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Original Article

Improvement of nutritional components and in vitro antioxidative properties of soy-powder yogurts using *Lactobacillus plantarum*

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ABSTRACT

This research was the first to demonstrate changes in nutritional compositions (isoflavone and CLA) from the 50% methanol extracts of soy-powder milk (SPM) and soy-powder yogurt (SPY) through fermentation using *Lactobacillus plantarum* S48 and P1201 strains. The radical scavenging activities and protective effects against oxidative stress in LLC-PK₁ cells were also investigated. The average physicochemical characteristics including acidity and viable cell number as well as β-glucosidase activity increased with 0.2 → 0.7%, 7.5 → 9.8 log cfu/mL, and 0.03 → 1.75 U/g in SPYs. Total average isoflavones were considerably reduced (3180.3 → 2018.3 μg/g) with the increase of aglycone contents (191.8 → 770.2 μg/g), especially, daidzein exhibited the most remarkable increase rate (98.6 → 460.9 μg/g; > 4.8 times) during fermentation. The CLA and total phenolics also increased with significant differences (ND → 1.6 mg/g; 2.4 → 3.6 mg/GAE/g) between SPM and SPY. Interestingly, the cis-9, trans-11 CLA showed approximately 90% in total content. Moreover, the scavenging capacities against three radicals markedly increased with about 30% in SPYs, as the following order: ABTS > hydroxyl > DPPH. The protective effects on oxidative stress (pyrogallol: O₂, SNP: NO, and SIN-1: ONOO⁻) were also observed high cell viabilities (>10%) under LLC-PK₁ cellular system. Our results suggest that SPY may be utilized as a potent source regarding natural antioxidants and beneficial components for health food and medical uses.

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1. Introduction

For several decades, soybean [*Glycine max* (L.) Merrill, Leguminous family] has been used as commercial crop in the world because of its valuable health benefits such as anticancer, antiatherosclerotic, anti-inflammatory, and antioxidant [1–4]. This species plays an important role in the manufacture of functional foods for human health regarding the nutritional qualities in recent years [5–7]. Many literatures have confirmed that the secondary metabolites (isoflavone, saponin, anthocyanin, phenolics, triterpenoid, pterocarpan, etc.) of soybean are associated with the pharmacological properties [3,5,7–9]. Among various metabolites, isoflavone of main composition is divided into four groups (malonylglucoside, acetylglucoside, glucoside, and aglycone) with three types (daidzein, genistein, and glycitein), exerting health protection effects on chronic diseases [4,6,10,11]. Specifically, isoflavone aglycones are considered as excellent metabolites than glucoside groups due to their bioavailability, absorption, and estrogenic properties against cancer and coronary heart diseases [3,12,13]. Moreover, the antioxidant and anti-allergic activities of daidzein and glycitein and the anticancer abilities of genistein are well known with previously published data [14]. A number of researches have demonstrated that the compositions and contents of soybean isoflavones depend on the processing skills such as enzymatic hydrolysis, cooking, heat treatment, germination, and fermentation [15–17]. In recently, there has been an increasing interest in fermented soybeans (soybean cook: *cheonggukjang*, soybean cake: *meju*, soybean sauce: *kanjang* and soybean paste: *doenjang*) from different processing methods [18,19]. Also, fermented soybeans and their process products have attracted the attention in food manufactures due to the increase of health-promoting metabolites and pharmacological properties [12,18,19]. Interestingly, fermentation is renowned as an effective and inexpensive technique to increase the nutritional qualities of soybeans and soy based foods [16,18]. This technique is increasingly of interest in food industry owing to the beneficial aspects as safety, non-toxicity, and nutraceutical value from various natural sources (fruits, crops, vegetables, and medicine materials) [7,18,19]. Conjugated linoleic acid (CLA) in other metabolites of soybean has been revealed to possess beneficial biological activities (obesity, cancer, atherosclerosis, diabetes, etc.) [20,21] and its content is positively correlated with the bioconversion of linoleic acid and other substances for fermentation [22].

Continuously, several authors have examined the fluctuations of nutritional components and biological effects in fermented soybeans [16,23,24]. Although numerous studies have demonstrated that fermented soybean exerts beneficial health effects, including antimutagenic, antioxidant, anticancer and antidiabetic [19,25,26], there are few reports concerning the protective activities of free radical induced oxidative stress in soybean milk and yogurt through fermentation process. Furthermore, little information available about the variations of soybean isoflavone and CLA contents in various fermented sources. Therefore, our work was designed to evaluate the nutritional compositions as well as antioxidant capacities on radicals and oxidative stress in soybean

materials through fermentation. As far as we know, this is the first study to examine changes in compositional components and antioxidant properties regarding the direct scavenging effects of SPM and SPY using *L. plantarum* strains.

The main purpose of the present research was to document changes in isoflavone and CLA contents as well as direct antioxidant activities against radicals (DPPH, ABTS, and hydroxyl) in the 50% methanol extracts of SPM and SPY from fermentation process of different *L. plantarum* strains. In addition, we investigated fluctuations of the protective effects regarding oxidative stress such as superoxide anion (O_2^-), nitric oxide (NO), and peroxynitrite ($ONOO^-$) under cellular system using LLC-PK₁ cells. We also evaluated the comparison of physicochemical characteristics, including pH, acidity, viable cell number, and β -glucosidase activity.

2. Materials and methods

2.1. Plant material and chemicals

The yellow soybean cultivar (cv. *Daewon*) was collected from the National Institute of Crop Science of the Rural Development Administration (Milyang, Korea) in 2013. Safflower seeds were supplied from the Agricultural Processing Office at Hamyang of Gyeongsangnam province and their oil was extracted with supercritical carbon dioxide (N-TECH, Incheon, Korea) with $25 \pm 5^\circ C$ for 36 ± 12 h at a pressure of 75 kgf/cm^2 . The potential probiotics *L. plantarum* S48 and P1201 strains were previously isolated by the method of Hwang et al. [27]. Mann Rogosa Sharp (MRS) broth/agar (MRSB/MRSA, Difco, Becton Dickinson Co., Sparks, MD, USA) was used as a microbial culture medium. For isoflavone analysis, aglycone and glucoside types were isolated from soybean seeds, as described in our earlier study [10] and malonyl and acetyl isoflavones were obtained from Fujicco Co. (Ltd., Nacalai Tesque Inc., Kobe, Japan). Standard CLA and linoleic acid (LA) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Glacial acetic acid, Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, ferric chloride, sodium acetate, 2,4,6-tripyridyl-s-triazine (TPTZ), *p*-nitrophenyl β -D-glucopyranoside (*p*-NPG), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), pyrogallol, and 3-morpholinosydnonimine (SIN-1) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Sodium nitroprusside (SNP) was used to induce oxidative stress and was purchased from Wako Co. (Tokyo, Japan). The LLC-PK₁ porcine renal epithelial cells were obtained from ATCC Inc. (Solon, OH, USA). The cell culture media, i.e., Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). Analytical-grade H_2O , methanol, and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, USA). Other chemicals and solvents used in the current work were of analytical grade (Sigma–Aldrich).

2.2. Instruments

Antioxidant and β -glucosidase activities as well as total phenolic content were performed by UV–Vis absorption

spectra on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, USA). The quantification of isoflavone was evaluated using an HPLC Agilent 1100 system (Boeblingen, Germany) consisting of an Agilent 1100 diode-array detector, quaternary pump, and autosampler. The fatty acid contents were analyzed by gas chromatography (GC) system (Agilent 7890A, Boeblingen, Germany) with a flame ionisation detector (FID). Cell viabilities were measured using a microplate reader (SPECTRAMax 340PC, Molecular Devices, Sunnyvale, CA, USA).

