Multifunctional properties of soymilk fermented by *Enterococcus faecium* strains isolated from raw soymilk

C. MARTINEZ-VILLALUENGA¹, M.I. TORINO², V. MARTÍN³, R. ARROYO³, P. GARCIA-MORA¹, I. ESTRELLA¹, C. VIDAL-VALVERDE¹, J.M. RODRIGUEZ³, J. FRIAS^{1*}

¹Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Juan de la Cierva, 3 28006 Madrid, Spain

² CCT CERELA-CONICET, Chacabuco 145, 4000 SM Tucumán, Argentina

³ Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Universidad Complutense de Madrid, Av. Puerta de Hierro, s/n, 28040 Madrid, Spain

*Corresponding author:

Tlf: +34 912587510

Fax: +34 915644853

Email: frias@ictan.csic.es

ABSTRACT

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

Keywords: soymilk, *Enterococcus faecium*, isoflavones, peptides, hypertension, oxidative stress, inflammation.

INTRODUCTION

Cardiovascular diseases (CVD) remain the highest cause of deaths worldwide. According to the World Health Organization¹, more than 17 million people died from CVD in 2008. Although the current economic burden of CVD is enormous, the cost is projected to get higher in the future. Hypertension and atherosclerosis are two closely related pathological conditions, both of which are major risk factors of CVD². Blood pressure is regulated partially by rennin-angiotensin-aldosterone system. Angiotensin I converting enzyme (ACE) (E.C. 3.4.14.1) is the main component of this system³. ACE modulates arterial blood pressure converting angiotensin I, an inactive decapeptide, into angiotensin II, an octapeptide with potent vasoconstrictor action³. 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 humans⁴.

Scientific evidence links oxidative stress to the development of hypertension⁵ and atherosclerosis⁶. 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 lesions⁷. Therefore, increasing cellular antioxidant capacity, reducing oxidative stress and associated inflammation can provide the beneficial effect to improve vascular health and prevent and/or inhibit the development of CVD⁸.

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 CVD⁹. 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, soymilk contains bioactive compounds which may have beneficial roles in cardiovascular health promotion. Soymilk protein supplemented to a high-fat diet lowered concentration of plasma lipids, total cholesterol, triglycerides and free fatty acids in C57BL/6N mice¹⁰. Fermented soymilk 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 fermentation of soymilk¹¹. On the other hand, fermentation with β -glucosidase-producing LAB has shown a great potential for the enrichment of bioactive aglycone isoflavones in soymilk¹². The most abundant isoflavones in soybean are malonyl-, acetyl- and β -glucoside conjugates of genistein and daidzein¹³ which are poorly absorbed in the small intestine and less bioactive as compared with their aglycone isomers¹⁴. 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 of vascular tone, oxidative stress, antioxidant gene transcription^{9,15} as well as modulators of inflammatory processes¹⁶.

In this study, we explored for the first time the potential application of LAB isolated from soymilk 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 Extrashynthese 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, *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.

Soymilk preparation. Soymilk was prepared according to Champagne et al. (2009) with some modifications. 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 were ground in a Thermomix blender (Vorwerk, Germany) at 50 °C and maximum speed for 3 min. The slurry was vacuum-filtered on Whatman n.1 paper and autoclaved at 115 °C for 15 min.

Isolation and selection of LAB. Raw soymilk 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}) of fermented soymilk 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 *Streptotococcus thermophilus*. MRS and M17 plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at two different temperatures (30 °C 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 soymilk incubated at 37 °C for 16 h. Fermented soymilk 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 soymilk. 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. Further, cultures were transferred at 2% (v/v) to sterile soymilk 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, soymilk 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 soymilk was measured with a pHmeter (Crison Instruments S.A., Barcelona, Spain).

Proteolytic activity of LAB was assessed by measuring the free amino groups in the whey fraction of fermented soymilk following the method reported by Adler-Nissen¹⁷. The absorbance of the samples was measured at 340 nm in a microplate reader (Biotek, USA). An external calibration curve was prepared with L-Leucine from 0.2 to 4 mM. Unfermented soymilk 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 mg peptides/mL of sample.

Isoflavone extraction and analysis. Isoflavones from freeze-dried samples were analysed by RP-HPLC and HPLC-MS according to Dueñas et al.¹⁸. Chromatographic peaks were identified by comparison of retention times, 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 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 Collison¹⁹.

ACEI activity. ACEI activity was determined in whey fractions of fermented soymilk. Whey fraction was obtained by sample stirring and centrifugation at $20,000 \times g$ at 4 °C

for 10 min. ACEI activity was measured following the fluorescence-based protocol of Santandreu and Toldrá²⁰. 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 (IC₅₀). IC₅₀ 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 non-linear regression sigmoidal curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA). Protein concentration was measured by Biorad DCTM protein assay (Biorad, Spain) using bovine serum albumin as standard.

