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Fermentation as a Bio-Process To Obtain Functional Soybean Flours

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The effect of fermentation on the antioxidant compounds [vitamins C and E, total phenolic compounds (TPC), and reduced glutathione (GSH)], and antioxidant capacity [superoxide anion scavenging activity (SOD-like activity), peroxyl radical-trapping capacity (PRTC), inhibition of phosphatidylcholine (PC) peroxidation, and Trolox equivalent antioxidant capacity (TEAC)] of soybean (*Glycine max* cv. Merit) was studied. Fermentation was carried out in solid state in cracked seeds inoculated with *Aspergillus oryzae*, *Rhizopus oryzae*, *Bacillus subtilis*, and *Lactobacillus plantarum* and in liquid state either in cracked seeds or milled soybean flours fermented naturally by only the microorganisms present in the seeds or by inoculation with *L. plantarum*. Vitamin C was not detected in the studied samples. Fermentation caused a decrease in vitamin E activity, except when cracked seed was fermented with *A. oryzae*, *R. oryzae*, or *B. subtilis* that increased 31, 30, and 89%, respectively. Fermentation produced an increase in TPC content and did not affect or reduce the GSH content. Fermentation decreased SOD-like activity drastically, while PRTC increased except when it was carried out naturally in cracked seed. TEAC values rose sharply when soybeans were fermented with *B. subtilis.* Processed soybean extracts inhibited PC peroxidation in comparison with the control assay. On the basis of the results obtained, the relative contributions of vitamin E, TPC, and GSH to antioxidant capacity were calculated and results showed a very high TPC contribution and a low contribution of GSH and vitamin E activity. Optimum results for functional soybean flours were achieved when fermentation was carried out with *B. subtilis* inoculum.

KEYWORDS: Soybean; fermentation; SOD-like activity; PRTC; phosphatidylcholine peroxidation; TEAC; vitamin C; vitamin E; total phenolic compounds; reduced glutathione

INTRODUCTION

Soybean has been consumed in Asiatic countries for centuries and is now often included in Western diets, because of its beneficial nutritional effects (*1*). Soybean is an excellent source of protein and presents high level of lipids. Starch content is lower than for other legumes, although it is a good source of dietary fiber and provides vitamins and minerals (*2*, *3*) Soybean is unique among the legumes because it is a concentrated source of isoflavones (*4*, *5*). However, several non-nutritive factors are found in soybean, as well as in other legumes, which can be eliminated or reduced with processes such as soaking, cooking, enzyme addition, germination, and fermentation (*6*–*11*).

Fermentation consists of modifying food by microorganisms (bacteria, molds, and yeasts) that grow and reproduce and consume part of the substrate and enrich it with the products of their metabolism. It is an ancient technology that remains one of the most practical methods for preserving foods and enhancing their nutritional and organoleptic qualities. It is a desirable method for processing and preserving food because of its low cost, low energy requirements, and high yield, with acceptable and diversified flavors for human consumption. The traditional Asian fermented soyfoods, such as soy sauce, tempeh, miso, natto, and tofu largely consumed in Eastern countries, are now also being consumed in the West (*12*, *13*). Fermentation has been reported to cause a general improvement in the nutritional value of legumes, increasing protein digestibility (*14*, *15*), monosaccharide content (*16*–*18*), polyunsaturated fatty acid content (*13*), vitamin B family biosynthesis (*11*, *14*, *18*) and to decrease non-nutritive factors (*7*, *11*, *19*).

Therefore, functional legumes are obtained by reducing non-nutritive factors and increasing nutritional quality. A food can be regarded as functional if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to

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either the state of well-being and health or reduction in the risk of a disease (*20*). One of the properties pursued in functional foods is to increase antioxidant activity, which is attracting a great interest in foods and diets (*21*).

Antioxidants, such as vitamin C, vitamin E, carotenoids, phenolic compounds, and glutathione, are naturally present in vegetables, fruits, grains, and legumes. They have different beneficial functions in the body and the ability to reduce and prevent oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, asthma, hepatitis, liver injury, arthritis, immune deficiency diseases, and ageing (*22, 23*). Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. Methods show extreme diversity but have the advantage of regarding antioxidant capacity as a global characteristic of the product and can be used to characterize the raw material and its evolution during processes. One of the most used method is the Trolox equivalent antioxidant capacity (TEAC) assay based on food antioxidants' inhibition of the absorbance of the radical cation generated from 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (*21, 24*). Some other methods are more specific and try to measure specific radicals, such as peroxyl radicals generated by thermal decomposition of 2,2′-azobis(2-amidopropane)hydrochloride (AAPH) (peroxyl-radical trapping capacity, PRTC) (*25*), whereas determination of SOD-like activity permits the assessment of superoxide anion scavenging activity when a xanthine and xanthine oxidase system is used to originate superoxide radicals (*26*). There are methods to study lipid peroxidation in cell membrane models in vitro, and the antioxidant activity of food extracts in phospholipid bilayers is evaluated by measuring the inhibition of lipid peroxidation in large unilamellar vesicles composed of egg yolk phosphatidylcholine (PC). This method is often used as a model for studying the antioxidant activity in vitro because liposomes can be related to the lamellar structures of biological membranes found in vivo. 2,2′-Azobis(2-amidopropane)hydrochloride is used as a radical generator (*27, 28*).