2.3. Preparation of SPM and SPY

The SPM and SPY sources were prepared by following the modification methods described by Hwang et al. and Hati et al. [27,28]. Soybeans were chosen, washed in water for approximately 12 h, and drained to remove the water. Then, soybean (120 g) was immersed in distilled water and steam-treated for 30 min at 100 °C. The steamed soybeans were placed in a dry oven (HD07026-5003, Hyundai Household Appliances Co., Ltd., Korea) at 55 °C for 2–3 days to evaporate the water and then were pulverized by an ultra-precision grinder to create soy powder. This material (10 g) was transferred separately into a 250 mL flask containing 100 mL of 2% sucrose. The mixture was sterilized in an autoclave at 121 °C for 15 min. After cooling, the enzyme mixture (10 units; cellulose, protease, and esterase; 3:2:1, pH 6.4) was added and hydrolyzed for 24 h at 37 °C to SPM. The oil of safflower seeds was added to the above SPM extract at 1.0% concentration for obtain maximum amount of CLA through fermentation [16,22,28]. The pre-culture *L. plantarum* S48 and P1201 strains were inoculated at 2.0×10^7 CFU/mL into SPM with an inoculation rate of 5%, and then fermentation was carried out to make SPY. In the preliminary experiments, we were to demonstrate optimal conditions of CLA production and isoflavone aglycone in soybean milk and yogurt through the fermentation of *L. plantarum* strains at 35 ± 1 °C for 48 h from various microorganisms and manufacture skills. Therefore, the present study was investigated with the above fermentation conditions using *L. plantarum* strains. The SPM and SPY samples were stored at –70 °C until analysis.

2.4. Measurement of pH, acidity, and viable cell number

The pH, acidity and viable cell number in samples were evaluated according to Cho et al. [18]. The pH was measured with a pH meter (MP 200, UK). The acidity was determined by titrating the sample with a 0.01 N NaOH solution, and its value was expressed as lactic acid (%). To determine the viable cell number, each sample (1 mL) was dissolved in 9 mL of sterilized distilled water at 25 °C, and the diluted suspension was spread on MRS agar plates. The plate was incubated for 48 h at 37 °C, and then colony counting was conducted. Each experiment was characterized by the mean in triplicate.

2.5. Determination of β -glucosidase effect

The β -glucosidase ability was confirmed with previous reported method with some modifications [28]. The pulverized seeds were mixed with 20 mL of 50 mM sodium phosphate buffer

(pH 7.0) in a screw-top flask, stirred at 25 °C for 5 min. The mixture was centrifuged (14,000g) for 10 min at 4 °C and the supernatant was filtered through a 0.45 μ m syringe filter (Whatman Inc., Maidstone, UK) before analysis. The effects of this enzyme were determined by evaluating the rate of hydrolysis of *p*-NPG. The crude extract (250 μ L) was added to 250 μ L substrate (5 mM *p*-NPG) in sodium phosphate buffer (50 mM, pH 7.0), and then incubated at 37 °C for 30 min. The enzymatic reaction was stopped by adding 0.2 M glycine-NaOH (500 μ L, pH 10.5). The blank solution was comprised of 50 mM glycine-NaOH (2.5 mL), substrate solution (2.0 mL), and 50 mM citric buffer (0.5 mL, pH 4.5) containing 0.1 M NaCl. The released *p*-nitrophenol amount in sample was determined by measuring the calibration curve using pure *p*-nitrophenol through various concentrations by spectrophotometer (Thermo Electron Co., CL, USA) at 410 nm. The effect rate (1 unit) is defined as the amount of enzyme that releases 1 μ M of *p*-NPG.

2.6. Calibration curves and HPLC conditions for isoflavone quantification

The isoflavone contents were measured using HPLC equipped with a C_{18} column as previously described by Cho et al. [10] with a slight modification. Each isoflavone standard was accurately weighted (2 mg) and diluted using dimethyl sulfoxide (DMSO) to gain a 1000 μ g/mL. Calibration curves were prepared from plotting chromatographic peak areas through seven concentrations (1, 2, 5, 10, 20, 50, and 100 μ g/mL) by diluting the stock solution using DMSO at 254 nm and their correlation coefficients (r^2) was higher than 0.999. In order to analyze isoflavone, the dried SPM or SPY samples were grounded using a HR 2860 coffee grinder (Philips, Drachten, Netherlands) for 5 min. The powder (1.0 g) was extracted with 50% methanol (20 mL) in a shaking incubator at 25 °C for 6 h, and then the supernatant was centrifuged for 3 min at 3000g. The crude extract was filtered through a 0.45 μ m syringe filter (Whatman Inc., Maidstone, UK) before HPLC analysis. The filtered extract was injected into an analytical Lichrophore 100 RP-18e column (Merck KGaA, 125 mm \times 4 mm I. D., 5 μ m, Darmstadt, Germany). The mobile phase was carried out with 0.1% acetic acid (v/v) in water (eluent A) and acetonitrile (eluent B) as follows: 0–15 min, 15% B; 25 min, 20% B; 40 min, 35% B; 50 min, 10% B and finally reconditioned with 100% B for 5 min. The total running time was 50 min with 1.0 mL/min flow rate at 25 °C and the injection volume was 20 μ L for all samples. The quantification of isoflavones was expressed as micrograms per 1 g of dried seeds.

2.7. Measurement of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu spectrophotometric method as the reported in earlier research [18]. A 500 μ L of the SPM and SPY or gallic acid extracts, which dissolved in 50% methanol, was added to 2 N Folin-Ciocalteu's reagent (250 μ L) and incubated at 25 °C for 1 h. To stop the reaction, 1 mL of 15% Na_2CO_3 solution was added and kept in the dark for 30 min. The absorbance of the solution mixture was measured at 750 nm and gallic acid (0.01–1.0 mg/mL) was used as standard. The results are expressed as gallic acid equivalents (GAE/g).

2.8. Determination of CLA content

The CLA analysis was conducted in accordance with the method of Serafeimidou et al. [29] with some slight modification. Briefly, a 2 mL volume of sample (SPM or SPY) was added to methanolic extract with 3 mL of 0.5 N NaOH and heated to 100 °C for 10 min to hydrolyze of fatty acid. Thereafter, boron trifluoride (2 mL) was added and the mixture heated for 30 min for fatty acid methylation. After cooling at 25 °C, the solution was saturated with NaCl (6 mL). Next, isooctane (2 mL) was added followed by vortexing, and the supernatant was collected and dissolved in anhydrous sodium sulfate. The supernatant was measured by gas chromatography (GC) with an SP-2560 capillary column (100 m × 0.25 mm, 0.20 μm, Sigma–Aldrich Co.). The flow rate of nitrogen carrier gas was 1.0 mL/min with injecting sample (20 μL). The column oven temperature was programmed at 200 °C, and it was held for 30 min; the final temperature was raised to 230 °C. The CLA content was reported as mg/g.

2.9. Determination of antioxidant effects against radicals

Antioxidant properties of the sample (SPM or SPY) were measured by the direct scavenging assays against DPPH, ABTS, and hydroxyl radicals according to previously reported data [6,30,31]. The pulverized SPM and SPY seeds (1 g, 60 mesh) were extracted with 10 mL of 50% methanol for 6 h at 25 °C, in darkness. The extract was filtered through a Whatman No. 42 filter paper and the supernatants were immediately analyzed for scavenging of DPPH, ABTS, and hydroxyl radicals. For DPPH radical scavenging activity, the various concentrations of sample extracts (50% methanol, 0.1 mL) were added to 0.2 mL of methanol and 0.8 mL of a DPPH methanolic solution (1.5×10^{-4} mM). The mixture was vortexed vigorously and was allowed to stand for 30 min at 25 °C in the dark. The absorbance of the mixture was determined using a spectrophotometer at 517 nm and BHT was used as a positive control. This effect was evaluated as a percentage by the following formula (1):

$$\text{DPPH radical scavenging activity (\%)} = [(1 - A_{\text{sample}}/A_{\text{control}}) \times 100] \quad (1)$$

(A_{sample} : absorbance of test sample, A_{control} : absorbance control)

The ABTS^{•+} assay was carried out using the effect of different substances to scavenge ABTS radical cations in comparison with Trolox (positive control). The 7 mM ABTS^{•+} (5 mL) was dissolved in 5 mL of 2.45 mM K₂S₂O₈ and incubated for 10–14 h until the reaction was complete. The ABTS^{•+} stock solution was melted in ethanol to an absorbance of 0.70 at 734 nm. After the addition of 0.9 mL of diluted ABTS^{•+} to 0.1 mL of the sample, the reaction mixture was examined by a spectrophotometer. The scavenging effect was calculated as a percentage using the following formula (2):

$$\text{ABTS radical scavenging activity (\%)} = [(A_c - A_s)/A_c \times 100] \quad (2)$$

(A_{sample} : absorbance of test sample, A_{control} : absorbance control)

