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Effects of fermentation on the phytochemical composition and antioxidant properties of soy germ

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Abstract

Soy germ is a remarkable source of bioactive phytochemicals offering an interesting alternative as starting ingredient for fermented food. This work aimed to determine whether lactic acid bacteria fermentation of soy germ induces changes on its phytochemical composition. The antioxidant properties of fermented soy germ samples periodically taken during the fermentation process were evaluated and correlated with the concentration and structural modifications of isoflavones, saponins, phytosterols and tocopherols. Fermented soy germ extracts exhibited a higher inhibition effect against the superoxide anion radical, and lesser but significant ferric-reducing and DPPH radical scavenging effects compared with raw soy germ. By comparison to the traditional whole seed-based products, soy germ exhibits higher levels of isoflavones, saponins, phytosterols and tocopherols. All these phytochemicals contributed to the antioxidant capacity of soy germ and were conserved under lactic acid bacteria fermentation.

Keywords: Fermented soybean germ; Isoflavone; Soyasaponin; Tocopherol; β-Sitosterol; Antioxidant

1. Introduction

A large number of traditional fermented soybean products have been consumed for thousands of years in Asian countries and have reached progressively the occidental markets. Fermentation is widely used in the food industry not only to improve the sensory characteristics of a product, but also to eliminate certain undesirable constituents, make nutrients more accessible while preserving and even improving the nutritional properties. In addition to be natural, nutritious and safe, epidemiological studies have shown that traditional fermented soybean products exhibit potent anticarcinogenic effects (Nair, 2004). Other works conducted on animal models have attributed to fermented soy foods significant effects in the prevention of gastric disorders (Kiers et al., 2003), coronary heart diseases (Yokota, Hattori, Ohishi, Hasegawa, & Watanabe, 1996) and cancer (Ohta et al., 2000). These effects have partly been ascribed to the higher antioxidant properties of fermented soybean products compared with their unfermented counterparts (Ren, Liu, Endo, Takagi, & Hayashi, 2006). Most of the research conducted on the antioxidant properties of soybean compounds focused on the role of isoflavones (Ruiz-Larrea et al., 1997), even though soybean is an important source of other naturally occurring potential antioxidants, including soyasaponins, tocopherols and phytosterols.

Twelve isoflavones have been detected in soybean seeds (Wang & Murphy, 1994). The aglycone molecules daidzein, glycitein and genistein may be conjugated with a 7-*O*-β-glucoside, a 6″-*O*-malonylglucoside or a 6″-*O*-acetylglucoside group. These bioactive phenolic compounds may play a crucial role in the prevention of oxidation-related diseases

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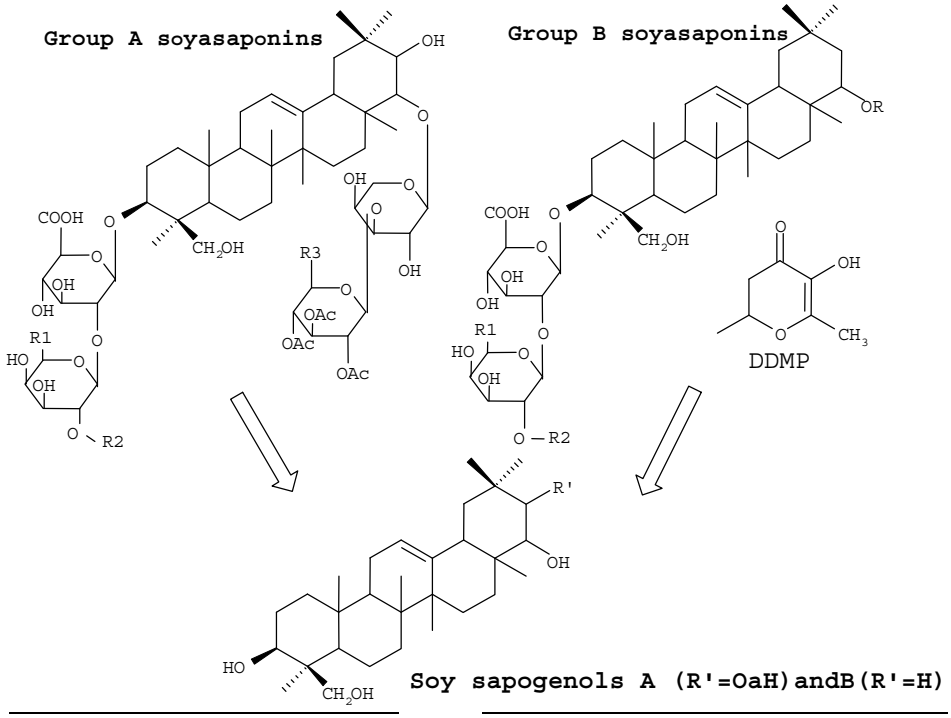
including atherosclerosis, hypertension (Park, Shin, Park, & Kang, 2005), breast cancer (DiSilvestro, Goodman, Dy, & Lavalle, 2005), or inflammatory bowel syndrome (Wiseman, 2006). Soyasaponins are another class of natural glycosides found in soybean seeds and soy-derived products. These triterpenoid molecules are classified in two major groups A and B, each containing an aglycone fraction linked to one or more oligosaccharide chains (Fig. 1). The effects of soyasaponins on human health have received much less attention than isoflavones, even though they have been shown to exert hypocholesterolemic, anticarcinogenic activities (Gurfinkel & Rao, 2003), as well as a beneficial effect against liver injury (Kuzuhara, Nishiyama, Minowa, Sasaki, & Omoto, 2000). Tocopherols (vitamin E) are lipid soluble molecules that are also present in significant amounts in soybean seeds. They have been extensively studied for their strong antioxidant activity, especially for their role in preventing disorders of the skin, eye, lungs, and other lipid-rich body constituents by inhibiting cell membrane and DNA damages induced by free radicals (Niki & Noguchi, 2004). It should be underlined that the lipid fraction of soybean also contains phytosterols in addition to the well-recognized antioxidant tocopherols. β-Sitosterol was found to decrease lipid peroxidation of platelet membranes *in vitro* (Van Rensburg, Daniels, Van Zyl, & Taljaard, 2000).