Oxygen radical absorbance capacity (ORAC). Oxygen Radical Absorbance Capacity (ORAC) was measured by fluorescence as described previously²¹. Results were expressed as mg Trolox equivalents (TE) per gram of dry matter (mg TE/g d.m.).

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 soymilk. Macrophages were cultured in DMEM containing 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO₂/95% air as described elsewhere²¹. The cells (5 × 10⁴/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 manufacturer's instructions.

Identification of the bacterial isolates. LAB isolates 2, 3, 4 and 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 non-hemolytic (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 per liter. 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 (*agg2*), biosynthesis of an extracellular metalloendopeptidase (*gelE*), biosynthesis of cytolysin (*cylA*) and immune evasion (*eps_{fs}*)²⁴. The primers couples and PCR conditions used to detect all the genes cited above were those proposed by Eaton and Gasson²⁵. 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*⁺), and *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 described²⁷⁻²⁹.

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

Data analysis. Experiments were performed in duplicate; each replicate was analysed at least in duplicate. Data were expressed as means $\pm$ standard deviation of two independent experiments. The statistical methods used were: 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, Norway) software package. PCA was applied to the data set after standardisation (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 standardised data to investigate similarities and sample types.

RESULTS

Growth, acidification and proteolytic activity of bacterial isolates in soymilk. Table 3 shows the bacterial growth, pH, and the proteolytic activity of LAB isolates and reference strains. Bacterial isolates grew well in soymilk, attaining values of $\Delta\text{Log CFU/mL}$ which 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 *Lb. plantarum* CECT 784 (LP), *Lb. delbrueckii* subsp. *lactis* CECT 372 (LL) and *S. thermophilus* CECT 986 (ST).

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 soymilk 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, soymilk fermentation with isolates 2, 3, 5 and 7 showed similar pH values to those observed in fermented soymilk 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 which was similar to ST and LP.

Biotransformation of isoflavones in fermented soymilk with bacterial isolates. The

concentration of isoflavone glucosides in non-fermented soymilk (NFS) was 85.48 µg/g fresh weight (f.w.) (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 f.w.) being genistein the most abundant aglycone found in NFS (11.12 µg/g f.w.). Biotransformation of isoflavones in soymilk during fermentation was strain specific. Isoflavone glucosides (glycitin, genistin and daidzin) markedly decreased ($P<0.05$) in soymilk fermented with isolate 3 and LP and, in a lesser extent in LL. In general, malonylglucoside content of soymilk was stable after fermentation, with the exception of isolate 3, 5 and LL. In contrast, lactic acid fermentation decreased ($P<0.05$) acetylglucoside isoflavone concentration of soymilk. 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 soymilk fermented with isolate 3. In soymilk fermented by isolate 3 and LP, the concentration of aglycones reached 71% and 82% of the total isoflavone concentration. The highest concentration of daidzein and genistein was found in soymilk fermented with isolate 3 (34.32 µg/g f.w. and 28.07 µg/g f.w., respectively) and LP (43.06 µg/g f.w., 31.99 µg/g f.w., respectively). Compared to other aglycones, the concentration of glycitein was much lower in all fermented soymilks. Higher glycitein concentrations were only found in soymilk fermented with LL (2.58 µg/g f.w.), isolate 3 (2.92 µg/g f.w.) and LP (5.6 µg/g f.w.).

ACEI, antioxidant and NOI activities of fermented soymilk with bacterial isolates.

Table 5 shows the ACEI activity, expressed as IC_{50} , of NFS soybean milk (101.6 μ g soluble protein/mL) and fermented soymilks (24.26 to 79.78 μ g 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 it is shown in the dose-response curves of some LAB isolates illustrated in Figure 2. With the exception of isolate 6 and 7, ACEI activity of fermented soymilk was higher ($P < 0.05$) compared to ST and LP, and similar to LL. Soymilk fermented with isolate 2 showed the highest ($P < 0.05$) ACEI activity ($IC_{50} = 24.26$ μ g protein/mL) followed by isolates 1, 3, 4 and 5 ($IC_{50} = 34.26$ -39.5 μ g protein/mL). The ACEI activity of fermented soymilks containing isolates 1-5 was up to 2-fold higher ($P < 0.05$) compared to a commercial fermented bovine milk containing ACE-inhibitory peptides ($IC_{50} = 49.62$ μ g protein/mL) (data not shown).

Soymilk fermentation with LAB isolates 1, 4, 5, 6 and 7 showed similar ORAC values (1.15-1.38 mg Trolox/g f.w.) to NFS (1.31 mg Trolox/g f.w.). In contrast, soymilk fermented with isolate 2, 3 and LP showed higher ($P < 0.05$) ORAC values (1.52, 1.75, 2.02 mg Trolox/g f.w.) compared to NFS.