Since fermented soybeans constitute a diverse range of products from many regions of the world, only a few studies have been carried out concerning the effect of fermentation process in antioxidant compounds and the antioxidant capacity. Therefore, the aim of the present study was to carry out a systematic study to find out the effect of fermentation on the antioxidant compounds and antioxidant capacity of soybean (*Glycine max* cv. Merit) with inoculum using *Aspergillus oryzae*, *Rhizopus oryzae*, *Bacillus subtilis*, and *Lactobacillus plantarum* or without inoculum using the natural seed microflora and further sterilization to determine the optimal conditions to obtain functional soybean flours with high levels of antioxidant capacity.

MATERIALS AND METHODS

Seeds. Soybeans (*G. max* cv. Merit) were provided by the Mang Fong Pacific Trading, S.A. Seeds were cleaned and stored in darkness in polyethylene containers at 4 °C until use.

Preparation of Cultures. *A. oryzae* 2094^T (ATCC 1011), *R. oryzae* CECT 2340 (ATCC 24563), *B. subtilis* CECT 39^T (ATCC 6051) and *L. plantarum* CECT 748^T (ATCC 14917) were purchased from the Spanish type Culture Collection (CECT) and used as inocula. Stock cultures were grown and maintained as follows:

A. oryzae and *R. oryzae* were grown for 7 days on potato dextrose agar (Difco Laboratories, Detroit, MI) at 30 °C, and the spores were collected and washed twice in sterile saline solution and used as inocula.

B. subtilis was grown aerobically in Brain Hearth Infusion (BHI) broth (Difco Laboratories, Detroit, MI) for 18 h at 30 °C. The pelleted cells were washed twice in sterile saline solution and used as inocula.

L. plantarum was grown in deMan Rogosa Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) for 18 h at 30 °C. The cells were washed twice in sterile saline solution (8.5% NaCl) and used as inocula.

Processed Seeds. *Solid-State Fermentations.* They were carried out using cracked soybeans (100 g) suspended in sterile distilled water (1:2 w/v) for 16 h and subsequently autoclaved at 121 °C for 15 min. The sterilized cracked seeds were inoculated with 5% (v/w) of the above cultures as follows: *A. oryzae* (10^5 spores/g), *R. oryzae* (10^5 spores/g), or *B. subtilis* (10^5 CFU/g). The suspensions were aseptically distributed over Petri dishes containing 30 g of beans and placed in a climatic incubator (Memmert, Germany) controlled at 30 °C and 90% relative humidity for 48 h. After fermentation, the fermented cracked beans were autoclaved at 121 °C for 15 min and freeze-dried. Solid-state fermentations were performed in triplicate.

Liquid-State Fermentations. They were carried out either in milled soybean flours (sieved at 0.250–0.5 mm) or in cracked soybeans. Suspensions were prepared in sterile distilled water (200 g/L) and were allowed to ferment spontaneously with the only microorganisms present on the seeds (natural fermentation) or were inoculated with 10% (v/v) of *L. plantarum* suspension (10^8 CFU/mL). Fermentation was carried out in a 2 L stirred fermentor (Infors AG, Switzerland) at 350 rpm for 48 h at 37 °C. After fermentation, the samples were autoclaved at 121 °C for 15 min and freeze-dried. Liquid-state fermentations were performed in duplicate.

Microbiological Analysis. Liquid-state fermentations were monitored by withdrawing samples before inoculation (0 h) and at the end of the fermentation (48 h), using plate counts to determine changes in viable cells of the following microorganisms: lactic acid bacteria were counted in MRS agar plates after incubation in an 5% CO_2 atmosphere during 72 h; aerobic mesophilic bacteria were grown in plate-count agar (Difco) incubated at 30 °C during 72 h; yeasts and molds were enumerated on chloramphenicol/bromophenol-blue agar after incubation at 25 °C for 5 days; *Enterobacteriaceae* were counted on violet red bile glucose (VRBG) agar (Oxoid) plates incubated at 30 °C for 24 h, the purple-red colonies being considered *Enterobacteriaceae*; and sulfite-reducing clostridia were determined in SPS agar (Difco) by incubating under anaerobic conditions at 37 °C for 48 h.