Depending on the pH and temperature conditions, a fermentation process may dramatically modify the content and the composition of these bioactive compounds (Rickert, Meyer, & Murphy, 2004). Most of the traditional fermented soy foods, such as tempeh, miso, soy sauces, natto, tofu, or soymilk are produced from whole soybean seeds or from purified soy proteins. By contrast, very few fermented foods are produced from soy germ, although this fraction is a higher source of antioxidant and antiproliferative phytochemicals than all other parts of the seed (Schryver, 2002). Soy germ, which accounts only for 2% of the total seed weight, is naturally 6- to 10-fold more concentrated in total isoflavones than cotyledons, with a specific isoflavone distribution containing 40–60% daidzein, 25–40% glycitein and 5–20% genistein, while genistein represents 45–60% of total isoflavones in the cotyledons (Daydé, Berger, & Theodorou, 2002). Several works have also reported very high concentrations of total soyasaponins in soy germ, with a 9-fold higher amount of group B soyasaponins in the germ compared with cotyledons, and



Group A soyasaponins			
	R_1	R_2	R_3
A1	CH_2OH	β-D-glu	CH_2OAc
A2	CH_2OH	H	CH_2OAc
A3	H	H	CH_2OAc
A4	CH_2OH	β-D-glu	H
A5	CH_2OH	H	H
A6	H	H	H

Group B soyasaponins			
R = HR = DDMP		R_1	R_2
I	βg	CH_2OH	α-L-rham
II	βa	H	α-L-rham
III	γg	CH_2OH	H
IV	γa	H	H
V	αg	CH_2OH	β-D-glu

Fig. 1. Structures of soyasaponins A and B and aglycone corresponding forms.

group A soyasaponins exclusively found in the germ (Hubert, Berger, & Daydé, 2005a). The oil extracted from the germ is also a better source of phytosterols and tocopherols than the oil extracted from the cotyledons.

However, the undesirable sensory properties provided by these compounds, including bitterness and astringency, have limited the consumption of soy germ-derived products for a long time (Okubo et al., 1992).

In this study, we investigated the chemical modifications occurring in the phytochemical profile of soy germ during a fermentation experiment conducted with lactic acid bacteria. Several reports have underlined the release of antioxidant compounds during a fermentation process (Pyo, Lee, & Lee, 2005). The enzymatic conversion of the conjugated isoflavones into the respective bioactive aglycone structures has been the focus of many studies (Tsangalis, Ashton, McGill, & Shah, 2002), but the effect of fermentation on other metabolites has never been described. The present work covers the changes in the concentration and composition of phytochemicals when soy germ was fermented by three different strains of lactic acid bacteria. The degradation kinetics of glucoside compounds including isoflavones and soyasaponins, as well as the evolution of phytosterol and tocopherol concentrations, were investigated as a function of incubation time during 48 h. Three different methods, including the DPPH radical scavenging activity, the inhibition of the superoxide anion radical $O_2^{\cdot-}$, and the ferric-reducing antioxidant activity, were performed on periodically taken fermented samples in order to establish a correlation between the compositional changes occurring in soy germ and the evolution of its antioxidant properties. Purified molecules were also tested in order to identify the substances potentially implicated in the antioxidant properties of soy germ, and to determine in which way a fermentation process may alter, maintain or improve these characteristics.

2. Materials and methods

2.1. Fermentation conditions

Soybean germs from an industrial load were provided by Genibio (Lorp-Sentaraille, France). A sample of finely ground powder was suspended in ultra-pure distilled water at 150 g/L. The mixture was heated and stirred for 30 min at 65 °C to dissolve solid particles. A 150 mL reconstituted solution was prepared for three fermentation experiments using three standardized mixtures of lactic acid bacteria (named A, B, and C). The three soy-based medium were inoculated at 1% (w/v) with each bacterial mixtures A, B, and C and incubated at 37 °C for 48 h under slow stirring at 60 rpm. Samples were removed periodically (1 h, 3 h, 5 h, 7 h, 10 h, 24 h, and 48 h). All samples were then pasteurized at 65 °C for 15 min, lyophilized and stored at −20 °C until isoflavones, saponins, phytosterols and tocopherols analyses and antioxidant assays.

2.2. Isoflavone analysis

Isoflavones were quantified as a function of fermentation time. Each sample (0.1 g) was dissolved in 80% aqueous methanol (10 mL) and extracted for 2 h at room temperature. The residue was removed by centrifugation at 12,000*g* for 10 min and subsequent decantation of the clear supernatant. Each extract was filtered (0.45 μm) and analyzed by reverse phase high performance liquid chromatography (RP-HPLC) with a P4000 pump controller, AS3000 autosampler and UV2000 detector monitored at 260 nm (Spectra Physics Analytical Inc., Fremont, CA, USA). The analytical column (250 × 4.6 mm i.d., 5 μm, Satisfaction RP-C_{18}-AB Cluzeau, Sainte- Foy-La-Grande, France) was kept at 30 °C and operated at a flow rate of 1.5 mL/min. The injection volume was 10 μL. The mobile phase was 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as reported in a previous study (Hubert, Berger, & Daydé, 2005b). This procedure was duplicated for each sample. The aglycone structures (daidzein, genistein, glycitein) and the corresponding β-glucoside conjugated forms (daidzin, genistin and glycitin) were quantified by an external calibration using their standard molecules (Chromadex, Santa Ana, CA). The response factors of the 6″-*O*-malonylglucosides and 6″-*O*-acetylglucosides were calculated from those of the corresponding β-glucoside isoflavones, correcting them in a molecular mass ratio (Hubert et al., 2005a, 2005b).

2.3. Soyasaponin analysis

Soyasaponins belonging to groups A and B were also analyzed as a function of incubation time. Soyasaponins B were first analyzed on the same hydro-alcoholic extract, with the same chromatographic column and solvents as used for isoflavone determination. UV absorbance was monitored at 205 nm. The gradient elution, separation, identification and calibration methods of all soyasaponins B were precisely described in a previous study (Hubert et al., 2005b). Total soyasaponins A were then quantified through the analysis of the corresponding aglycone fraction. Soyasapogenol A was analyzed after submitting the hydro-alcoholic extract to an acid hydrolysis at 85 °C for 6.5 h, to release the sapogenol from the conjugated saponin structures. The extraction and hydrolysis steps were duplicated for each sample. Soyasapogenols A and B were separated, detected at 205 nm, identified and quantified as described in a previous study (Hubert et al., 2005a). Purified standards of soysapogenols A and B were provided by Chromadex (Santa Ana, CA, USA).

2.4. Tocopherol analysis

Lipids containing tocopherols and phytosterols were extracted with *n*-hexane in a soxhlet apparatus under refluxing for 4 h. The solvent was then evaporated under

vacuum at 45 °C. The extracted oils were stored in the dark at −20 °C. For tocopherol quantification, duplicate samples of the extracted germ oil (50 mg) were transferred into a 5 mL brown volumetric flask, and dissolved in 100% *n*-hexane. The chromatographic separation of α-, β-, γ-, and δ-tocopherols was achieved on a normal phase equisorb silica column (250 × 4.0 mm i.d., 5 μm, Cluzeau, Sainte-Foy-La-Grande, France) coupled with a fluorimetric detector monitored at an excitation wavelength of 290 nm and at an emission wavelength of 330 nm (multi λ, Waters 2475, Milford, MA). The mobile phase was *n*-hexane/propan-2-ol (99.5/0.5 v/v) at a flow rate of 1 mL/min. The injection volume was 20 μL. Each tocopherol quantification was based on an external calibration using the standard molecules of α-, β-, γ-, and δ-tocopherols (Sigma–Aldrich, Steinheim, Germany).