Soymilk samples did not show significant effect on the macrophage RAW264.7 cell proliferation (data not shown) at the concentrations tested (10-fold dilutions of the whey fraction corresponding to 40 ± 0.39 μ g of soluble protein/mL of medium). Subsequently, macrophages activated with LPS (1 μ g/mL) were treated for 24 h with NFS and fermented samples (10-fold dilutions of the whey fraction corresponding to 40 ± 0.39 μ g 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 fermented soymilk with isolates 1, 2,

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 soymilk 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 analysis. The score and loading plots for PC1 vs. PC2 are superimposed in Fig. 3. PC1 explained 68% of the total variance in the data set while PC2 explained 30%. The location of soymilk fermented by isolate 3 and LP may be explained by their higher values in aglycones, ORAC and NOI activity. In contrast, soymilk fermented by isolates 1, 4, 5, 6, 7 and LL showed lower aglycones, ORAC and NOI activity, therefore, they are located diametrically opposite to LP and isolate 3. The location of fermented soymilk with isolate 2 may be explained by its high peptide content and ACEI activity which is opposite to the location of fermented soymilk 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 activity as evidenced their Pearson correlation coefficients ($r=0.898$ and 0.952 , respectively). In addition, ORAC and NOI activity were also positively correlated ($r=0.871$; $P<0.05$).

The results obtained following HCA are shown as a dendrogram in which four well-defined clusters are found (Figure 4). Samples are grouped in clusters based on their relative distance. Group I included soymilk fermented with isolate 2 which showed the highest ACEI activity. Group II comprised soymilk fermented with ST with low aglycone content, ACEI, ORAC and NOI activity. Fermented samples obtained with LP and isolate 3 were clustered in group III because of the highest levels of aglycones, ORAC and NOI activity. Finally, soymilks fermented with isolates 1, 5, 6, 4, 7 and LL

were clustered (Group IV) based on their moderate ACEI activity and low aglycone content, ORAC and NOI activity.

Identification and characterization of bacterial isolates. Isolates 1, 2, 3 and 5 were selected for identification based on PCA and HCA. The selected isolates were identified by classical morphological and biochemical tests, species-specific PCR and/or 16S rDNA sequencing. All the selected isolates were identified as *Enterococcus faecium*. None of the *E. faecium* strains was haemolytic or showed gelatinase activity and none of them contained any virulence determinant (*ccf*, *cpd*, *cad*, *cob*, *efaA_{fs}*, *efaA_{fm}*, *agg2*, *gelE*, *cylA*, *eps_{fs}*). All the *E. faecium* strains were susceptible to low concentrations (≤ 4 $\mu\text{g/mL}$) of penicillin, ampicillin, ciprofloxacin, fosfomycin, nitrofurantoin, 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 substance, gelatinase, extracellular superoxide, and extracellular surface protein³⁰. Therefore, in order 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 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 fermented soymilks with isolates 2 and 3 were quite different from soymilks fermented with isolates 1 and 4-7 which indicates that different strains of *E. faecium* were isolated from soymilk. *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 was 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 diarrhea³⁵. *E. faecium* also produced heat stable enterocins capable of inhibiting food-spoiling or pathogenic bacteria such as *Helicobacter pylori*, *Listeria* sp., or *Staphylococcus aureus*³⁶.

All studied isolates grew well in soymilk, attaining almost the same cell counts (8.5 log CFU/mL) found for other LAB³⁷. From screening, all studied isolates demonstrated proteolytic activity in soymilk at 37 °C, although enterococcus species are not generally considered highly proteolytic³⁸. Differences of peptides accumulated in soymilk during fermentation among LAB isolates might be strain-specific as it has also been observed by other researchers³⁷. Recently, intra and extracellular proline, arginine, lysine, leucine, valine and cysteine aminopeptidase activities have been observed in different species of enterococci which were found strain-dependent³¹. These aminopeptidase activities are associated with flavor development in fermented products³⁸, however, they could play an important role in the release of bioactive peptides. Our results have shown that soymilk fermented with pure cultures of *E. faecium* exhibit ACEI activity significantly higher to other LAB such as *S. thermophilus* and *L. plantarum*. Moreover, IC₅₀ values of soymilk fermented by *E. faecium* showed in

this study were markedly lower to those reported by other authors in fermented milk with *Lb. helveticus*³⁹ 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^{32,39}. Our results show for first time the application of *E. faecium* as starter culture to produce a fermented soymilk 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 soymilk processed by different thermal methods⁴⁰. Soymilk heated at 115 °C for 15 min showed lower total glucosides but higher total aglycones than soymilks processed by direct steam injection (100 °C 20 min) as well as direct and indirect Ultra-High temperatures (143 °C, 60 s). Thermal processing may cause the intertransformation and degradation of isoflavones which explains different isoflavone contents in soymilks 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 soymilk fermentation. Total aglycones reached 71% of the total isoflavone content in soymilk fermented by *E. faecium* isolate 3 which was close to that found in fermented soymilk with *L. plantarum*, considered a high β -glucosidase-producing LAB⁴¹. β -glucosidase activity has been found to be strain and time dependent¹². For instance, β -glucosidase activity of *E. faecium* 35 and *L. paraplantarum* KM rapidly increase up to 6 h of fermentation⁴². In contrast, Pyo et al.⁴³ 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 soymilk fermentation was the ultrasound treatment of probiotic cultures at 100 W for 3

min⁴⁴. 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 soymilk via passive diffusion across the intestinal brush border⁴⁵.