Chemical Analysis. The vitamin C content was determined by capillary electrophoresis using a P/ACE system 2050 (Beckman Instruments, Fullerton, CA, USA) and UV detection at 254 nm (*29*).

Analysis of tocopherol isomers (α-, β-, γ-, and δ-tocopherol) was carried out by HPLC according to Frias et al., (*29*) using a Water 470 scanning fluorescence detector (Water Associates, Mildford, CT, USA) at $\lambda_{exc} = 296$ nm and $\lambda_{em} = 320$ nm. Vitamin E activity was calculated as α-TE/100 g of dry matter (*30*).

The content of total phenolic compounds (TPC) was determined according to Fernandez-Orozco et al. (*31*). TPC from samples extracted using 80% methanol (1/10 m/v) for 2 h at room temperature and then extracts were centrifuged at 4000 rpm with MPW-360 centrifuge (Factory of precise mechanics, Warsaw, Poland). The method is based on the colour reaction of Folin-Ciocalteu reagent with hydroxyl groups. Absorbance was measured at 725 nm using a spectrophotometer (UV-160 1PC, Shimadzu, Japan).

The extraction and analysis of reduced (GSH) and oxidized (GSSG) glutathione was conducted according to Fernandez-Orozco et al. (*31*). Since soybeans contain a high lipid content, samples were submitted to a previous extraction with HPLC-grade *n*-heptane until lipid content was removed (extract should remain clear and not opaque). GSH and GSSG were determined with a spectrofluorometric method based on the reaction of *o*-phthalaldehyde (OPT) as a fluorescent reagent with GSH at pH 8.0 and GSSG at pH 12.0. GSH was complexed to *N*-ethylmaleimide (NEM) to prevent interference of GSH with measurement of GSSG. The fluorescence was measured at $\lambda_{em} = 420$ nm and $\lambda_{exc} = 350$ nm in a luminescence spectrometer (LS 50B Perkin Elmer). The GSH/GSSG ratio was also calculated.

Table 1. Changes in Viable Cell Count during Liquid-State Fermentations of *Glycine max* cv. Merit

Glycine max cv. Merit	fermentation[b]	fermentation time (h)	population (log_{10} CFU/mL)[a] lactic acid bacteria	aerobic mesophilic bacteria	yeasts and molds[c]	enterobacteria	sulfite-reducing clostridia
flour	NF	0	2.90 ± 0.14	3.45 ± 0.21	<2	<1	<1
		48	10.45 ± 0.63	11.45 ± 0.63	<2	<1	<1
	LP	0	3.15 ± 0.21	3.40 ± 0.14	<2	<1	<1
		48	7.50 ± 0.00	7.65 ± 0.21	<2	<1	<1
cracked seed	NF	0	3.30 ± 0.42	3.60 ± 0.14	<2	<1	<1
		48	10.00 ± 0.00	10.38 ± 0.60	2.75 ± 0.35	<1	<1
	LP	0	2.80 ± 0.28	3.25 ± 0.35	<2	<1	<1
		48	9.50 ± 0.70	9.65 ± 0.91	<2	<1	<1

[a] Mean value ± standard deviation of two fermentations. [b] NF = natural fermentations; LP = fermentations inoculated with *L. plantarum*. [c] Indicates that the dilution shown was the lowest dilution tested.

SOD-like activity determinations were carried out according to Fernandez-Orozco et al. (*31*) Samples were extracted in PBS and then treated with HPLC-grade *n*-heptane to remove lipid content. The SOD-like activity of raw and fermented seed extracts was measured with the SOD Ransod kit (Ransod, Cat. No. SD 125, Randox Laboratories Ltd., Antrim, UK). The assays were performed using a thermostated recording spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu) adjusted to 37 °C inside the chamber. The test required 25 μL of sample, with a read time of 3 min. The superoxide dismutase activity was measured by the degree of inhibition of this reaction at 505 nm.

For PRTC determinations, raw and fermented seeds were extracted with 80% aqueous methanol (1/10 w/v) on an electromagnetic stirrer with continuous mixing for 2 h at ambient temperature in the dark. The assay was carried out according to Fernandez-Orozco et al. (*31*), and thermal decomposition of ABAP (2,2′-azobis(2-amidopropane)hydrochloride) is the source of free radicals (peroxyl radicals) in this method. Spectrophotometric measurements were obtained at 37 °C and 414 nm.

