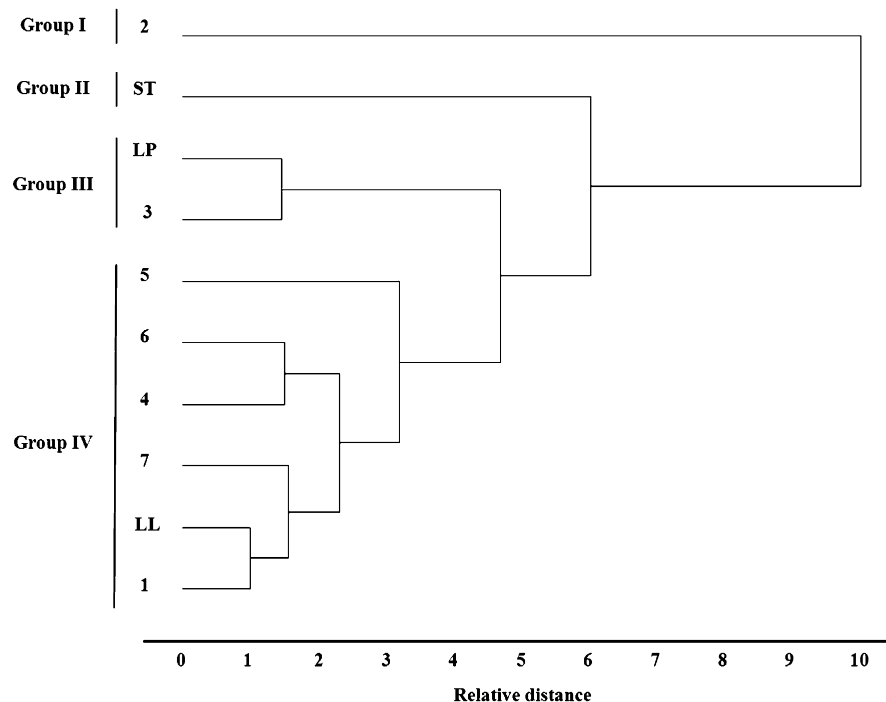

Figure 4. Dendogram of hierarchical cluster analysis of the fermented soy milk with LAB isolates and reference strains.

showed the highest ACEI activity. Group II comprised soy milk fermented with ST with low aglycone content and ACEI, ORAC, and NOI activities. Fermented samples obtained with LP and isolate 3 were clustered in group III because of the highest levels of aglycones and ORAC and NOI activities. Finally, soy milks fermented with isolates 1 and 4−7 and LL were clustered (group IV) on the basis of their moderate ACEI activity, low aglycone content, and low ORAC and NOI activities.

Identification and Characterization of Bacterial Isolates. Isolates 1−3 and 5 were selected for identification on the basis of PCA and HCA. The selected isolates were identified by classical morphological and biochemical tests, species-specific PCR, and/or 16S rDNA sequencing. All of the selected isolates were identified as *E. faecium*. None of the *E. faecium* strains was hemolytic or showed gelatinase activity, and none of them contained any virulence determinant (*ccf, cpd, cad, cob,* $efaA_{fs}$, $efaA_{fm}$, agg_2, *gelE, cylA,* eps_{fs}). All of the *E. faecium* strains were

susceptible to low concentrations (≤4 μg/mL) of penicillin, ampicillin, ciprofloxacin, fosfomycin, nitrofurantoine, tetracycline, erythromycin, vancomycin, teicoplanin, chloramphenicol, and rifampicin.

■ DISCUSSION

The use of enterococci in the food industry is still controversial. Enterococci have been found as opportunistic pathogens that cause nosocomial infections in patients with underlying diseases and in neonates. Factors contributing to pathogenesis are their resistance to a variety of antibiotics and virulence factors such as aggregation susbstance, gelatinase, extracellular superoxide, and extracellular surface protein.[30] Therefore, to select an enterococcal strain as a potential starter candidate, the susceptibility to clinically relevant antibiotics and the presence of virulence determinants were thoroughly investigated in the present study. The *E. faecium* strains selected in this study showed an absence of virulence determinants and/or any other factor of clinical significance, such as the antibiotic resistance pattern or gene transfer potential, which indicates that the isolated *E. faecium* strains is safe.