2.5. Phytosterol analysis

Duplicate samples of the extracted soy germ oil (100 mg) were diluted in a 5% KOH solution in ethanol (10 mL) and submitted to saponification at 65 °C for 15 min. The mixture was cooled by adding distilled water (5 mL) and the nonsaponifiable matter was extracted on an aluminum oxide (10 g) column (MP Biochemicals, Eschwege, Germany) by eluting 5 mL ethanol and 30 mL ether. The extracted solution was evaporated under vacuum and dissolved in 1-methylimidazole (5 μL) and *N*-methyl-*N*-trimethylsilylheptafluoroacetamide (100 μL) (Sigma–Aldrich, Steinheim, Germany). Silylation was achieved at 105 °C for 15 min. The different soybean phytosterols (campesterol, stigmasterol, β-sitosterol, and Δ-7-avenasterol) were quantified by capillary gas chromatography (GC 8000 series, Fisons, Milano, Italy) using a flame ionization detector (FID) at $P(H_2) = 100$ kPa and $P(\text{air}) = 60$ kPa. Each sample was directly injected (1 μL) into a 30 m × 0.25 mm i.d. × 0.25 μm film thickness Zebron capillary GC column (Phenomenex, Paris, France). The initial oven temperature of 240 °C increased at 4 °C/min to 320 °C where it was held for 10 min. Inlet and flame-ionization detector temperatures were 320 °C. The carrier gas was N_2 at 130 kPa. Betulin was used as internal standard (Sigma–Aldrich, Steinheim, Germany).

2.6. Extraction of the antioxidant polar fraction

The periodically taken fermented and non-fermented soybean powders (50 mg) were diluted in a MeOH/H_2O solution (500 μL, 2/1 v:v) and extracted for 30 min at room temperature. After centrifugation (1500*g*, 5 min, 4 °C), the supernatant was filtered (0.45 μm). This extraction step was repeated three times. The collected supernatants were evaporated and dissolved either in a MeOH–H_2O solution (2/1 v:v, 4 mL) for the DPPH assay, or in ultra pure distilled water (4 mL) for the other tests measuring the reducing power or the superoxide anion radical scavenging activity.

2.7. Measurement of the DPPH radical-scavenging activity

The antioxidant activity was first evaluated using the stable 1,1-diphenyl-2-picrylhydrazyl nitrogen-centered radical (DPPH·) (Sigma–Aldrich, Saint-Louis, MO, USA) as a scavenging target. Each polar extract was submitted to serial dilution (0.2, 0.5, 1, 1.5, and 2 mg/mL) in a MeOH/H_2O solution (1 mL, 2/1 v:v), and mixed with an ethanolic DPPH solution (1 mL, 400 μM). Following a 30 min incubation period in the dark at room temperature, the ability of the extracts to scavenge the DPPH radical was assessed by measuring the absorbance at 523 nm (UVIKON 931 spectrophotometer, Trappes, France). The inhibition percentage of the DPPH absorbance was calculated by the following equation: scavenging activity (%) $= [A_a - A_b]/A_a$, where A_a is the absorbance of the control mixture, and A_b is the absorbance of the solution containing the tested extract. This method was also tested on the individual purified standards of isoflavones aglycones (daidzein, genistein, and glycitein), and β-glucosides (daidzin, genistin, and glycitin), soyasaponins I, II and III (Chromadex, Santa Ana, CA, USA), β-sitosterol, stigmasterol, α-tocopherol and δ-tocopherol (Sigma–Aldrich, Steinheim, Germany). Each standard was diluted (20, 50, 75, 100, and 150 μg/mL) in a MeOH/H_2O solution (1 mL, 2/1 v:v), and submitted to the DPPH assay as for the fermented soy germ extracts.

2.8. Measurement of the reducing activity

The reducing activity of the extracts was determined according to the method of Oyaizu (1986). Each extract was submitted to serial dilution (0.2, 0.5, 1, 1.5, and 2 mg/mL) in distilled water. The resulting samples or distilled water as control (0.5 mL) were mixed with 0.5 mL of a 1% potassium ferricyanide solution (Prolabo, Paris, France) and 0.5 mL of a sodium phosphate buffer solution (0.02 M, pH 7.0). The mixture was incubated at 50 °C for 20 min. Then 0.5 mL of a 10% trichloroacetic acid solution (w/v) was added, and the mixture was centrifuged at 780*g* for 5 min. The upper layer (1.5 mL) was mixed with 0.2 mL of a 0.1% ferric chloride solution. The absorbance was measured at 700 nm. A higher absorbance of the mixture indicated a higher reducing activity. The reducing activity of cysteine was used as reference. This method was also tested on the individual purified standards of isoflavones, soyasaponins, phytosterols and tocopherols cited above. Each standard was submitted to serial dilution (20, 50, 75, 100, and 150 μg/mL) in distilled water and tested for its reducing activity.

2.9. Measurement of superoxide anion radical scavenging activity

The ability of the extracts to scavenge the superoxide anion radical $O_2^{\cdot-}$ was determined according to the method of Robak and Gryglewski (1988). All reagents were prepared in a sodium phosphate buffer solution (100 mM,

pH 7.4). Each soy germ extract was submitted to serial dilution (0.2, 0.5, 1, 1.5, and 2 mg/mL) in distilled water. The resulting samples or distilled water as control (50 μL) were mixed with a nitrobluetetrazolium solution (NBT, 50 μL, 300 μM), a β-nicotinamide adenine dinucleotide solution (β-NADH, 50 μL, 936 μM) and a PMS solution (methyl phenazonium methosulphate, 50 μL, 120 μM). The final mixtures were incubated at room temperature for 5 min. The absorbance was measured at 560 nm. NBT is a yellow compound that, under double reduction, generates an insoluble blue diformazan precipitate. NBT is reduced directly by superoxide radicals generated by β-NADH. The inhibition of the NBT reduction in the solution shows the capacity of an extract to inhibit superoxide anion radical production. The superoxide anion radical scavenging activity of the sample was calculated according to the following equation: Scavenging activity (%) = $[A_a - A_b]/A_a$, where A_a is the absorbance of the control mixture, and A_b is the absorbance of the solution containing the tested extract. This method was also tested on the individual purified standards of isoflavones, soyasaponins, phytosterols and tocopherols cited above. The three antioxidant activity measurement methods were validated by an assay of reproducibility conducted on two reference samples: one fermented soy germ powder and one raw soy germ sample from an industrial load (Genibio, Lorp-Sentaraille, France). By repeating three times during three different days the extraction of the polar fraction followed by the three antioxidant assays, we found a variation coefficient lower than 10%, whatever the method tested.

3. Results

3.1. Modification of isoflavone composition during fermentation

The structural changes of isoflavones caused by enzymatic hydrolysis occurring in the soy germ extracts during fermentation are shown in Fig. 2. The initial raw soy germs contained 39.0 ± 2.5 μmol/g of total isoflavones, with β-glucosides, malonylglucosides, aglycones and acetylglucosides accounting for 46.9%, 44.5%, 7.3% and 1.3%, respectively. The distribution of the total soy germ isoflavones, including 62.4% daidzein derivatives, 25.7% glycitein derivatives and 11.9% genistein derivatives remained constant over 48 h, with a standard deviation of less than 3.7% for the three isoflavone families over the whole incubation period, indicating that isoflavones were not converted into other microbially-induced metabolites, and that the strains A, B and C were not able to metabolize daidzein into equol. The HPLC analysis of the periodically taken fermented samples revealed an increase of the aglycone forms (Fig. 2), with various kinetics depending on the microorganism composition. After 48 h incubation at 37 °C with the strains A, B, and C, the aglycones accounted for 62.7%, 75.2%, and 75.3% of total isoflavones, respectively. At the same time, the concentrations of the malonyl-, acetyl- and β-glucosides isoflavones were significantly reduced. The final fermented soy germ extracts contained less than 22% malonylglucosides, 15% β-glucosides, and 2% acetylglucosides regardless of the strain tested.