The hydroxyl radical was generated by the Fenton reaction in a system of FeSO₄ and H₂O₂. The reaction mixture contained of 0.15 mL FeSO₄-EDTA (10 mM), 0.15 mL H₂O₂ (10 mM), 0.15 mL 2-deoxyribose (10 mM), 0.15 mL H₂O, and 0.05 mL sample solution of various concentrations in an Eppendorf tube. After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% 2-thiobarbituric acid in 50 mL of 50 mM NaOH. The solution was boiled for 10 min, and then cooled in water at 25 °C. The absorbance of the solution was measured at 510 nm. Ascorbic acid was used as a positive control and calculated as a percentage using the following formula (3):

$$\text{Hydroxyl radical activity (\%)} = [1 - (A_{\text{sample}} - A_{\text{nhp}})/A_{\text{control}} \times 100] \quad (3)$$

(A_s : absorbance of test sample, A_c : absorbance control, and A_{nhp} : absorbance without hydrogen peroxide)

2.10. Cell culture and MTT cell viability assay

LLC-PK₁ renal tubular epithelial cells were retained in a culture flask containing 5% FBS-supplemented Delbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) medium (pH 7.2) in a humidified atmosphere of 5% CO₂ at 37 °C [32]. The cells were sub-cultured weekly with 0.05% trypsin–EDTA in phosphate buffered saline (PBS). The cells were seeded into 96-well plates at 10⁴ cells/mL and allowed to adhere for 2 h. Next, the cells were treated with 0.25 mM of pyrogallol, 1.2 mM of SPM, and 1.0 mM of SIN-1 to generate superoxide (O₂^{•-}), nitric oxide (NO), and peroxynitrite (ONOO⁻), respectively. After incubation for 24 h, the 50% methanol extract of SPN or SPY was added in the test wells with various concentrations for 2 h. Cell viability was demonstrated by the MTT colorimetric method, as described previously [33]. A 50 μL of MTT solution (1 mg/mL) was added to each well of a 96-well culture plate. After incubation at 37 °C for 4 h, the medium containing MTT was removed. The resultant crystal was solubilized with 100 μL DMSO and the absorbance of each well was measured using a microplate reader at 540 nm.

2.11. Statistical analysis

All of the measurements were carried out three independent experiments with each triplicate sample ($n = 3$). The results were subject to variance analyses using Sigma Plot 2001 (Systat Software Inc., Chicago, IL, USA). The nutritional component contents (isoflavone, CLA, and total phenolic) and biological properties (β -glucosidase activity, radical scavenging effect, and cell viability) were expressed as the mean \pm SD values.

3. Results and discussion

3.1. Comparisons of physicochemical characteristics such as pH, acidity, cell number, and β -glucosidase activity in SPM and SPY through different *L. plantarum* strains

The physicochemical characteristics including pH, acidity, cell number, and β -glucosidase activity in SPM and SPY through

Table 1 – Comparisons of pH, acidity, viable cell number, and β -glucosidase activity in SPMs and SPYs through fermentation using *L. plantarum* strains.

Strain	Sample	pH	Acidity (%, as lactic acid)	Viable cell number (log cfu/ml)	β -glucosidase activity (U/g)
<i>L. plantarum</i> S48	SPM ^b	6.4 ± 0.2	0.2 ± 0.0	7.4 ± 0.2	0.02 ± 0.0
	SPY ^c	4.3 ± 0.1	0.7 ± 0.0	9.6 ± 0.3	1.70 ± 0.1
<i>L. plantarum</i> P1201	SPM	6.3 ± 0.3	0.2 ± 0.0	7.5 ± 0.2	0.03 ± 0.0
	SPY	4.2 ± 0.2	0.7 ± 0.0	9.9 ± 0.3	1.80 ± 0.1

^a All values of factors are expressed as mean ± SD of triplicate experiments.
^b SPM, soy-powder milk (before fermentation).
^c SPY, soy-powder yogurt (fermentation by *L. plantarum* strain).

two *L. plantarum* strains are shown in Table 1. A decrease in the pH value and increase in acidity and viable cell number were observed after 48 h of fermentation. In more detail, the pH values were found to be decreases in the range of 6.4 → 4.3 and 6.3 → 4.2 after fermentation using S48 and P1201 strains and the acidity data were slightly increased with 0.2 (SPM) → 0.7 (SPY) (Table 1). The viable cell number exhibited considerable differences between SPM and SPY, and their numbers were increased from 7.4 to 9.6 and 7.5 to 9.9 log cfu/mL, respectively (Table 1). Generally, commercial yogurts were reported to have a pH range of 4.2–4.4 [34]. It is also well documented that an appropriate acid property is one of the most factors to ensure a good quality of fermented yogurt [29]. The pH values of SPY samples were approximately 4.3 and 4.2 in this research. Thus, we believe that the SPY samples using *L. plantarum* S48 and P1201 strains may be recommended as excellent natural sources for health and functional foods. Moreover, the β -glucosidase activities showed remarkable differences during fermentation and their effects were markedly increased as 0.02 → 1.70 (S48) and 0.03 → 1.80 (P1201) in comparison with SPM sources (Table 1). It has also been recently established that the degrees of β -glucosidase capacity can be responsible for the conversion of phenolic compounds [35,36]. Based on the above evidences, this effect may be an important factor of nutraceutical source regarding fermented foods.

3.2. Change in isoflavone contents of SPM and SPY samples during fermentation

Soybean has commonly twelve isoflavones of four groups (aglycone, glucoside, acetylglucoside, and malonylglucoside) (Fig. 1A) and their chemicals were documented by the order of individual isoflavone in our published data [10,18]. Moreover, their precise structures were confirmed by comparison of retention times in earlier literatures according to the representative HPLC chromatogram of 12 isoflavone standards [10,18]. As illustrated in Fig. 2A, the each isoflavone' retention time was in the following order: peak 1 (daidzin, t_R = 22.7 min), peak 2 (glycitin, t_R = 23.8 min), peak 3 (genistin, t_R = 26.5 min), peak 4 (malonyldaidzin, t_R = 27.3 min), peak 5 (malonylglycitin, t_R = 27.6 min), peak 6 (acetyldaidzin, t_R = 28.2 min), peak 7 (acetylglycitin, t_R = 30.3 min), peak 8 (malonylgenistin, t_R = 31.4 min), peak 9 (daidzein, t_R = 34.2 min), peak 10 (glycitein, t_R = 35.3 min), peak 11 (acetylgenistin, t_R = 36.4 min), and peak 12 (genistein, t_R = 41.7 min). The current study was to investigate the isoflavone contents in soybean milk and yogurt under fermentation using *L. plantarum* strains.

Isoflavones were evaluated from the peak areas by HPLC analysis and their contents were shown in Table 2. Although the concentration and distribution of isoflavones varies in genetic factors and environmental conditions [10,18,28], soybean commonly present in the order of malonylglucoside (70–80%), glucoside (25%), acetylglucoside (5%), and aglycone (2%) types [37]. However, the glucoside type displayed the highest isoflavone contents with 2507.6 μ g/g in SPM, followed by malonylglucoside (476.8 μ g/g) and aglycone (201.3 μ g/g), whereas acetylglucoside isoflavones exhibited the lowest contents with 19.7 μ g/g (Table 2). In other words, two major and ten minor isoflavone peaks were detected in the SPM chromatogram (Fig. 2A) and the most predominant isoflavone was genistein (3) (1091.8 μ g/g), representing about 34.1% of the total content. In addition, daidzein (1) was the second major isoflavone (899.3 μ g/g) with approximately 28.1%, followed by glycitin (2) (516.5 μ g/g at 16%), malonylgenistin (8) (240.8 μ g/g at 7.5%), and malonyldaidzin (4) (185.6 μ g/g at 6%) (Table 2). The remaining compositions were measured with 271.4 μ g/g (8.4%). As a result, malonylglucoside may be converted to glucoside or aglycone types due to the environmental factors of steamed or heated process [15,19]. Our results were similar to the previous researches that isoflavone glucosides were considerably increased with change in malonylglucoside isoflavones owing to the heat sensitive [28,36]. To measure the isoflavone contents in fermentation, the SPM sample was carried out at 35 ± 1 °C for 48 h, and then were inoculated with *L. plantarum* strains as previously reported data [18,27]. Representative HPLC chromatograms of SPY extracts are shown in Fig. 2B–C. The individual and total isoflavone contents of fermentation using S48 strain exhibited significant differences in comparison with SPM samples. Isoflavones in SPY of S48 strain accumulated in the order of glucoside, aglycone, malonylglucoside, and acetylglucoside groups (Table 2). In more detail, isoflavone glucoside form was the predominant form, representing approximately 49.3% of the total content, and the remaining types showed the following order: aglycone (38%) > malonylglucoside (12%) > acetylglucoside (0.7%). Among the 12 individual isoflavones, daidzein (9) had the highest content (436.1 μ g/g), followed by genistin (3) (422.7 μ g/g), glycitin (2) (420.4 μ g/g), and genistein (12) (258.2 μ g/g), while the lowest content was observed in acetylgenistin (11) (1.3 μ g/g). The total isoflavone content (1923.7 μ g/g) was 1.6 times lower than that of SPM source (3205.2 μ g/g). According to the results shown above, the isoflavone content may be affected with fermentation technique in environment factors. This phenomenon was in agreement