Results from PCA and HCA showed that soy milks fermented with isolates 2 and 3 were quite different from soy milks fermented with isolates 1 and 4−7, which indicates that different strains of *E. faecium* were isolated from soy milk. *E. faecium* strains have also been isolated from other traditional fermented foods in which *E. faecium* strains play an important role in the organoleptic characteristics of these products.[31,32] Moreover, certain strains of *E. faecium* have contributed to the health benefits of fermented foods. Fermented products containing *E. faecium* CRL 183 as adjunct starter culture were found to be effective in reducing serum total cholesterol and atherosclerotic lesions in animal models.[33,34] *E. faecium* SF68 has been proposed to be clinically effective against antibiotic-associated diarrhea. *E. faecium* also produced heat-stable enterocins capable of inhibiting food-spoiling or pathogenic bacteria such as *Helicobacter pylori*, *Listeria* sp., or *Staphylococcus aureus*.[35]

All studied isolates grew well in soy milk, attaining almost the same cell counts (8.5 log CFU/mL) found for other LAB.[36] From screening, all studied isolates demonstrated proteolytic activity in soy milk at 37 °C, although enterococcus species are not generally considered to be highly proteolytic.[37] Differences of peptides accumulated in soy milk during fermentation among LAB isolates might be strain-specific as it has also been observed by other researchers.[36] Recently, intra- and extracellular proline, arginine, lysine, leucine, valine, and cysteine aminopeptidase activities have been observed in different species of enterococci, which were found to be strain-dependent.[31] These aminopeptidase activities are associated with flavor development in fermented products;[37] however, they could play an important role in the release of bioactive peptides. Our results have shown that soy milk fermented with pure cultures of *E. faecium* exhibit ACEI activity significantly higher that those of other LAB such as *S. thermophilus* and *L. plantarum*. Moreover, IC_{50} values of soy milk fermented by *E. faecium* shown in this study were markedly lower than those reported by other authors in milk fermented with *Lactobacillus helveticus*,[38] which is commercially used for the production of fermented dairy products claiming hypotensive effects. Recently, *E. faecalis* and *E. faecium* have been used as starters for the production of fermented milk and cheese with ACEI activity.[32,38] Our results show for the first time the application of *E. faecium* as starter culture to produce a fermented soy milk with ACEI activity.

Isoflavone contents found in the present work in NFS (heated at 115 °C for 15 min) were different from those found in the literature for soy milk processed by different thermal methods.[39] Soy milk heated at 115 °C for 15 min showed lower total glucosides but higher total aglycones than soy milks processed by direct steam injection (100 °C 20 min) as well as direct and undirect ultrahigh temperatures (143 °C, 60 s). Thermal processing may cause the intertransformation and degradation of isoflavones, which explains different isoflavone contents in soy milks processed by different thermal methods. Besides thermal processing, fermentation with LAB may have a significant impact on the transformation of isoflavones. Among the bacterial isolates tested, only *E. faecium* 3 showed a significant bioconversion of the glucoside isoflavones into their corresponding aglycones after 16 h of soy milk fermentation. Total aglycones reached 71% of the total isoflavone content in soy milk fermented by *E. faecium* isolate 3, which was close to that found in fermented soy milk with *L. plantarum*, considered a high β-glucosidase-producing LAB.[40] β-glucosidase activity has been found to be strain and time dependent.[12] For instance, β-glucosidase activity of *E. faecium* 35 and *L. paraplantarum* KM rapidly increase up to 6 h of fermentation.[41] In contrast, Pyo et al.[42] observed that β-glucosidase activity of *L. plantarum* and *L. delbrueckii* increased up to 24 h of fermentation. A recent approach to enhance isoflavone bioconversion during soy milk fermentation was the ultrasound treatment of probiotic cultures at 100 W for 3 min.[43] The ultrasound treatment facilitates β-glucosidase excretion from the cells and the transfer of substrates (glucosides) and products (aglycones) through cell membranes. It is well established that the synthesis of isoflavone aglycones improves the bioavailability and biological functionality of soy milk via passive diffusion across the intestinal brush border.[44]

Oxidative stress has been implicated as a causal factor in diseases such as hypertension and atherosclerosis. Superoxide radicals react with nitric oxide, forming peroxynitrite that promotes inflammatory responses by activation of the transcriptional factor NF-κB, which results in the formation of atherosclerotic lesions.[7] Therefore, decreasing oxidative stress and inflammation have been suggested as strategies for the prevention and/or amelioration of CVD. Hence, in our study antioxidant and NOI activities were screened to select starter cultures suitable for the production of multifunctional fermented soy milk to target CVD. The current study shows that soy milk fermented by *E. faecium* isolate 3 and *L. plantarum* exhibit a significant oxygen radical absorbance capacity. ORAC was positively correlated with isoflavone concentration in fermented soy milk, which indicates that the scavenging activity of fermented soy milk might be attributed to aglycones. The free radical scavenging activities of the flavonoids is well documented.[45] It has been also described that the degree of hydroxylation is positively correlated with the antioxidant potential,[46] which explains that aglycone isomers of isoflavones exhibit higher radical scavenging activity than glucoside isomers. Therefore, higher ORAC values observed in soy milk fermented with *E. faecium* isolate 3 and *L. plantarum* could be attributed to an effective bioconversion of isoflavones, which agrees with previous studies.[42]