3.2. Modification of soyasaponin composition during fermentation

The total amount of soyasaponins B in the initial non-fermented soy germ powder was 20.1 μmol/g, with the total 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated forms accounting for 14.8 μmol/g. The major soyasaponin pairs {I + βg} and {V + αg} accounted for 13.9 and 5.4 μmol/g in the native soy germ, respectively. The other soyasaponins II, III, IV, βa, γg, and γa represented together only 4.2% of total soyasaponins belonging to group B. As for isoflavones, the conjugation profile of soyasaponins B was modified during the incubation period. The amount of DDMP-conjugated soyasaponins decreased drastically from 14.8 to 2.1, 9.0, and 2.9 μmol/g when soy germ was fermented with the strains A, B, and C, respectively, indicating that all the lactic acid bacteria tested were able to cleave the DDMP-moieties, releasing a maltol molecule. From the beginning to 10 h of incubation, the total concentration of soyasaponins B decreased from 20.1 to 11.2 (strain A) and 14.4 μmol/g (strain C), mainly because of the degradation of soyasaponin I and its DDMP corresponding form βg (Fig. 3). These trisaccharidic glycosilated soyasaponins may have lost their terminal sugar by enzymatic hydrolysis and convert into other unidentified structures. From 10 h to 48 h, the total content of soyasaponins B remained unchanged when soy germ was incubated with the strains A and C, while an unexpected increase in the major soyasaponins {I + βg} and {V + αg} was observed during incubation with the strain B, reaching a final concentration of 19.8 μmol/g total soyasaponins. This could be explained by the deglycosilation of particular soyasaponins belonging to group A. It has been reported that after deglycosilation, soyasaponins A4 and A1 become structurally similar to soyasaponin V belonging to group B and elute at the same retention time during HPLC analyses, what can explain the increased concentration of soyasaponins {V + αg} (Rickert et al., 2004). Unfortunately, due to the lack of purified standard molecules and due to the difficulty to quantify the various structures of conjugated soyasaponins A, the degradation kinetics of the glycosidic fraction was not studied. We only access to the total amount of group A soyasaponins, that is 27.3 μmol/g, by quantifying their corresponding aglycone form soyasapogenol A. This amount was unchanged at the end of the fermentation experiment, whatever the strain tested.

3.3. Effect of fermentation on tocopherol and phytosterol contents

Tocopherol and phytosterol contents were significantly reduced during fermentation in the three culture systems

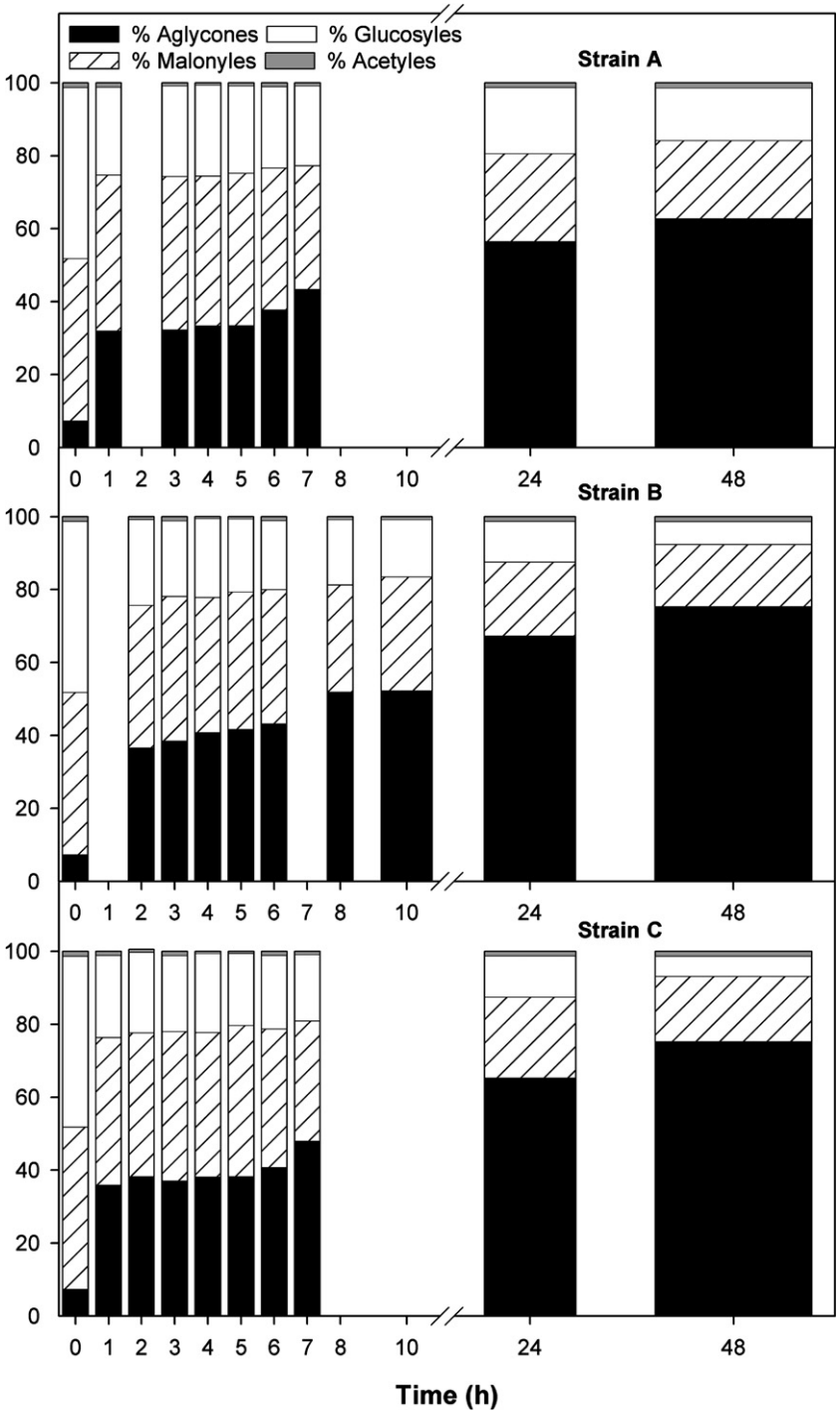


Fig. 2. Degradation kinetics of glycosilated isoflavones in soy germ fermented with three lactic acid bacteria strains.