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- κ B which results in the formation of atherosclerotic lesions⁷. Therefore, decreasing oxidative stress and inflammation have been suggested as strategy for prevention and/or amelioration of CVD. Hence, in our study antioxidant and NOI activities were screened in order to select starter cultures suitable for the production of multifunctional fermented soymilk to target CVD. The current study shows that soymilk 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 soymilk which indicates that scavenging activity of fermented soymilk might be attributed to aglycones. The free radical scavenging activities of the flavonoids is well documented⁴⁶. It has been also described that the degree of hydroxylation is positively correlated with the antioxidant potential⁴⁷ which explains that aglycone isomers of isoflavones exhibit higher radical scavenging activity than glucoside isomers. Therefore, higher ORAC values observed in soymilk fermented with *E. faecium* isolate 3 and *L. plantarum* could be attributed to an effective bioconversion of isoflavones which agrees with previous studies⁴³.

Biomarkers of inflammation have been applied to predict the risk of atherosclerosis. This study have used macrophages RAW267.4 induced by treatment

with LPS (1 µg/mL) and the nitric oxide concentration released to the medium was measured as 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 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 response⁴⁸. 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 lead to formation of peroxynitrite in high amount which may further increase inflammatory response. Fermented soymilk with *E. faecium* isolate 3 and *L. plantarum* markedly inhibited nitric oxide production (47% and 49%, respectively) in LPS-activated macrophages. Therefore, soymilk fermented by *E. faecium* isolate 3 may be potentially helpful for the prevention or alleviation of inflammatory processes associated to 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 soymilk. In consistency with our results, previous studies have reported the role of genistein, daidzein and daidzein metabolites such as equol in mediating inflammation. Kao et al.⁴⁹ 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.⁵⁰ found that genistein and daidzein inhibited COX-2/PGE2 and iNOS/NO pathways in LPS-stimulated macrophages. More recently, Di Cagno et

al.⁵¹ observed that organic fermented soymilk inhibited the inflammatory status of Caco-2 cells which were explained by the concomitant activities of aglycones and equol contained in the soymilk 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 reports showing the blood-pressure and lipid-lowering effects of aglycones in animal models^{33,34,52}. Cardioprotective effects of isoflavones aglycones have been even observed at nanomolar concentrations (10 nM-300 nM)¹⁵. The isoflavones aglycones concentration in soymilk fermented with isolate 3 was 65.6 µg/g f.w. On the basis of this, the total intake of isoflavone aglycones from two portions of 125 g f.w. of this fermented soymilk per day would provide 16.4 mg/day (7 mg genistein + 8.6 mg daidzein). The intake of 50 mg aglycone equivalents reveal a plasma maximum concentration of 2 µM in healthy volunteers⁵³. Based on these results, it can be assumed that consumption of 250 g of fermented soymilk by *E. faecium* isolate 3 may provide benefits to human health.

In summary, *E. faecium* isolate 3 is a safe culture which efficiently produces peptides and isoflavone aglycones providing to soymilk with a combination of inhibitors of angiotensin I-converting enzyme, antioxidant and antiinflammatory potential. The application of *E. faecium* isolate 3 for multifunctional food production from soymilk could be a promising strategy in the prevention therapy against CVD.

Abbreviations list

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)-2H-tetrazolium; NO: nitric oxide; NFS: Non-fermented soymilk; 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

Acknowledgements

This work was supported by the European Comission (Marie Curie action PIOF-GA-2008-219860), FUN-C-FOOD (Consolider-Ingenio 2010) and AGL2010-15420 and AGL2010-16310 projects from the Ministerio de Ciencia e Innovación (Spain). The authors would like to thank CONICET (Argentina) for the Foreign Postdoctoral Fellowship awarded to M.I. Torino under the Scholarship Program for Young Researchers 2010.

References

- (1) World Health Organization. 2008. Causes of death. http://www.who.int/healthinfo/global_burden_disease/cod_2008_sources_methods.pdf. (Access date 12 April 2012).
- (2) Hui, X.; Lam, K.; Vanhoutte, P.M.; Xu, A. Adiponectin and cardiovascular health: an update. *Brit. J. Pharmacol.* **2012**, *165*, 574-590.
- (3) Skeggs, L. T.; Kahn, J. R.; Shumway, N. P. The preparation and function of the hypertension-converting enzyme. *J. Exp. M.* **1956**, *103*, 295-299.