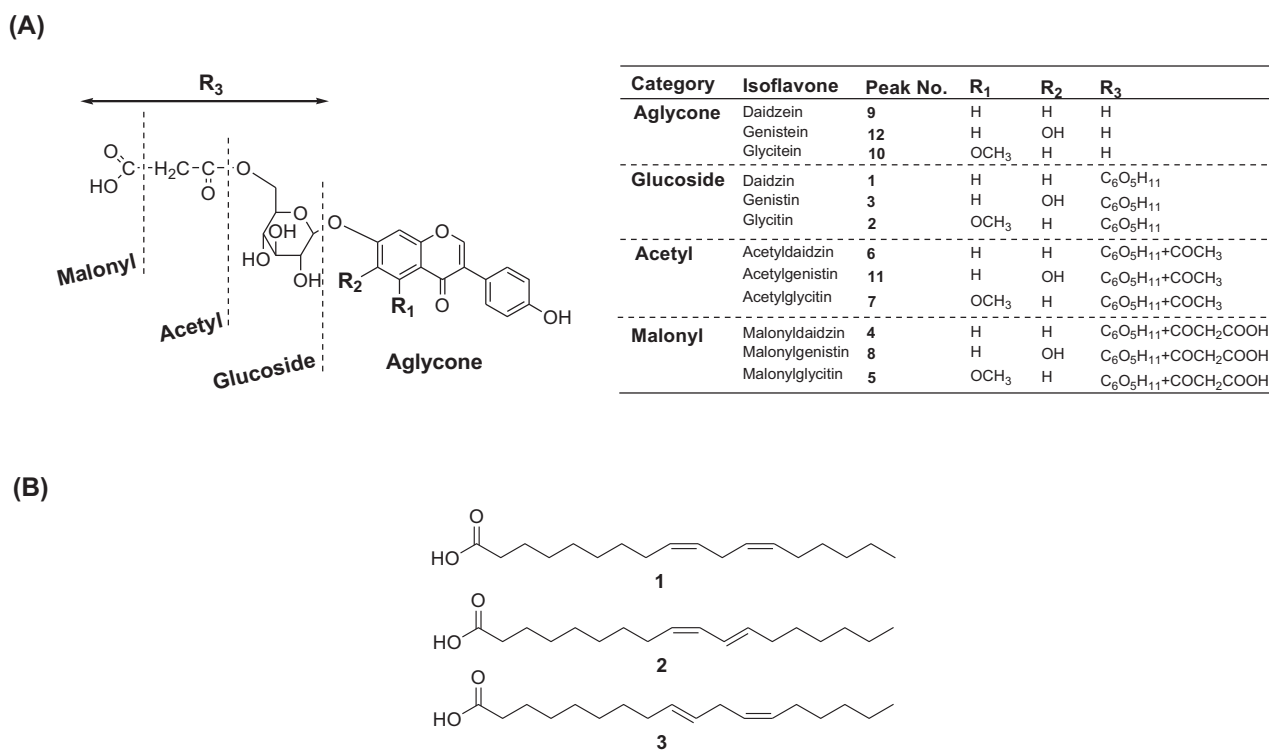


Fig. 1 – Chemical structures of isoflavone and CLA derivatives. (A) 12 isoflavones in SPM and SPY, (B) 2 CLA derivatives in SPY.

with a previous report that isoflavone glucosides (malonylglucoside and glucoside types) were converted into aglycones for fermentation [15,16,18]. To gather more information for fermentation, we evaluated the fluctuations of isoflavone contents in SPY using *L. plantarum* P1201. The average total isoflavone of SPM was measured to be 3155.4 $\mu\text{g/g}$ and was similar to the above results (3205.2 $\mu\text{g/g}$) using S48 strain (Table 2). The four isoflavone types also exhibited similar patterns as the following order: glucoside (2457.3 $\mu\text{g/g}$) > malonylglucoside (497.2 $\mu\text{g/g}$) > aglycone (182.3 $\mu\text{g/g}$) > acetylglucoside (18.7 $\mu\text{g/g}$). The isoflavone content of SPY (2112.8 $\mu\text{g/g}$) showed slightly differences in comparison with that of S48 strain (1923.7 $\mu\text{g/g}$). Furthermore, the distributions of 12 isoflavones in 4 types were observed similar patterns by comparing those of S48 strain (Fig. 2C). The isoflavone types were in the following order: glucoside > aglycone > malonylglucoside > acetylglucoside with 997.2, 811.9, 292.2, and 11.5 $\mu\text{g/g}$, respectively. In the individual composition, daidzein (9) of aglycone type was detected the highest content (485.6 $\mu\text{g/g}$), following by glycitin (2) (450.1 $\mu\text{g/g}$), genistin (3) (433.7 $\mu\text{g/g}$), and genistein (12) (289.3 $\mu\text{g/g}$). The remaining 8 isoflavone accounted for less than 22% of total content and their contents exhibited no remarkable differences by comparing the SPY sample of S48 strain. Especially, when SPM were fermented under P1201 strain, the contents of total isoflavone aglycone increased approximately 100 $\mu\text{g/g}$ than SPY sample using S48 (Table 2). As a result, this technique using *L. plantarum* may be an important factor in demonstrating the qualities of soy products for nutraceutical sources due to the accumulation of

isoflavone aglycones. Our work provides available information that soybean fermentation is considered as an effective food manufacture technology to improve the nutritional qualities. The quantification and comparison of isoflavone aglycones in SPM and SPY were firstly evaluated from fermentation process using *L. plantarum*.

3.3. Changes in CLA and total phenolic contents of SPM and SPY samples during fermentation

It is well-known that soybean fatty acid has been recognized as an important nutritional composition owing to the potential human beneficial properties [38]. Among various components, the CLA isomers (cis-9, trans-11 and trans-10, cis-12) (Fig. 1B) are renowned as excellent health materials on chronic diseases including anticarcinogenic and antiobesity effects [20,21,29]. Although many studies have shown that fermented products contained higher CLA contents than non-fermented food products [22,39], there are only few reports on useful information concern to CLA contents in soy milk and soy yogurt from fermentation using *L. plantarum* strains. The typical GC chromatogram of linoleic acid (peak 1, $t_R = 18.3$ min) in SPM is shown in Fig. 2D and the produced amounts of CLA isomers in SPYs through fermentation with *L. plantarum* S48 and P1201 are represented in Fig. 2E–F.