(Table 1). Raw soy germs contained 0.36 mg/g of total tocopherols, with 47.5% α-tocopherol, 0.3% β-tocopherol, 2.2% γ-tocopherol, and 50.0% δ-tocopherol. At the end of the incubation period, the total amount of tocopherols accounted only for less than 0.08 mg/g whatever the strain tested. Reduced phytosterol levels were also observed, with an initial total concentration of 4.17 mg/g reaching 2.31, 3.01 and 1.09 mg/g after 48 h incubation with the strains A, B and C, respectively (Table 1). The relative distribution of the individual phytosterols remained globally constant during the whole experiment, β-sitosterol accounting for 75.9–79.0%, campesterol for 3.1–4.3%, stigmasterol for 6.8–7.2%, and Δ-7-avenasterol for 9.7–12.9% of total phytosterols. Several factors might be attributed to the phytosterol loss, including oxidation or dehydration.

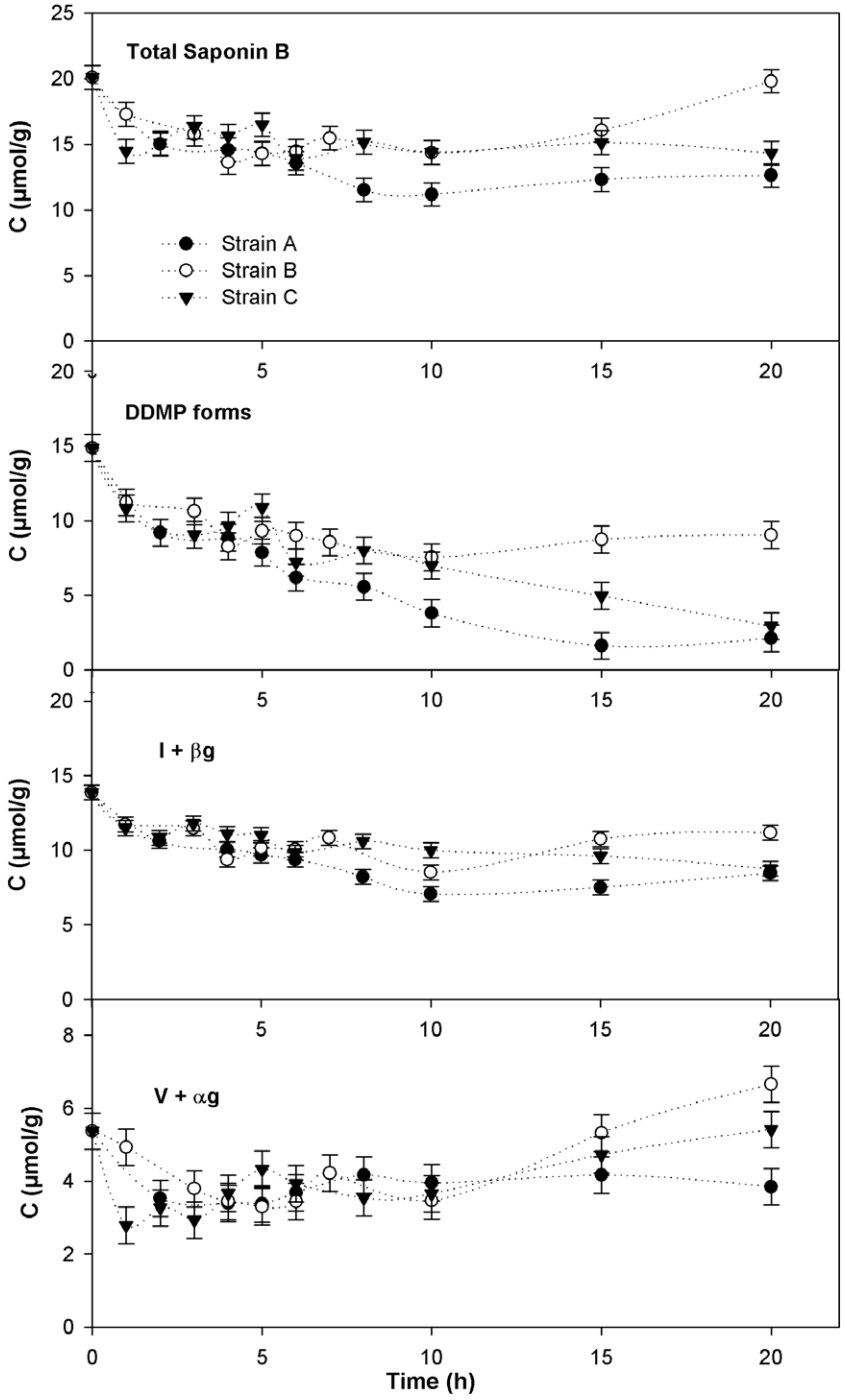


Fig. 3. Group B soyasaponin profile evolution during soy germ lactic acid fermentation. DDMP: 2,3-Dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one, A, B and C are three standardized mixtures of lactic acid bacteria, βg, βa, γg, γa, and αg are the five DDMP-conjugated soyasaponins, and I, II, III, IV and V are the corresponding non-DDMP corresponding structure.

3.4. DPPH radical scavenging activity of fermented soy germ extracts

The DPPH radical scavenging activities of the three fermented soy germ culture medium as a function of incubation time are shown in Fig. 4. Raw soy germ inhibited the absorbance of DPPH by 39% at a concentration of 1 g/L ($\lambda = 523$ nm). After 1 h, the scavenging effect was slightly increased when soy germ was inoculated by the strains B and C, while remained constant when inoculated by the strain A. Then, the DPPH scavenging effect decreased of about 20% whatever the strain and a slight increase was finally observed from 24 h to 48 h. Soy germ fermented with the strain B showed the strongest final scavenging effect with a 45% DPPH absorbance inhibition compared with 26% and 34% with the strains A and C, respectively.

Table 1
Tocopherol and phytosterol contents after lactic acid fermentation of soy germ determined by HPLC (see experimental section)

Tocopherols	α (mg/g)	β (mg/g)	γ (mg/g)	δ (mg/g)	Total (mg/g)	Total (μmol/g)
Molecular weight (g/mol)	430.7	416.7	416.7	402.7		
Unfermented	0.17	ND	0.01	0.18	0.36	0.87
Strain A	0.03	ND	0.01	ND	0.05	0.09
Strain B	0.03	0.01	0.04	0.01	0.08	0.19
Strain C	0.04	ND	0.01	ND	0.05	0.12
Phytosterols (mg/g)	Campesterol (mg/g)	Stigmasterol (mg/g)	β-Sitosterol (mg/g)	Δ-7 -Avenasterol (mg/g)		
Molecular weight (g/mol)	400	412	414	412		
Unfermented	0.13	0.2	3.7	0.13	4.17	10.06
Strain A	0.1	0.16	1.83	0.22	2.31	5.59
Strain B	0.13	0.21	2.29	0.39	3.01	7.31
Strain C	0.03	0.08	0.87	0.11	1.09	2.64

ND: Non-detected. Values are the mean of duplicate analyses.