- 487 (4) Hong, F.; Ming, L.; Yi, S.; Zhanxia, L.; Yongquan, W.; Chi, L. The
488 antihypertensive effect of peptides: a novel alternative to drugs? *Peptides* **2008**, *29*,
489 1062-1071.
- 490 (5) Berry, C.; Brosnan, M. J.; Fennell, J.; Hamilton, C. A.; Dominiczak, A. F. Oxidative
491 stress and vascular damage in hypertension. *Curr. Opin. Nephrol. Hypertens.* **2001**, *10*,
492 247-255.
- 493 (6) Kunsch, C.; Medford, R. M. Oxidative stress as a regulator of gene expression in the
494 vasculature. *Circ. Res.* **1999**, *85*, 753-766.
- 495 (7) Lusis, A. J. Atherosclerosis. *Nature*, **2000**, *407*, 233-241.
- 496 (8) Wu, L.; Ashraf, M. H. N.; Facci, M.; Wang, R.; Paterson, P. G.; Ferrie, A.; Juurlink,
497 B. H. J. Dietary approach to attenuate oxidative stress, hypertension, and inflammation
498 in the cardiovascular system. *PNAS*, **2004**, *101*, 7094-7099.
- 499 (9) Cordova, A.C.; Sumpio, B.J.; Sumpio, B.E. Perfecting the plate: adding
500 cardioprotective compounds to the diet. *J. Am. Coll. Surgeons* **2012**, *214*, 97-114.
- 501 (10) Choi, J.; Jeon, J.; Jang, S.; Jeong, Y.; Jeong, S.; Park, H.; Choi, M. Differential
502 effects of powdered whole soy milk its hydrolysate on antiobesity and
503 antihyperlipidemic response to high-fat treatment in C57BL/6N mice. *J. Agric. Food*
504 *Chem.* **2011**, *59*, 2584-2591.
- 505 (11) Yeo, S-K.; Liong M-T. Angiotensin I-converting enzyme inhibitory activity and
506 bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics.
507 *Int. J. Food Sci. Nutr.* **2010**, *61*, 161-181.
- 508 (12) Chien, H-L.; Huang, H-Y.; Chou, C-C. Transformation of isoflavone
509 phytoestrogens during the fermentation of soymilk with lactic acid bacteria and
510 bifidobacteria. *Food Microbiol.* **2006**, *23*, 772-778.

- 511 (13) Wang, H.J.; Murphy, P.A. Isoflavone content in commercial soybean foods. *J.*
512 *Agric. Food Chem.* **1994**, *42*, 1666-1673.
- 513 (14) Setchell, K.D.R. Absorption and metabolism of soy isoflavones-from food to
514 dietary supplements and adults to infants. *J. Nutr.* **2000**, *130*, 654S-655S.
- 515 (15) Siow, R.; Li, F.; Rowlands, D. J.; de Winter, P.; Mann, G. E. Cardiovascular
516 targets for estrogen and phytoestrogens: Transcriptional regulation of nitric oxide
517 synthase and antioxidant defense genes. *Free Radical Bio. Med.* **2007**, *42*, 909-925.
- 518 (16) Pan, M-H.; Lai C-S.; Dushenkov, S.; Ho C-T. Modulation of inflammatory genes
519 by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467-4477.
- 520 (17) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein
521 hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256-
522 1262.
- 523 (18) Dueñas, M.; Hernández, T.; Estrella, I.; Fernández, D. Germination as a process to
524 increase the polyphenol content and antioxidant activity of lupin seeds (*Lupinus*
525 *angustifolius* L.). *Food Chem.* **2009**, *117*, 599-607.
- 526 (19) Collison, M.W. Determination of total soy isoflavones in dietary supplements,
527 supplement ingredients, and soy foods by high-performance liquid chromatography
528 with ultraviolet detection; collaborative study. *J. AOAC Int.* **2008**, *91*, 489-500.
- 529 (20) Santandreu M. A., Toldrá, F. A. fluorescence-based protocol for quantifying
530 angiotensin-converting enzyme activity. *Nat. Protoc.* **2006**, *1*, 2423-2427.
- 531 (21) Martinez-Villaluenga, C.; Peñas, E.; Sidro, B.; Ullate, M.; Frias, J.; Vidal-
532 Valverde, C. White cabbage fermentation improves ascorbigen content, antioxidant and
533 nitric oxide production inhibitory activity in LPS-induced macrophages. *LWT - Food*
534 *Sci. Technol.* **2012**, *46*, 77-83.