As can be seen, two CLA isomers have been confirmed by comparing the retention times of standard materials: peak 2 (cis-9, trans-11 CLA, $t_R = 20.4$ min) and peak 3 (trans-10, cis-12 CLA, $t_R = 20.6$ min). There are no remarkable differences in individual and total CLA contents of SPM samples (cis-9,

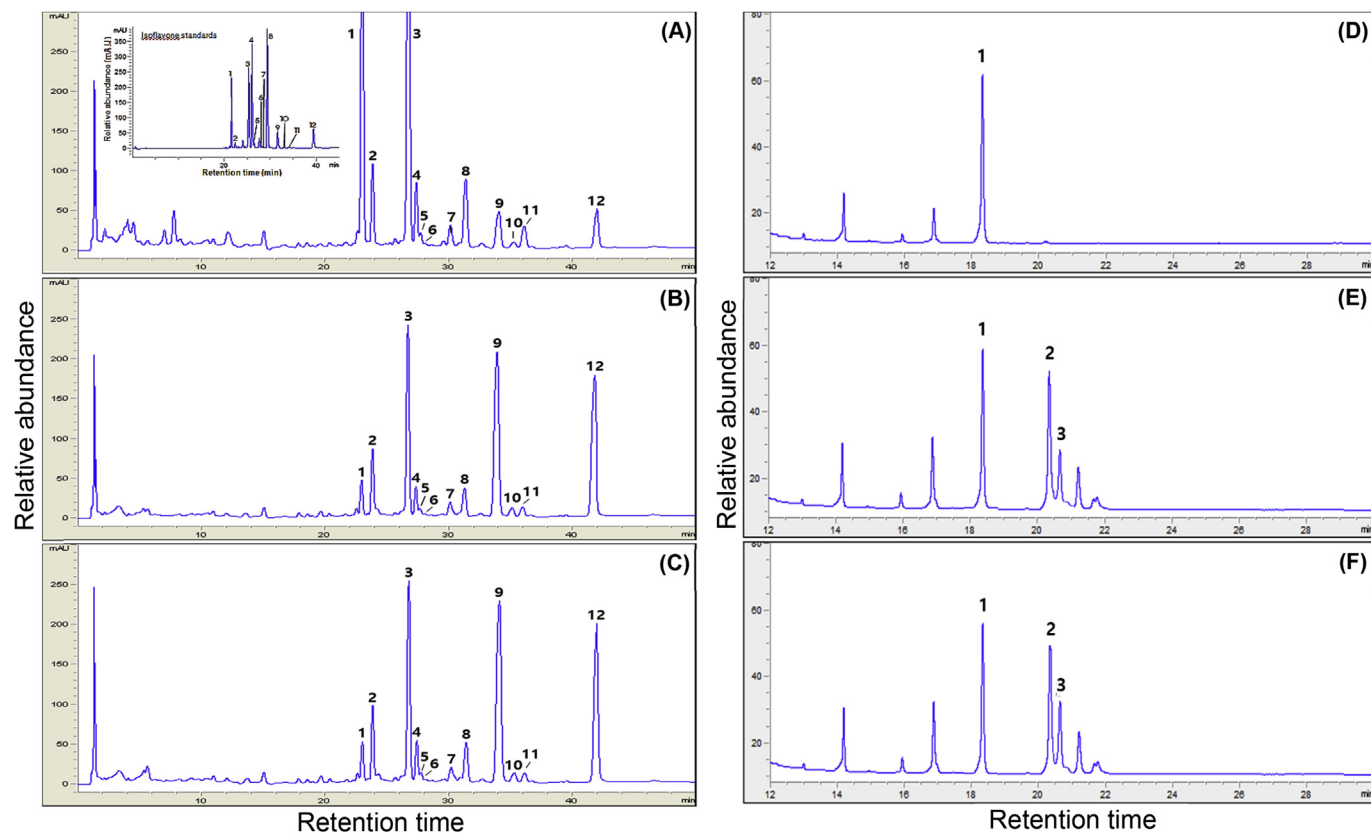


Fig. 2 – Comparisons of HPLC and GC chromatograms of isoflavone and CLA derivatives from the 50% methanol extracts of SPM and SPY samples. HPLC chromatograms; (A) SPM extract, (B) SPY extract using *L. plantarum* S48, (C) SPY extract using *L. plantarum* P1201; 1. daidzin, 2. glycitin, 3. genistin, 4. malonyldaidzin, 5. malonylglycitin, 6. acetyldaidzin, 7. acetylglycitin, 8. malonylgenistin, 9. daidzein, 10. glycitein, 11. acetylgenistin, and 12. genistein, GC chromatograms (D) SPM extract, (E) SPY extract using *L. plantarum* S48, (F) SPY extract using *L. plantarum* P1201. 1. linoleic acid, 2. *cis*-9, *trans*-10 CLA, and 3. *trans*-10, *cis*-12 CLA.

Table 2 – Changes in isoflavone contents from SPMs and SPYs through fermentation using different *L. plantarum* strains.

Isoflavone content ($\mu\text{g/g}$) ^a	Strain			
	<i>L. plantarum</i> S48		<i>L. plantarum</i> P1201	
	SPM ^b	SPY ^c	SPM	SPY
Glucoside				
Daidzin	899.3 \pm 44.9 ^a	106.1 \pm 5.3	878.7 \pm 42.9	113.4 \pm 5.6
Glycitin	516.5 \pm 25.8	420.4 \pm 21.0	521.8 \pm 26.0	450.1 \pm 22.5
Genistin	1091.8 \pm 55.0	422.7 \pm 21.1	1056.8 \pm 121.8	433.7 \pm 21.6
Malonylglucoside				
Daidzin	185.6 \pm 9.2	83.0 \pm 4.1	195.8 \pm 8.2	116.7 \pm 5.8
Glycitin	50.4 \pm 2.5	29.0 \pm 1.4	42.2 \pm 2.1	35.3 \pm 1.7
Gensitin	240.8 \pm 12.0	114.8 \pm 5.7	259.2 \pm 9.4	140.2 \pm 7.0
Acetylglucoside				
Daidzin	13.7 \pm 0.6	5.5 \pm 0.2	13.6 \pm 0.6	6.4 \pm 0.3
Glycitin	4.0 \pm 0.2	12.6 \pm 0.6	3.4 \pm 0.1	3.4 \pm 0.1
Gensitin	2.0 \pm 0.1	1.3 \pm 0.3	1.7 \pm 0.9	1.7 \pm 0.0
Aglycone				
Daidzein	101.4 \pm 5.0	436.1 \pm 21.8	95.7 \pm 5.2	485.6 \pm 24.2
Glycitein	26.7 \pm 1.3	34.1 \pm 1.7	23.3 \pm 1.1	37.0 \pm 1.8
Genistein	73.2 \pm 3.6	258.2 \pm 12.9	63.3 \pm 3.1	289.3 \pm 14.4
Total (mean value)	3205.2	1923.7	3155.4	2112.8

^a All values are presented as the mean \pm SD of triplicate determination.

^b SPM, soy-powder milk (before fermentation).

^c SPY, soy-powder yogurt (fermentation by *L. plantarum* strain).

trans-11 CLA: ND and *trans*-10, *cis*-12 CLA: ND). However, their contents were considerably higher in the SPY extracts (total contents: 1.5 and 1.6 mg/g) than those of SPMs (Table 3). The individual and total CLA contents in two SPYs were not significantly varied in the range of 1.3–1.4 (*cis*-9, *trans*-11 CLA) and 1.5–1.6 mg/g (*trans*-10, *cis*-12 CLA). In particular, the *cis*-9, *trans*-11 CLA was detected with approximately 87% in total average content, whereas the *trans*-10, *cis*-12 isomer showed low content (13%) (Table 3).

The overall results suggested that the increase of CLA content may be related to the degradation of polyunsaturated acids such as linoleic acid during fermentation as the procedure described by Li et al. and Shantha et al. [40,41]. These above results were consistent with previously reported data that the *cis*-9, *trans*-11 CLA isomer was observed with a very high content in comparison with *trans*-10, *cis*-12 CLA in Greek yogurts [29]. Many researchers have also documented that the CLA concentrations are correlated with the bioconversion of linoleic acid and other materials for fermentation [39–42]. We believe that the *L. plantarum* S48 and P1201 are the strong

potential candidate strains for biotransformation of various components in soybeans and their products. Therefore, the basic results of our study indicate that fermentation is a key factor influencing the degradation of linoleic acid in SPM. In this work, we demonstrated for the first time the variations of the CLA contents in SPYs of fermentation using *L. plantarum* strains. Secondly, total phenolic content is positively associated with potential human health properties including antioxidant effects [18,19,30] and is attributable to functional bioactive compositions regarding health-promoting characteristics of foods, vegetables, crops, and other edible plants [16,27,30]. Changes in total phenolic contents of SPM and SPY samples are shown in Table 3 and their results were markedly increased in SPYs compared with those of SPMs. In brief, total phenolic contents exhibited considerable differences as 2.4 \rightarrow 3.6 (S48 strain) and 2.3 \rightarrow 3.6 mg/GAE/g (P1201 strain) after fermentation. Soybean metabolites can be commonly transformed into other compounds due to the high temperature and steam treatment of fermentation process [15–17]. Moreover, their compounds have changed in conjugated types

Table 3 – Changes in CLA and total phenolic contents from SPMs and SPYs through fermentation using different *L. plantarum* strains.