3.5. Ferric-reducing power of fermented soy germ extracts

The ferric-reducing antioxidant activities of the periodically taken fermented soy germ extracts were evaluated for each strain as a function of incubation time. As shown in Fig. 4, the three culture media had similar trends during the whole experiment, but the reducing activity evolution was different compared with the DPPH scavenging effect. The initial ferric-reducing power of raw soy germ, expressed as an equivalent amount of cystein (59.7 μM), was globally maintained until 6 h incubation, and followed by a significant decrease until the end of the experiment. From 6 h to 48 h incubation, the reducing power decreased from 61.5 to 9.7 μM cystein equivalents when soy germ was incubated with the strain A, from 55.4 to 27.3 μM with the strain B, and from 61.1 to 17.9 μM with the strain C. Thus, our fermentation conditions induced an important reduction of soy germ reducing power if the incubation period is longer than 6 h.

3.6. Superoxide anion radical ($O_2^{\cdot-}$) scavenging activity of fermented soy germ extracts

The ability of the fermented soy germ extracts to scavenge the superoxide anion radicals was also investigated. Raw soy germ inhibited the production of superoxide anion radicals by 25% at 0.2 g/L. As shown in Fig. 4, the evolution trend of oxygen-radical scavenging activity in the three culture medium were very similar during the whole incubation period, with a high increase from the beginning to 6 h, followed by a weaker, but significant increase until 48 h. The final values of the superoxide radical absorbance inhibition were 80.2%, 92.9%, and 86.9% when soy germ was incubated with the strains A, B, and C, respectively.

3.7. Antioxidant activity of purified standard molecules

In an attempt to correlate the antioxidant activity of the periodically taken extracts with the evolution of soy germ phytochemicals during fermentation, we also tested the antioxidant activity of purified standard molecules present in high levels in soy germ (Table 2). These compounds included α- and δ-tocopherols, β-sitosterol, daidzein, glycitein, genistein, and their β-glucoside corresponding forms daidzin, glycitin and genistin, as well as soyasapogenols A and B, soyasaponins I, II and III. It was found that δ-tocopherol was the most effective in scavenging the DPPH radical with a 74% absorbance inhibition at a concentration of 0.5 mg/mL, followed by soyasapogenol B and α-tocopherol which exerted, respectively 44% and 41% absorbance inhibition at the same concentration. Soyasapogenol A was less effective than soyasapogenol B, with only a 10% absorbance inhibition. The β-glucoside isoflavones daidzin, genistin and glycitin, as well as the non-DDMP conjugated soyasaponins I and II and β-sitosterol were also poorly active towards the DPPH radical. Different trends were observed between the DPPH radical scavenging and the ferric-reducing activities. β-Sitosterol, which was not able to scavenge the DPPH radical, showed the highest reducing activity, followed by soyasaponin II, glycitein, and soyasapogenol B. By contrast with the DPPH assay, α- and δ-tocopherols did not exhibit any reducing activity under these assay conditions. Nevertheless, these two compounds were the most effective molecules in scavenging the superoxide anion radical $O_2^{\cdot-}$ with an absorbance inhibition higher than 90% at a concentration of 0.05 mg/mL, followed by the β-glucoside isoflavones and by their aglycone corresponding forms. In comparison, the soyasaponins and phytosterols were poorly active towards $O_2^{\cdot-}$.

4. Discussion

The health benefits of fermented soy foods have been attributed to the antioxidant capacity of particular compounds structurally modified or released after bacterial hydrolysis. Numerous soy-derived fermented products are commercially available, most of which are made from the whole soybean seeds or from soy protein isolates. Extracts issued from isolated soy germ, normally a waste product from soybean processing due to its off-flavor, could offer an interesting alternative as starting material for fermented

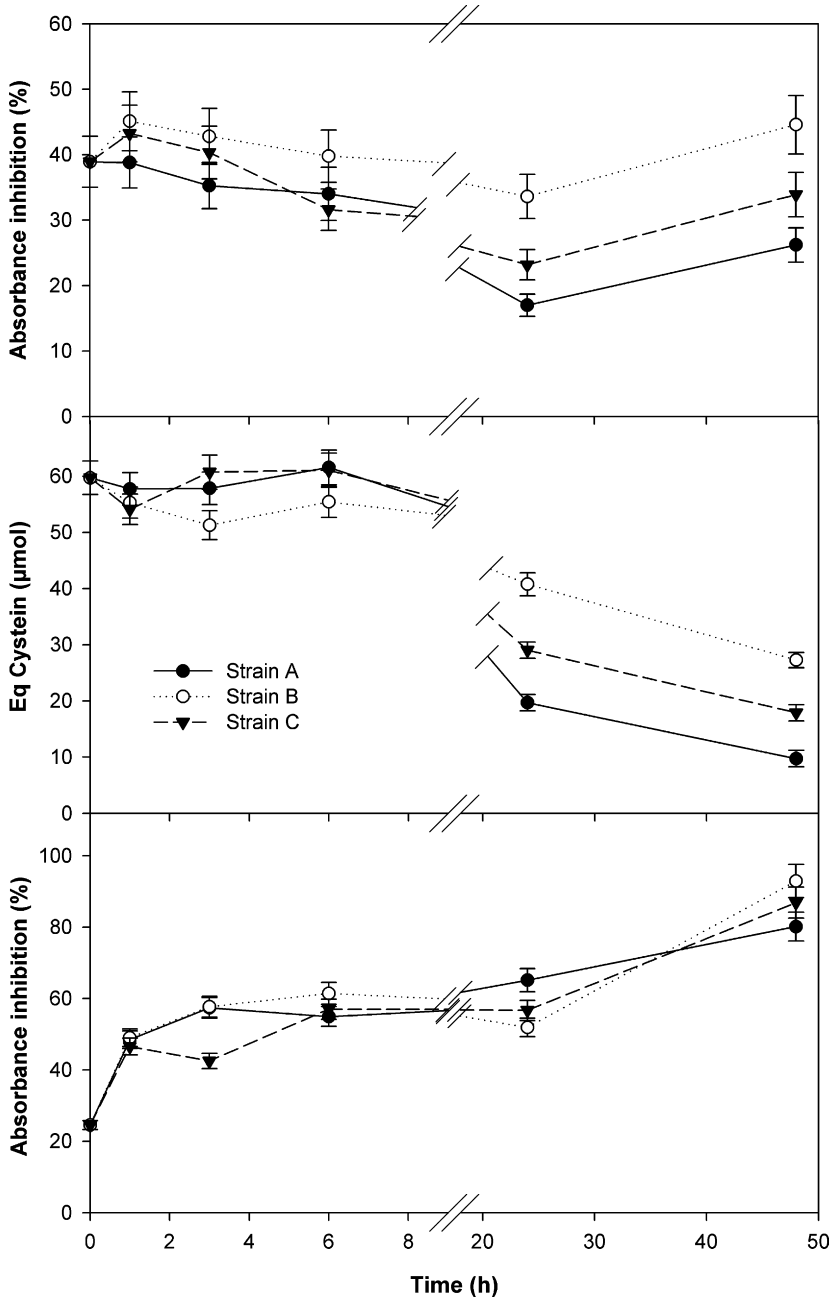


Fig. 4. Antioxidant capacity of fermented soy germ extracts. (A) DPPH radical scavenging activity ($c = 1$ g/L, $\lambda = 523$ nm). (B) Time-dependent reducing activity. (C) Oxygen-radical scavenging activity ($c = 0.2$ g/L).

ingredients, because of its naturally high levels of bioactive phytochemicals, including isoflavones, saponins from the groups A and B, phytosterols and tocopherols, which all have demonstrated an antioxidant activity. Moreover, the radical scavenging capacity of soy germ extracts was reported much higher than those of cotyledon extracts (Monje et al., 2006).