- (22) Dutka-Malen, S.; Evers S.; Courvalin P. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* **1995**, 33, 24-27.
- (23) Kullen, M.J.; Sanozky-Dawes, R.B.; Crowell, D.C.; Klaenhammer, T.R. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. *J. Appl. Microbiol.* **2000**, 89, 511-516.
- (24) Jiménez, E.; Delgado, S.; Maldonado, A.; Arroyo, R.; Albújar, M.; García, N.; Jarod M.; Fernández, L.; Gómez, A.; Rodríguez, J.M. *Staphylococcus epidermidis*: a differential trait of the fecal microbiota of breast-fed infant. *BMC Microbiol.* **2008**, 8, 143.
- (25) Eaton, T.; Gasson M.J. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* **2001**, 67, 1628-1635.
- (26) Ramos-Trujillo, E.; Pérez-Roth, E.; Méndez-Alvarez, S.; Claverie-Martín, F. Multiplex PCR or simultaneous detection of enterococcal genes *vanA* and *vanB* and staphylococcal genes *meca*, *ileS-2* and *femB*. *Int. Microbiol.* **2003**, 6, 113-115.
- (27) Perichon, B.; Reynolds, P.; Courvalin P. VanD-type glycopeptide-resistant *Enterococcus faecium* BM 4339. *Antimicrob. Agents Chemother.* **1997**, 41, 2016-2018.
- (28) Fines, M.; Perichon, B.; Reynolds, P.; Sahm, D.F.; Courvalin, P. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. *Antimicrob. Agents Chemother.* **1999**, 43, 2161-2164.
- (29) McKessar, S.J.; Berry, A.M.; Bell, J.M.; Turnidge, J.D.; Paton, J.C. Genetic characterization of *vanG*. A novel vancomycin resistance locus of *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **2000**, 44, 3224-3228.

- (30) Foulquié-Moreno, M.R.; Sarantinopoulos, P.; Tsakalidou, E.; De Vuyst, L. The role and application of enterococci in food and health. *Int. J. Food Microbiol.* **2006**, *106*, 1-24.
- (31) Serio, A.; Chaves-López, C.; Paparella, A.; Suzzi, G. Evaluation of metabolic activities of enterococci isolated from Pecorino Abruzzese cheese. *Int. Dairy J.* **2010**, *20*, 459-464.
- (32) Chaves-López, C.; Serio, A.; Martuscelli, M.; Paparella, A.; Osorio-Cadavid, E.; Suzzi, G. Microbiological characteristics of kumis, a traditional fermented Colombian milk, with particular emphasis on enterococci population. *Food Microbiol.* **2011**, *28*, 1041-1047.
- (33) Cavallini, D.C.; Suzuki, J.Y.; Abdalla, D.S.; Vendramini, R.C.; Pauly-Silveira, N.D.; Roselino, M.N.; Pinto, R.A.; Rossi, E.A. Influence of a probiotic soy product on fecal microbiota and its association with cardiovascular risk factors in an animal model. *Lipids Health Dis.* **2011**, *10*, 126.
- (34) Rossi, E.A.; Cavallini, D.C.U.; Carlos, I.Z.; Vendramini, R.C.; Dâmaso, A.R.; De Valdez, G.F. Intake of isoflavone-supplemented soy yogurt fermented with *Enterococcus faecium* lowers serum total cholesterol and non-HDL cholesterol of hypercholesterolemic rats. *Eur. Food Research and Technol.* **2008**, *228*, 275-282.
- (36) De Vuyst, L.; Foulquié-Moreno, M.R.; Revets, H. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. *Int. J. Food Microbiol.* **2003**, *84*, 299-318.
- (37) Donkor, O.N.; Henriksson, A.; Vasiljevic, T.; Shah, N. P. Probiotic strains as starter cultures to improve angiotensin-converting enzyme inhibitory activity in soy yogurt. *J. Food Sci.* **2005**, *70*, M375-M381.

- (38) Macedo, A.C.; Vieira, M.; Poças, R.; Malcata, F. X. Peptide hydrolase system of lactic acid bacteria isolated from Serra da Estrela cheese. *Int. Dairy J.* **2000**, *10*, 769-774.
- (39) Muguerza, B.; Ramos, M.; Sánchez, E.; Manso, M.A.; Miguel, M.; Aleixandre, A.; Delgado, M.A.; Recio, I. Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk. *Int. Dairy J.* **2006**, *16*, 61-69.
- (40) Xu, B.; Chang, A.; Liu, Z.; Yuan, S.; Zou Y.; Tan, Y. Comparative studies on the chemical and cell-based antioxidant activities and antitumor cell proliferation properties and antitumor cell proliferation properties of soy milk manufactured by conventional and commercial UHT methods. *J. Agric. Food Chem.* **2010**, *58*, 3558-3566.
- (41) Rekha, C.R.; Vijayalakshmi, G. Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *J. Applied Microbiol.* **2010**, *109*, 1198-1208.
- (42) Chun, J.; Jeong, W.J.; Kim, J.-S.; Lim, J.; Park, C.-S.; Kwon, D.Y.; Choi, I.; Kim, J.H. Hydrolysis of isoflavone glucosides in soymilk fermented with single or mixed cultures of *Lactobacillus paraplantarum* KM, *Weissella* sp. 33, and *Enterococcus faecium* 35 isolated from humans. *J. Microbiol. Biotechnol.* **2008**, *18*, 573-578.
- (43) Pyo, Y-H.; Lee, T-C.; Lee, Y-C. Effect of lactic acid fermentation on enrichment of antioxidant properties and bioactive isoflavones in soybean. *J. Food Sci.* **2005**, *70*, S215-S220.
- (44) Yeo, S.; Liong, M. Effect of ultrasound on the growth of probiotics and bioconversion of isoflavones in prebiotic-supplemented soymilk. *J. Agric. Food Chem.* **2011**, *59*, 885-897.