Biomarkers of inflammation have been applied to predict the risk of atherosclerosis. This study has used macrophages RAW 267.4 induced by treatment with LPS (1 μg/mL), and the nitric

oxide concentration released to the medium was measured as a biomarker of inflammation. LPS activates the transcription factor NF-κB, which translocates to the nucleus regulating gene expression involved in the synthesis of pro-inflammatory mediators such as prostaglandins (PG), cytokines, and nitric oxide (NO). Under physiological conditions, NO is synthesized by constitutive nitric oxide synthase (cNOS) at nanomolar concentrations, acting as a cellular messenger and regulating a broad range of biological functions such as smooth muscle relaxation, cardiac and skeletal muscle contractility, platelet adhesion and aggregation, metabolism of lipids, glucose, and amino acids, neuronal activity, and immune response.[47] In the immune system, NO may exert both anti- and pro-inflammatory effects. During inflammation, a greatly increased NO level produced from induced NOS (iNOS) in immune cells leads to the formation of peroxynitrite in high amount, which may further increase inflammatory response. Fermented soy milk with *E. faecium* isolate 3 and *L. plantarum* markedly inhibited nitric oxide production (47 and 49%, respectively) in LPS-activated macrophages. Therefore, soy milk fermented by *E. faecium* isolate 3 may be potentially helpful for the prevention or alleviation of inflammatory processes associated with CVD. This biological effect was positively correlated (r = 0.898) with aglycone concentration, which suggests that aglycones are the bioactive compounds responsible for the NOI activity observed in fermented soy milk. Consistent with our results, previous studies have reported the role of genistein, daidzein, and daidzein metabolites such as equol in mediating inflammation. Kao et al.[48] demonstrated that isoflavone powder produced from soybean cake inhibited LPS-induced inflammation in BALB/c mice by lowering the secretions of interleukin-1β, interleukin-6, NO, and PGE2. Similarly, Dia et al.[49] found that genistein and daidzein inhibited COX-2/PGE2 and iNOS/NO pathways in LPS-stimulated macrophages. More recently, Di Cagno et al.[50] observed that organic fermented soy milk inhibited the inflammatory status of Caco-2 cells, which was explained by the concomitant activities of aglycones and equol contained in the soy milk preparation.

In addition to antioxidant and anti-inflammatory activities of isoflavone aglycones, other beneficial effects have been reported with regard to cardiovascular health. There are studies showing the blood pressure- and lipid-lowering effects of aglycones in animal models.[33,34,51] Cardioprotective effects of isoflavones aglycones have been even observed at nanomolar concentrations (10−300 nM).[15] The isoflavone aglycone concentration in soy milk fermented with isolate 3 was 65.6 μg/g fw. On the basis of this, the total intake of isoflavone aglycones from two portions of 125 g fw of this fermented soy milk per day would provide 16.4 mg/day (7 mg of genistein + 8.6 mg of daidzein). The intake of 50 mg aglycone equivalents reveals a plasma maximum concentration of 2 μM in healthy volunteers.[52] On the basis of these results, it can be assumed that the consumption of 250 g of soy milk fermented by *E. faecium* isolate 3 may provide benefits to human health.

In summary, *E. faecium* isolate 3 is a safe culture that efficiently produces peptides and isoflavone aglycones providing to soy milk a combination of inhibitors of angiotensin I converting enzyme and antioxidant and anti-inflammatory potentials. The application of *E. faecium* isolate 3 for multifunctional food production from soy milk could be a promising strategy in prevention therapy against CVD.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; ACEI, angiotensin I converting enzyme inhibition/inhibitory activity; CVD, cardiovascular disease; DMEM, Dulbecco's modified Eagle medium; HCA, hierarchical cluster analysis; HPLC-PAD, high-performance liquid chromatography with photodiode array detection; LAB, lactic acid bacteria; LL, *Lactobacillus delbrueckii* subsp. *lactis* CECT 372; LP, *Lactobacillus plantarum* CECT 784; LPS, lipopolysaccharide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NO, nitric oxide; NFS, nonfermented soy milk; NOI, nitric oxide inhibitory; ORAC, oxygen radical absorbance capacity; PCA, principal component analysis; PCR, polymerase chain reaction; ROS, reactive oxygen species; ST, *Streptococcus thermophilus* CECT 986; TE, Trolox equivalents.

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