After fermentation, the total amounts of isoflavones and soyasaponins were globally maintained, conferring to the final soy germ extracts a more interesting phytochemical composition than the traditional fermented soy foods, which are largely less concentrated in these bioactive components. The incubation of soy germ with lactic acid bacteria led to considerable changes in isoflavone and soyasaponin conjugation profiles. The observed conversion of glycosylated isoflavones into aglycones during the whole incubation period, revealed a β-glucosidase activity of the microorganisms tested. These results are in agreement with previous works reporting that fermented soybean foods contain aglycones as predominant isoflavone structures (Tsangalis et al., 2002). This is an important fact since the bioavailability and metabolism of isoflavones are

Table 2
Antioxidant activity of purified soy germ secondary metabolites

Nom	DPPH absorbance inhibition (%)	Ferric-reducing antioxidant activity (mM cystein equivalent)	Oxygen-radical scavenging activity (%)
α-Tocopherol	41	0.35	>90
δ-Tocopherol	74	0.19	>90
β-Sitosterol	<10	1.51	<10
Daidzein	16	0.68	40
Glycitein	<10	1.07	53
Genistein	13	0.39	46
Daidzin	17	0.10	63
Glycitin	<10	0.07	59
Genistin	<10	0.63	73
Sapogenol A	10	0.37	41
Sapogenol B	44	0.85	<10
Saponin I	<10	0.42	18
Saponin II	<10	1.18	<10
Saponin III	<10	0.14	<10

Values are the mean of duplicate analyses.

strongly linked to their molecular structures. Several human studies have reported that dietary isoflavones are absorbed more efficiently as aglycones than their respective glucoside structures (Izumi, Osawa, Obata, Tobe, & Saito, 2000). By contrast, other works have suggested that isoflavones should be preferentially ingested as conjugated forms because the β-glucosidic moiety may act as protecting group to prevent biodegradation of isoflavones before reaching the gut (Zubik & Meydani, 2003). In this fermentation experiment, it becomes possible to control the final concentration of the bioactive aglycones by choosing a specific incubation time for the bioconversion of conjugated isoflavones.

Saponins are another class of soybean glycosylated compounds that were structurally influenced by the fermentation process, what is also interesting since the biological activity of soyasaponins depends highly on their overall conformation, mainly given by the length, the number, and the composition of the sugar side chain (Gurfinkel & Rao, 2003). We observed that the native soyasaponins conjugated with a 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) group were progressively converted into their non-DDMP counterpart during the incubation period. All the lactic acid bacteria tested were able to cleave

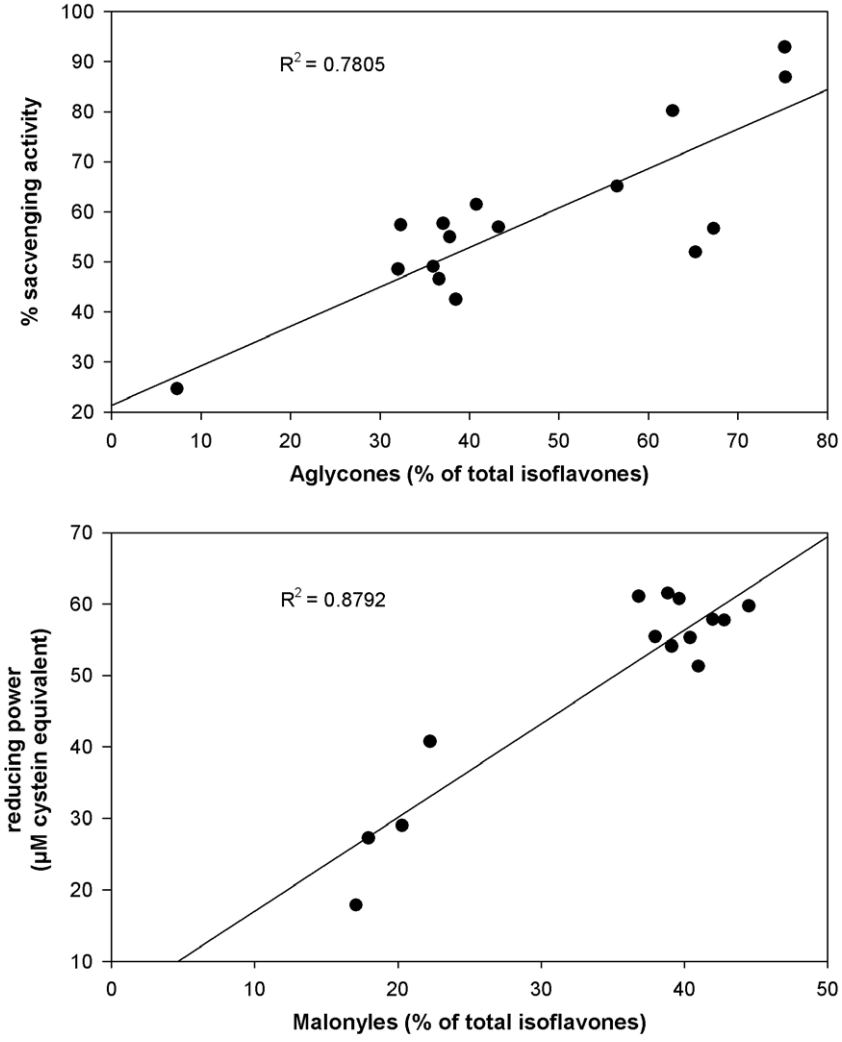


Fig. 5. Correlation between antioxidant activity and the contents of aglycone isoflavones.

the DDMP-moieties of group B soyasaponins to release a maltol molecule. It would be interesting to further investigate the taste improvement of the final fermented soy germ extract, since a sweet flavor has been attributed to free maltol molecules (Okubo et al., 1992). The absence of soyasapogenols A and B in the three culture media at the end of the experiment revealed that soyasaponins were not fully deglycosilated. We only observed a slight decrease of the trisaccharidic glycosilated soyasaponins {I + βg} and {V + αg}, probably due to the loss of their terminal sugar by enzymatic hydrolysis and conversion into other unidentified structures. The well-recognized bioactive phytosterols and tocopherols were significantly altered under our fermentation conditions. After 48 h incubation, more than 85% of the initial tocopherols were destroyed, whatever the strain tested. This loss could be attributed to the incubation temperature (37 °C) and to the presence of oxygen initially dissolved in the incubation medium, which might have promoted their alteration. This oxidation process occurs in any oxygen-enriched environment where substrates are exposed to ultraviolet light or heat. A significant proportion of phytosterols was also removed during fermentation depending on the culture system. In an attempt to maintain the content and composition of phytosterols in a fermented soy germ product, the strain B was the most suitable.