607 (45) Larkin, T.; Price, W.E.; Astheimer, L. The key importance of soy isoflavone
608 bioavailability to understanding health benefits. *Cr. Rev. Food Sci. Nutr.* **2008**, *48*, 538-
609 552.

610 (46) Korkina, L.G.; Afanasev, I.B. Antioxidant and chelating properties of flavonoids.
611 *Adv. Pharmacol.* **1997**, *38*, 151–163.

612 (47) Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygens
613 scavenging and antioxidative effects of flavonoids. *Free Radical Bio. Med.* **1994**, *16*,
614 845–850.

615 (48) Gao, Y. The multiple actions of NO. *Eur. J. Physiol.* **2010**, *459*, 829-839.

616 (49) Kao, T.H.; Wu, W.M.; Hung, C.F.; Wu, W.B.; Chen, B.H. Anti-inflammatory
617 effects of isoflavone powder produced from soybean cake. *J. Agric. Food Chem.* **2007**,
618 *55*, 11068-11079.

619 (50) Dia, V.P.; Berhow, M.A.; De Mejia, E.G. Bowman-birk inhibitor and genistein
620 among soy compounds that synergistically inhibit nitric oxide and prostaglandin E2
621 pathways in lipopolysaccharide-induced macrophages. *J. Agric. Food Chem.* **2008**, *56*,
622 11707-11717.

623 (51) Di Cagno, R.; Mazzacane, F.; Rizzello, C.; Vincentini, O.; Silano, M.; Giuliani, G.;
624 De Angelis, M.; Gobbetti, M. Syntesis of isoflavone aglycones and equol in soy milks
625 fermented by food-related lactic acid bacteria and their effect on human intestinal Caco-
626 2 cells. *J. Agric. Food Chem.* **2010**, *58*, 10338-10346.

627 (52) Song, X.; Pan, W.; Noguchi, T.; Yasui, N.; Negishi, H.; Takebe, M.; Yamori, Y.;
628 Ikeda, K. Isoflavone aglycones promote the mRNA expression of endothelial nitric
629 oxide synthase in aorta and reduced blood pressure in stroke-prone spontaneously
630 hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* **2007**, *34*, S53-S54.

(53) Donovan, J.L.; Manach, C.; Faulks, R. M.; Kroon, P. A. Absorption and Metabolism of Dietary Plant Secondary Metabolites. In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*, Crozier, A., Clifford, M. N., Ashihara, H., Ed.; Blackwell Publishing: Oxford (UK), 2006.

FIGURE CAPTIONS

Figure 1. HPLC chromatograms comparing the isoflavones chromatographic profile of non-fermented soymilk (- - -) and fermented soymilk with isolate 3 (—). Peak 1: Daidzin; Peak 2: Glycitin; Peak 3: Genistin; Peak 4: daidzein malonylglucoside; Peak 5: daidzein acetylglucoside; Peak 6: genistein malonylglucoside; Peak 7: daidzein; Peak 8: glycitein; Peak 9: genistein acetylglucoside; Peak 10: genistein.

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

Figure 3. Principal component analysis (PCA) score and loading biplot. Samples (score plot) correspond to the soymilks 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. Dendrogram of hierarchical cluster analysis of the fermented soymilk with LAB isolates and reference strains.

Table 1.-Identification of major isoflavones in non-fermented and fermented soymilk samples by HPLC-MS.

Peak No.	UV Wavelength (nm)	[H] ⁻ (<i>m/z</i>)	Fragment ions (<i>m/z</i>)	Isoflavones
1	249, 313	415	253	daidzein 7- <i>O</i> -glucoside (daidzin)
2	256, 320	445	283	glycitein 7- <i>O</i> -glucoside (glycitin)
3	260, 327	431	269	genistein 7- <i>O</i> -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

Table 3. Cell growth, acidification and proteolytic activity (expressed as the content of peptides released during soymilk fermentation in mg/mL) of LAB isolates and reference strains in soymilk incubated at 37°C for 16 h.