Strain	sample	CLA content (mg/g) ^a			Total phenolic content (mg/GAE/g) ^a
		<i>cis</i> -9, <i>trans</i> -11	<i>trans</i> -10, <i>cis</i> -12	Total	
<i>L. plantarum</i> S48	SPM ^b	nd ^d	nd	nd	2.4 \pm 0.1
	SPY ^c	1.3 \pm 0.0	0.2 \pm 0.0	1.5 \pm 0.0	3.6 \pm 0.1
<i>L. plantarum</i> P1201	SPM	nd	nd	nd	2.3 \pm 0.1
	SPY	1.4 \pm 0.0	0.2 \pm 0.0	1.6 \pm 0.0	3.5 \pm 0.1

^a All values are presented as the mean \pm SD of triplicate determination.

^b SPM, soy-powder milk (before fermentation).

^c SPY, soy-powder yogurt (fermentation by *L. plantarum* strain).

^d nd: not detected.

Table 4 – Comparisons of radical scavenging activities and protective effects on viability of LLC-PK₁ cells treated with pyrogallol, SNP, and SIN-1 in SPMs and SPYs through fermentation using different *L. plantarum* strains.

Strain	Sample	Radical scavenging effect (%) ^a			Cell viability (%) ^a		
		DPPH	ABTS	Hydroxyl	Pyrogallol	SNP	SIN-1
Control	Normal	–	–	–	100.0	100.0	100.0
	Treated	–	–	–	63.8 ± 3.6	70.3 ± 4.0	62.7 ± 3.6
<i>L. plantarum</i> S48	SPM ^b	39.3 ± 2.3 ^d	59.9 ± 3.5	69.4 ± 4.1	58.7 ± 2.9	60.3 ± 3.0	67.2 ± 3.3
	SPY ^c	65.4 ± 3.9	92.1 ± 5.5	89.4 ± 5.3	75.8 ± 3.7	85.6 ± 4.2	71.3 ± 3.5
<i>L. plantarum</i> P1201	SPM	34.7 ± 2.0	57.9 ± 3.4	68.6 ± 4.7	58.3 ± 2.5	60.2 ± 3.0	63.1 ± 3.6
	SPY	68.3 ± 4.1	95.6 ± 5.7	89.9 ± 5.7	76.9 ± 3.8	84.1 ± 4.1	74.4 ± 4.2
–	Positive control	82.1 ± 5.3 (BHT)	89.7 ± 5.7 (Trolox)	56.3 ± 2.9 (Ascorbic acid)	–	–	–

^a All values of samples positive controls are presented as the mean ± SD of triplicate determination.

^b SPM, soy-powder milk (before fermentation).

^c SPY, soy-powder yogurt (fermentation by *L. plantarum* strain).

^d Radical scavenging activities of the 50% methanol extracts of samples and positive controls as well as protective effects in the 50% methanol extracts of samples treated with pyrogallol, SNP, and SIN-1 in LLC-PK1 were carried out at 200 µg/mL.

from hydroxyl groups of glucosides [18,18,23]. The contents of phenolic compounds in various soybeans and their products also showed considerable differences according to the environmental conditions [7,11,37] and their contents in fermentation using *L. plantarum* strains displayed similar patterns as the earlier reported data [16,18,28]. We confirmed that the *L. plantarum* can be important candidate strain for the biotransformation of soybean metabolites on the human health benefits.

3.4. Comparisons of antioxidant properties against three radicals in SPM and SPY samples during fermentation

It is well-known that total phenolic contents and antioxidant activities in soybeans and their products can be considerably affected by fermentation [19,36,43]. Numerous researches have also reported that the fermentation can be used to promote the nutritional values owing to the increase of isoflavone aglycone and other phenolic contents [18,19,28,35]. In several biological properties, the direct scavenging effects on radicals are used to evaluate the antioxidant status of crops, vegetables, and fruits [6,8,11]. The earlier works have focused on DPPH, ABTS, and hydroxyl radicals due to their simple quality control and reproducibility [10,30,31]. For these reasons, we investigated antioxidant activities against radicals in SPMs and SPYs through fermentation using *L. plantarum* strains. Their capacities were evaluated by comparing the inhibition rate (percentage) on the formation of three radicals from the 50% methanol extracts of samples and positive controls (BHT, Trolox, and ascorbic acid). Many researchers have documented that antioxidant properties against ABTS radical in foods exhibited the predominant scavenging activities, followed by DPPH and hydroxyl radicals [44,45]. We revealed that the ABTS radical scavenging abilities of SPM and positive control increased with increasing concentrations (50, 100, 200, 300, 400, and 500 µg/mL) in the preliminary experiments. The SPM effects at different concentrations were as follows: 100% at 500 µg/mL, 100% at 400 µg/mL, 58.7% at 200 µg/mL, 46.3% at 100 µg/mL, and 35.4% at 50 µg/mL. In these above regards, the antioxidant capacities on the radical scavenger were evaluated at 200 µg/mL regarding the dose-dependent variations.

The antioxidant activities of SPMs and SPYs were observed significant differences in radical sources (Table 4). First, the SPY samples by two strains exhibited higher DPPH radical scavenging activities than SPMs. The inhibition percentage of potent DPPH radical in SPYs exhibited higher effects with 65.4 (S48 strain) and 68.3% (P1201 strain) than those of SPMs (39.3 and 34.7%) at a concentration of 200 µg/mL. These results suggest that the isoflavone aglycone and other phenolic contents in the 50% extracts of SPY sources may be affected to the major portion of antioxidant properties against DPPH radical, as described in previous studies [18,41,43]. Although the positive control (BHT: 82.1%) showed higher activities by comparing the SPY samples, their extracts can be considered as excellent natural sources for healthy foods and nutraceuticals. In the ABTS radical assay, the 50% methanol extracts of SPYs were also detected higher effects than SPMs, and their capacities were observed with 92.1 (S48 strain) and 95.6% (P1201 strain). The SPY extracts showed significant degrees in the ABTS radical scavenging effects by comparison with those of SPMs (S48: 59.9 → 92.1% and P1201: 57.9 → 95.6%). Two SPYs had higher activities than Trolox (89.7%) of positive control and their effects increased with increasing concentrations (Table 4). Specifically, these sources had higher radical scavenging capacities with the ABTS when compared to the DPPH radical. This result indicates that ABTS radical may be attributed to chain breaking and hydrogen donating antioxidants of many phytochemicals in SPY by comparing the hydrogen donating capacities through the DPPH radical [46]. Our effects were similar to the results obtained by previous researches [8,10]. To gather information on antioxidant properties, we examined the hydroxyl radical scavenging effects. This radical react with 2-deoxyribose and the degradation products are measured from absorption values. The SPY and SPM samples were examined using the deoxyribose skill as completion between deoxyribose and extracts for radical generated from Fenton reaction method [31]. The scavenging activities on this radical were also similar to the results obtained in the DPPH and ABTS radicals. As seen in Table 4, the average hydroxyl radical scavenging ability of SPMs was observed higher value (69.0%) than those of the DPPH (37.0%) and ABTS (58.9%) radicals. The SPY extracts had

considerably higher scavenging effects toward hydroxyl radical with significant differences (S48: 69.4 → 89.4%; P1201: 68.6 → 89.9%) than SPMs. These differences suggest that antioxidant capacities against the hydroxyl radical may be importantly correlated to the molecular figurations on aromatic hydroxylation of phenolics in SPM during fermentation [31,44]. The isoflavone aglycone contents may be also a key factor in these radical scavenging effects because daidzein and genistein are considered as potent hydroxyl radical scavenger [43,47]. In the present research, although the SPY sample using P1201 strain displayed higher total isoflavone and aglycone contents than soybean product of S48, their antioxidant capacities exhibited similar patterns in each radical. According to the our results, the SPY extracts may be recommended as an excellent source of human health foods and nutraceuticals due to the higher activities by comparing the ascorbic acid (positive control, 56.3% at 200 µg/mL). Consequently, the antioxidant properties against radicals in the 50% methanol extracts of SPYs occurred in the following order: ABTS > hydroxyl > DPPH at a concentration of 200 µg/mL. We assumed that isoflavone aglycones and other phenolics in SPY are responsible for the antioxidant abilities of scavenging activities against DPPH, ABTS, and hydroxyl radicals. The variations of the antioxidant effects in SPM and SPY through fermentation using *L. plantarum* were demonstrated for the first time.