The total antioxidant capacity of the periodically taken fermented soy germ extracts was evaluated using three different assays. The first model of scavenging the stable radical DPPH was sensitive enough to show that both crude and fermented soy germ extracts contain particular hydrogen-donor substances which may convert free radicals into harmless substances. However, this activity was modulated by the incubation time and the nature of microorganism. Similarly, the ferric-reducing power of the periodically taken fermented soy germ extracts was strongly related to the incubation time and choice of lactic acid bacteria. The fermented samples tested in the present study exerted a stronger effect against the superoxide radical anion radical ($O_2^{\cdot-}$) than against the stable radical DPPH. Similar results were previously observed, showing that conjugated and unconjugated isoflavones exhibited a low scavenging potency for DPPH free radicals due to their poor hydrogen-donor capacity compared with other phenolic compounds (Kao & chen, 2006; Mitchell et al., 1998). After fermentation, soy germ extracts exerted a more potent scavenging effect towards $O_2^{\cdot-}$. We observed a good linear correlation between the concentration of isoflavone aglycones and the scavenging effect of the superoxide anion radical ($r = 0.781$) in the three culture medium (Fig. 5), what concords with a recent study where isoflavones inhibited ultraviolet light-induced oxidative DNA damage by a superoxide dismutase-like mechanism (Russo, Cardile, Lombardo, Vanella, & Acquaviva, 2006). Thus, the increasing superoxide scavenging activity during fermentation might be partly attributed to the increasing concentration of isoflavones aglycones progressively released from their corresponding conjugated structures. These results are of particular interest because, despite their mild reactive properties in biological systems, superoxide radical anions are potential intracellular precursors of more aggressive and deleterious reactive oxygen species.

Isoflavones are recognized as potential protective compounds against various oxidative stress-related diseases. Their activity should depend highly on the number and position of available hydroxyl groups determining the stabilization of the resulting phenoxyl radicals formed by hydrogen donation. By testing the antioxidant capacity of purified standards, we found that genistein and daidzein provide similar trends regarding the three selected antioxidant activity assays. In comparison, glycitein exhibited different activities, probably due to the presence of a methoxyl group on its phenolic ring. This effect of blocking hydroxyl groups through methylation, as for glycitein compared with daidzein and genistein, on the reduction of the antioxidant capacities was reported in a recent study in which the importance of phenolic hydroxyl groups for the antioxidant actions of isoflavones was underlined (Ruffer & Kulling, 2006).

The results of this work also showed that isoflavones were not the unique molecular family contributing to the antioxidant properties of soy germ. When looking into the antioxidant activity of purified standard molecules, we demonstrated that soyasaponins, phytosterols and tocopherols also exhibited potent antioxidant activities. Regarding the DPPH assay, δ- and α-tocopherols were the most effective among all the purified standards tested, suggesting that the reduction of DPPH scavenging capacity observed during the fermentation experiment in the three culture media could be related to the drastic degradation of tocopherols. By contrast, δ- and α-tocopherols, which were the most active free radical scavengers, showed the lowest reducing power, indicating that vitamin E functions primarily as a chain-breaking antioxidant rather than a powerful reductor compound. This is in agreement with a previous study demonstrating that vitamin E rapidly scavenged hydroxyl radicals, but scavenged other oxidant species at a relatively low rate (Yu et al., 2002). The small difference between δ-tocopherol and α-tocopherol is probably related to the position of hydroxyl groups on the phenolic ring. Among the other soy germ phytochemicals, β-sitosterol was a very bad free radical scavenger but showed the highest reducing activity toward potassium ferricyanide. We also observed that soyasapogenol B had a more potent activity in scavenging DPPH than isoflavones and conjugated soyasaponins. It would be interesting in further research to find specific microorganisms that would be able to cleave the glucuronide bond of glycosylated soyasaponins to release the free aglycones soyasapogenols A and B.

These results showed that in addition to isoflavones, other phytochemicals including soyasaponins, phytosterols, and tocopherols may contribute together to the overall antioxidant properties of soy germ. Although fermentation process results in a slight loss of antioxidant activities, the antioxidant pool of fermented soy germ remains much

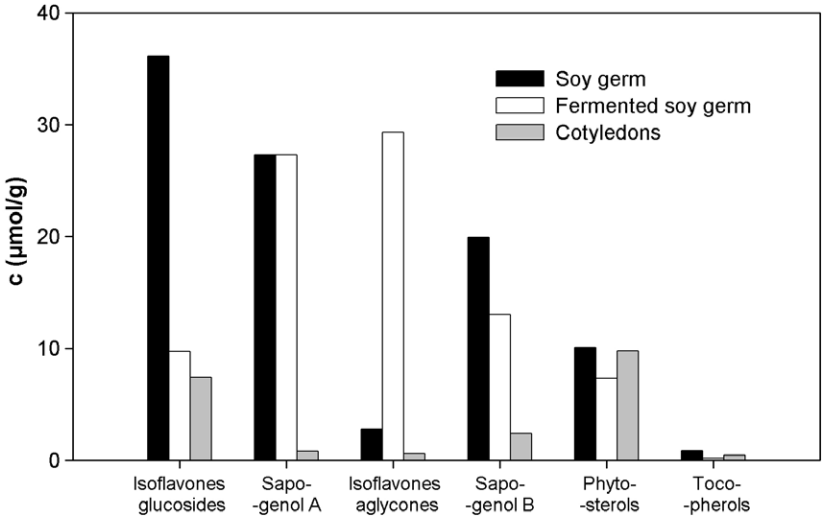


Fig. 6. Comparison of antioxidant phytochemical composition in raw soy germ, fermented soy germ and soy cotyledons. Concentration values related to fermented soy germ correspond to the results obtained with the lactic acid strain B.

higher than the antioxidant pool of the other usual fermented foods made from cotyledons. As shown in Fig. 6, both fermented and non-fermented soy germ exhibit higher levels of antioxidant phytochemicals including isoflavones, soyasaponins, tocopherols and phytosterols compared with cotyledons. Isolated soy germ may be regarded as a suitable natural ingredient for the elaboration of fermented products due to its remarkable content of bioactive compounds and significant antioxidant capacity. In our fermentation conditions, it appeared that a 6 h incubation period of soy germ with the three lactic bacteria tested led to a soy germ extract possessing very interesting characteristics, that is, a low pH reflecting a good stability of the final product and higher levels of bioactive aglycone isoflavones, conserved amounts of soyasaponins, phytosterols and tocopherols, and effective antioxidant properties.

5. Conclusion

Soy germ provides an interesting combination of several potential antioxidant substances that may find some protective applications against the decline of physiological function involving oxidative damage. The fermentation process resulted in considerable changes in the conjugation profile of the glycosylated isoflavones and soyasaponins, thus modifying significantly the biological activity of the fermented extracts. By controlling each step of the process, particularly the incubation time, it should be possible to predict the final content and composition of isoflavones and saponins, but also to prevent the degradation of tocopherols and phytosterols, thus maintaining the antioxidant capacity of the final product.

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