	Log₁₀ (CFU/mL)	pH	Peptides (mg/mL)
<i>NFS</i>	6.32 ± 0.24 ^a	6.50 ± 0.12 ^g	
<i>Isolate 1</i>	8.32 ± 0.1 ^{cd}	4.82 ± 0.08 ^f	0.71 ± 0.03 ^{cd}
<i>Isolate 2</i>	8.09 ± 0.3 ^c	4.44 ± 0.23 ^{bcd}	0.87 ± 0.04 ^{ef}
<i>Isolate 3</i>	8.27 ± 0.10 ^{cd}	4.54 ± 0.10 ^{cde}	0.61 ± 0.01 ^{bc}
<i>Isolate 4</i>	8.24 ± 0.14 ^{cd}	4.75 ± 0.01 ^{ef}	0.72 ± 0.09 ^{bc}
<i>Isolate 5</i>	8.42 ± 0.12 ^d	4.27 ± 0.01 ^b	0.63 ± 0.06 ^{bc}
<i>Isolate 6</i>	8.88 ± 0.03 ^e	4.78 ± 0.09 ^{ef}	0.63 ± 0.04 ^{bc}
<i>Isolate 7</i>	8.96 ± 0.09 ^e	4.33 ± 0.01 ^{bc}	0.49 ± 0.06 ^a
ST	7.48 ± 0.03 ^b	4.39 ± 0.22 ^{bc}	0.67 ± 0.09 ^{bcd}
LL	8.43 ± 0.09 ^d	3.98 ± 0.01 ^a	0.94 ± 0.08 ^f
LP	8.37 ± 0.15 ^d	3.99 ± 0.01 ^a	0.58 ± 0.05 ^b

Data indicate the mean ± standard deviation of two independent experiments. Means with different superscript letters in the same column are significantly different (P<0.05 in one-way ANOVA)

NFS = Inoculated non-fermented soymilk; ST = *S. thermophilus* CETC 986; LL= *L. delbrueckii* subsp. *lactis* CETC 372; LP = *L. plantarum* CETC 784.

Table 4. Isoflavone content ($\mu\text{g/g}$ f.w.) of fermented soymilks with LAB isolates and reference strains.

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

Data indicate the mean $\pm$ standard deviation of two independent experiments. Means with different superscript letters in the same row are significantly different ($P < 0.05$ in one-way ANOVA)

NFS = Inoculated non-fermented soymilk; ST = *S. thermophilus* CETC 986; LL = *L. delbrueckii* subsp. *lactis* CETC 372; LP = *L. plantarum* CETC 784.

Table 5. ACEI activity (expressed as IC₅₀ in mg protein/mL), ORAC (expressed in mg Trolox equivalents/ g f.w.) and NOI (expressed as % inhibition of NO production in macrophages) activities of fermented soymilk with LAB isolates and reference strains.

	ACEI	ORAC	NOI
NFS	101.6 ± 7.09 ^f	1.31 ± 0.1 ^{bc}	23.87± 0.92 ^a
<i>Isolate 1</i>	35.11 ± 1.50 ^b	1.31 ± 0.07 ^{bc}	24.44± 2.93 ^a
<i>Isolate 2</i>	24.26 ± 0.30 ^a	1.52 ± 0.07 ^e	25.43± 2.78 ^{abc}
<i>Isolate 3</i>	39.5 ± 3.45 ^b	1.75 ± 0.27 ^f	46.64± 4.28 ^d
<i>Isolate 4</i>	34.26 ± 4.91 ^b	1.15 ± 0.06 ^{ab}	24.00± 0.73 ^a
<i>Isolate 5</i>	34.45 ± 2.67 ^b	1.38 ± 0.06 ^{cd}	30.38± 3.40 ^b
<i>Isolate 6</i>	79.78 ± 8.87 ^e	1.24 ± 0.02 ^{abc}	23.58± 3.41 ^a
<i>Isolate 7</i>	41.98 ± 2.96 ^{bc}	1.28 ± 0.12 ^{bc}	30.51± 2.18 ^{bc}
ST	66.73 ± 0.56 ^d	1.46 ± 0.10 ^{de}	26.76± 2.45 ^{abc}
LL	39.86 ± 3.64 ^b	1.09 ± 0.10 ^a	26.10± 1.25 ^{abc}
LP	51.37 ± 0.95 ^c	2.02 ± 0.10 ^g	48.78± 3.83 ^d

Data indicate the mean ± standard deviation of two independent experiments. Means with different superscript letters in the same column are significantly different (P<0.05 in one-way ANOVA)

NFS = Inoculated non-fermented soymilk; ST = *S. thermophilus* CETC 986; LL= *L. delbrueckii* subsp. *lactis* CETC 372; LP = *L. plantarum* CETC 784.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

TOC