3.5. Comparisons of protective properties against oxidative stress of LLC-PK₁ cells in SPM and SPY samples during fermentation

Several researchers documented that the cellular oxidative stress in LLC-PK₁ cells was induced by free radical generators, such as pyrogallol, SNP, and SIN-1 [32,48]. However, little information has been examined on the protective abilities concerning oxidative stress in fermented soybean products by *L. plantarum* strains. Thus, we conducted the protective effects of the SPM and SPY extracts against oxidative stress in LLC-PK₁ cells. As illustrated in Table 4, the treatments of pyrogallol, SNP, and SIN-1 led to the significant decreases with 100 → 63.8, 100 → 70.3, and 100 → 62.7% in cell viabilities, indicating oxidative stress by superoxide anion (O₂⁻), nitric oxide (NO), and peroxy nitrite (ONOO⁻) generators. The cell viability considerably decreased, falling to 70.3% owing to the generation of NO by SNP in comparison of normal state (not treated with SNP). The cell viabilities of two SPM extracts (200 µg/mL) decreased with 60.3 and 60.2% after NO generation, when cells were treated. However, when the cells were treated with the SPY extracts by fermentation at the same concentration, their viabilities markedly increased approximately 25% with 85.6 (S48 strain) and 84.1% (P1201 strain), respectively (Table 4). Therefore, the SPY source may be considered as potential agent for the prevention and treatment of oxidative stress-related diseases. In our continuing survey of the effects against oxidative damage in SPYs, we measured the protective properties on SIN-1 under the cellular system to generate ONOO⁻. While the treatment of SIN-1 (ONOO⁻ generator), the cell viability significantly reduced 37.3% (100 → 62.7%) as compared with cells not treated with SIN-1 (Table 4). The addition of SPM extracts was

slightly higher than SIN-1 treated control with 62.7 → 67.2 and 63.1% at a concentration of 200 µg/mL. The treatment of SPY extracts also exhibited the increase with cell viabilities of 71.3 and 74.4%. As a result, the protective activities of the SPY extracts were observed higher cell viabilities than SPMs in LLC-PK₁ cells treated with SIN-1. These above results suggest that the protective effects on viability of LLC-PK₁ cells may be responsible for the phenolic and isoflavone aglycone contents as well as viable cell numbers in SPY when compared to the SPM extract, as reported previously [48,49]. To obtain more information of the protective activities, we examined cell viabilities regarding pyrogallol under the cellular system to generate O₂⁻ (Table 4). According to the treatment (200 µg/mL) of SPM samples, the cell viabilities were decreased with 58.7 and 58.3% by comparing the pyrogallol-induced oxidative stress (63.8%). However, when the cells were treated with SPY extracts, the cell viabilities remarkably increased with 75.8 (S48 strain) and 76.9% (P1201 strain). This phenomenon indicate that many phenolic compounds except isoflavone glucosides in the 50% methanol extracts may be mainly responsible for the major portion of protective effects in free radical-induced oxidative stress [48,49]. It is commonly revealed that soybean extracts significantly reduced the oxidative stress created by H₂O₂ on PC12 neuron cells [50]. Also, the methanol extract of *chungkukjang* remarkably inhibited the oxidative stress on LLC-PK₁ cells due to the increase of anthocyanin and phenolic compound contents during fermentation [51]. Thus, our results are similar to those of previous researches [50,51]. Consequently, the protective effects of SPY extracts under cellular system were as follows, in decreasing order: SNP > pyrogallol > SIN-1. The present work was the first to demonstrate that the SPY extract have potent protective effects from oxidative stress induced by free radicals (pyrogallol, SNP, and SIN-1) under cellular oxidative damage.

4. Conclusion

The current study documented for the first time that changes occur in nutritional compositions such as isoflavone and CLA in SPM and SPY extracts of fermentation using *L. plantarum*. The isoflavone, CLA, and total phenolic contents displayed significant differences between SPM and SPY. Total isoflavones were remarkably reduced with 3205.2 → 1923.7 (S48 strain) and 3155.4 → 2112.8 (P1201 strain) µg/g after fermentation, isoflavone aglycones (daidzein > genistein > glycitein) considerably increased with large variations of 201.3 → 728.4 (S48 strain) and 182.3 → 811.9 (P1201 strain) µg/g. The CLA contents also markedly increased (ND → 1.6 mg/g) with the predominant *cis*-9, *trans*-11 isomer (approximately 90%) in SPYs. Furthermore, the antioxidant properties against DPPH, ABTS, and hydroxyl radicals were remarkably increased in SPYs, especially, ABTS radical were observed the highest effects. Interestingly, the average ABTS and hydroxyl radical scavenging properties of two SPY samples (ABTS: 93.9%, hydroxyl: 89.7%) had higher effects than positive controls (Trolox: 89.7%; ascorbic acid: 56.3%, 200 µg/mL). The protective properties of SPYs against oxidative stress showed higher cell viabilities than those of SPMs with significant differences

under LLC-PK₁ cells treated with free radical generators such as pyrogallol, SNP, and SIN-1. Specifically, the treatment of SPYs with SNP led to the highest increase rate in cell viabilities with 60.3 → 85.6% (S48 strain) and 60.2 → 84.1% (P1201 strain). On the basis of the above results, we believe that isoflavone aglycone and CLA contents as well as antioxidant activities on radicals and protective effects under LLC-PK₁ cells may be important factors for the quality of SPY through fermentation. We also confirmed that SPY can be utilized as commercial soy foods owing to the scientific evidences for nutraceuticals and pharmaceuticals.

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REFERENCES

- [1] Kumar V, Rani A, Dixit AK, Pratap D, Bhatnagar D. A comparative assessment of total phenolic content, ferric reducing-anti-oxidative power, free radical-scavenging activity, vitamin C and isoflavones content in soybean with varying seed coat colour. *Food Res Int* 2010;43:323–8.
- [2] Zhang RF, Zhang FX, Zhang MW, Wei ZC, Yang CY, Zhang Y, et al. Phenolic composition and antioxidant activity in seed coats of 60 Chinese black soybean (*Glycine max* L. Merr.) varieties. *J Agric Food Chem* 2011;59:5935–44.
- [3] Lee JH, Lee BW, Kim JH, Jeong TS, Kim MJ, Lee WS, et al. LDL-Antioxidant pterocarpanes from roots of *Glycine max* (L.) Merr. *J Agric Food Chem* 2006;54:2057–63.
- [4] Messina M. Soy foods, isoflavones, and the health of postmenopausal women. *Am J Clin Nutr* 2014;100:423S–30S.
- [5] Brummer E, Graef GL, Orf J, Wilcox JR, Shoemaker RC. Mapping QTL for seed protein and oil content in eight soybean populations. *Crop Sci* 1997;37:370–8.
- [6] Lee YH, Kim B, Kim S, Kim MS, Kim H, Hwang SR, et al. Characterization of metabolite profiles from the leaves of green perilla (*Perilla frutescens*) by ultra high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry and screening for their antioxidant properties. *J Food Drug Anal* 2017;25:776–88.
- [7] Masilamani M, Wei J, Sampson HA. Regulation of the immune response by soybean isoflavones. *Immunol Res* 2012;54:95–110.
- [8] Lee JH, Cho KM. Changes occurring in compositional components of black soybeans maintained at room temperature for different storage periods. *Food Chem* 2012;131:161–9.
- [9] Ha TJ, Lee BW, Park KH, Jeong SH, Kim HT, Ko JM, et al. Rapid characterisation and comparison of saponin profiles in the seeds of Korean *Leguminous* species using ultra performance liquid chromatography with photodiode array detector and electrospray ionisation/mass spectrometry (UPLC-PDA-ESI/MS) analysis. *Food Chem* 2014;146:270–7.
- [10] Cho KM, Ha TJ, Lee YB, Seo WD, Kim JY, Ryu HW, et al. Soluble phenolics and antioxidant properties of soybean (*Glycine max* L.) cultivars with varying seed coat colours. *J Funct Foods* 2013;5:1065–76.
- [11] Balisteiro DM, Rombaldi CV, Genovese MI. Protein, isoflavones, trypsin inhibitory and in vitro antioxidant capacities: comparison among conventionally and organically grown soybeans. *Food Res Int* 2013;51:8–14.
- [12] Izumi T, Piskula M, Osawa S, Obata A, Tobe K, Saito M, et al. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J Nutr* 2000;130:1695–9.
- [13] Antony MS, Clarkson TB, Hughes CL, Morgan TM, Burke GL. Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. *J Nutr* 1996;126:43–50.
- [14] Piskula MK, Yamakoshi J, Iwai Y. Daidzein and genistein but not their glucosides are absorbed from the rat stomach. *FEBS Lett* 1999;447:287–91.
- [15] Shi H, Nam P, Ma A. Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. *J Agric Food Chem* 2010;58:4970–6.
- [16] Yang HL, Zhang L, Xiao G, Feng J, Zhou H, Huang F. Changes in some nutritional components of soymilk during fermentation by the culinary and medicinal mushroom *Grifola frondosa*. *LWT-Food Sci Technol* 2015;62:468–73.
- [17] Yang H, Gao J, Yang A, Chen H. The ultrasound-treated soybean seeds improve edibility and nutritional quality of soybean sprouts. *Food Research International*. *Food Res Int* 2015;77:704–10.
- [18] Cho KM, Lee JH, Yun HD, Ahn BY, Kim H, Seo WT. Changes in phytochemical constituents (isoflavones, flavanols, and phenolic acids) during *cheonggukjang* soybeans fermentation using potential probiotics *Bacillus subtilis* CS90. *J Food Comp Anal* 2011;24:402–10.
- [19] Shukla S, Park J, Kim DH, Hong SY, Lee JS, Kim M. Total phenolic content, antioxidant, tyrosinase and α -glucosidase inhibitory activities of water soluble extracts of noble starter culture *Doenjang*, a Korean fermented soybean sauce variety. *Food Control* 2016;59:854–61.
- [20] Dizler A, Park Y. Implication of conjugated linoleic acid (CLA) in human health. *Crit Rev Food Sci Nutr* 2012;52:488–513.
- [21] Lau DS, Archer MC. The 10t,12c isomer of conjugated linoleic acid inhibits fatty acid synthase expression and enzyme activity in human breast, colon, and prostate cancer cells. *Nutr Cancer* 2010;62:116–21.
- [22] Trigueros L, Sendra E. Fatty acid and conjugated linoleic acid (CLA) content in fermented milks as assessed by direct methylation. *LWT-Food Sci Technol* 2015;60:315–9.
- [23] Cho KM, Lim HJ, Kim MS, Kim DS, Hwang CE, Nam SH, et al. Time course effects of fermentation on fatty acid and volatile compound profiles of *Cheonggukjang* using new soybean cultivars. *J Food Drug Anal* 2017;25:637–53.
- [24] Hwang YH, Lai YJ, Chou CC. Fermentation temperature affects the antioxidant activity of the enzyme-ripened sufu, an oriental traditional fermented product of soybean. *J Biosci Bioeng* 2011;112:49–53.
- [25] Sanjukta S, Rai AK, Muhammed A, Jeyaram K, Talukdar NC. Enhancement of antioxidant properties of two soybean varieties of Sikkim Himalayan region by proteolytic *Bacillus subtilis* fermentation. *J Funct Foods* 2015;14:650–8.
- [26] Ademiluyi AO, Oboh G, Boligon AA, Athayde ML. Effect of fermented soybean condiment supplemented diet on α -amylase and α -glucosidase activities in Streptozotocin-induced diabetic rats. *J Funct Foods* 2014;9:1–9.

- [27] Hwang CE, An MJ, Lee HY, Lee BW, Kim HT, Ko JM, et al. Potential probiotic *Lactobacillus plantarum* P1201 to produce soy-yogurt with enhanced antioxidant activity. *Korean J Food Sci Technol* 2014;46:556–65.
- [28] Hati S, Vij S, Singh BP, Mandal S. β -glucosidase activity and bioconversion of isoflavones during fermentation of soymilk. *J Sci Food Agric* 2015;95:216–20.
- [29] Serafeimidou A, Zlatanov S, Laskaridis K, Sagredos A. Chemical characteristics, fatty acid composition and conjugated linoleic acid (CLA) content of traditional Greek yogurts. *Food Chem* 2012;134:1839–46.
- [30] Liao DY, Chai YC, Wang SH, Chen CW, Tsai MS. Antioxidant activities and contents of flavonoids and phenolic acids of *Talinum triangulare* extracts and their immunomodulatory effects. *J Food Drug Anal* 2015;23:294–302.
- [31] Herraiz T, Galisteo J. Hydroxyl radical reactions and the radical scavenging activity of β -carboline alkaloids. *Food Chem* 2015;172:640–9.
- [32] Song YB, Kim B, Choi MJ, Song YO, Cho EJ. Protective effect of arabinose and sugar beet pulp against high glucose-induced oxidative stress in LLC-PK₁ cells. *Food Chem* 2012;134:189–94.
- [33] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
- [34] Pinthony R, Macrae R, Dick J. The development of a soya-based yoghurt. III Analysis of oligosaccharide. *J Fd Technol* 1980;15:641–67.
- [35] da Silva LH, Celeghini RMS, Chang YK. Effect of the fermentation of whole soybean flour on the conversion of isoflavones from glycosides to aglycones. *Food Chem* 2011;128:640–4.
- [36] Marazza JA, Nazareno MA, de Giori GS, Garro MS. Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *J Funct Foods* 2012;4:584–601.
- [37] Yamabe S, Kobayashi-Hattori K, Kaneko K, Endo H, Takita T. Effect of soybean varieties on the content and comparison of isoflavone in rice-koji miso. *Food Chem* 2007;101:1247–53.
- [38] Chatterjee D, Bhattacharjee P. Comparative evaluation of the antioxidant efficacy of encapsulated and un-encapsulated eugenol-rich clove extracts in soybean oil: shelf-life and frying stability of soybean oil. *J Food Eng* 2013;117:545–50.
- [39] Yadav H, Jain S, Sinha PR. Production of free fatty acids and conjugated linoleic acid in probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* during fermentation and storage. *Int Dairy J* 2007;17:1006–10.
- [40] Li H, Liu Y, Bao Y, Liu X, Zhang H. Conjugated linoleic acid conversion by six *Lactobacillus plantarum* strains cultured in MRS broth supplemented with sunflower oil and soymilk. *J Food Sci* 2012;77:330–6.
- [41] Shantha NC, Ram LN, O'leary J, Hicks CL, Decker EA. Conjugated linoleic acid concentrations in dairy products as affected by processing and storage. *J Food Sci* 1995;60:695–7.
- [42] Van Nieuwenhove CP, Oliszewski R, Gonzalez SN, Perez Chaia AB. Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk. *Lett Appl Microbiol* 2007;44:467–74.
- [43] Juan MY, Chou CC. Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis* BCRC 14715. *Food Microbiol* 2010;27:586–91.
- [44] Zalibera M, Staskoško A, Šlebodová A, Jančovičová V, Čermáková T, Brezová V. Antioxidant and radical-scavenging activities of Slovak honeys—an electron paramagnetic resonance study. *Food Chem* 2008;110:512–21.
- [45] Zhang Q, Yu J, Zhang L, Hu M, Xu Y, Su W. Extraction, characterization, and biological activity of polysaccharides from *Sophora flavescens* Ait. *Int J Biol Macromol* 2016;93:459–67.
- [46] Gülçin İ. Antioxidant activity of food constituents: an overview. *Arch Toxicol* 2012;86:345–91.
- [47] Kladna A, Berczyński P, Kruk I, Piechowska T, Aboul-Enein HY. Studies on the antioxidant properties of some phytoestrogens. *Luminescence* 2016;31:1201–6.
- [48] Yamabe N, Park JY, Lee S, Cho EJ, Lee S, Kang KS, et al. Protective effects of protocatechuic acid against cisplatin-induced renal damage in rats. *J Funct Foods* 2015;19:20–7.
- [49] Piao XL, Piao XS, Kim SW, Park JH, Kim HY, Cai SQ. Identification and characterization of antioxidants from *Sophora flavescens*. *Biol Pharm Bull* 2006;29:1911–5.
- [50] Kwak JH, Jo YN, Jeong JH, Kim HJ, Jin SI, Choi SG, et al. Protective effects of black soybean seed coat extracts against oxidative stress-induced neurotoxicity. *Korean J Food Sci Technol* 2013;45:257–61.
- [51] Yi N, Seo KC, Choi JM, Cho EJ, Song YO, Han JS. Protective effects of Chungkookjang extract on high glucose induced oxidative stress in LLC-PK₁ cells. *J Food Sci Nutr* 2008;13:84–9.