TEAC assays were carried out according to Fernandez-Orozco et al. (*31*). The antioxidant capacity of phosphate buffer saline extracts of raw and fermented soybean products was determined using potassium persulfate as a free radical generator and spectrophotometric measurements at 734 nm at 37 °C for 10 min.

To determine peroxidation in unilamellar liposomes soy products extracts were prepared in 80% methanolic using the method described by Fernandez-Orozco et al. (*31*). A model system consisting of unilamellar liposomes of egg yolk PC and the water-soluble azo compound AAPH as a free radical generator was used. The amount of phosphatidyl choline peroxides (PC-OOH) formed at 37 °C during 7 h of incubation was determined at 235 nm at 60 min intervals by the HPLC method using the Shimadzu system, C_8 Nova Pack column, and methanol–water mixture (96:4 v/v) as a mobile phase. The amount of peroxides was calculated from the standard curve prepared with PC-OOH according to the method described by Frias et al., (*29*). The antioxidant capacity in this in vitro system was calculated as inhibition of peroxidation according to the equation of Azuma et al. (*32*), as follows:

$$\frac{[\text{PC-OOH}_p] - [\text{PC-OOH}_{inh}] \times 100}{[\text{PC-OOH}_p]} \quad (1)$$

where $[\text{PC-OOH}_p]$ is the concentration of PC-OOH after the reaction for 2 h without a soybean extract and $[\text{PC-OOH}_{inh}]$ is the concentration of PC-OOH after the reaction for 2 h with soybean extract.

Statistical Methods. Data were subjected to multifactor ANOVA using the least-squared difference test with the Statgraphic 4.0 for Windows Program (Statistical Graphics Corporation, Rockville, MD, USA), and multiple correlations were obtained using Statistica 5.1 Program (Statsoft, Tulsa, OK 74104 USA) for Windows using a PC.

RESULTS AND DISCUSSION

Soybean flour or cracked seeds (*G. max* cv. Merit) were processed either by solid or liquid-state fermentations. Afterwards, the fermented samples were sterilized by autoclaving, and the effect of processing on the content of antioxidant compounds and antioxidant capacity measured by different methods was studied.

Table 1 shows the microbial population present in the liquid-state fermentations carried out naturally (NF) or by using *L. plantarum* as inoculum (LP). Lactic acid bacteria (LAB) and aerobic mesophilic bacteria increased after 48 h of fermentation. However, yeast and mold growth was inhibited during soybean fermentation, with the exception of the NF of cracked soybeans, which reached 2.75 CFU/mL after 48 h. In fermentations inoculated with *L. plantarum*, microscopic observations of the isolated colonies revealed that these LAB were able to dominate over the microbial population present naturally in soybeans. The count of *Enterobacteriaceae* and sulfite-reducing clostridia was lower than 1 CFU/mL in all the LAB soybean fermentations.

Table 2 shows the effect of fermentations on the content of vitamin C, tocopherol isomers, and vitamin E activity of soybean. Vitamin C was not detected either in raw soybean or in the fermented products. These results coincide with those reported by Doblado et al. (*21*), Frias et al. (*29*), and Torres et al. (*33*), who did not find vitamin C in raw and fermented cowpeas, lupins, and pigeon peas, respectively. Different authors have found vitamin C content in soybean and legume grains ranging from none detected to 10 mg/100 g d.m. (*21, 29, 33–38*), and fermentation processes cause noticeable reductions (*21, 29*). In raw seeds, the γ-tocopherol was the main isomer (4.1 mg/100 g d.m.), followed by δ- and α-tocopherol (1.65 and 1.0 mg/100 g d.m., respectively), while β-tocopherol was present in a minor amount (0.25 mg/100 g d.m.). The fermentation process affected the isomers differently depending on the conditions. While solid-state fermentations brought about a sharp increase in δ-, γ-, and β-tocopherols (709–884, 166–275, and 32–128%, respectively), while for α-tocopherol reductions between 21 and 84% were observed. As a consequence of the modifications in tocopherol isomers, vitamin E activity increased significantly ($P \leq 0.05$) after solid-state fermentation (31, 30, and 89%, for *A. oryzae*, *R. oryzae*, and *B. subtilis*, respectively). However, when the process was carried out in the fermentor (liquid-state fermentation NF or LP), α-tocopherol suffered a sharp reduction (99%), β- and γ-tocopherol decreased slightly (12–16 and 16–19%, respectively) but δ-tocopherol increased sharply (259–274%). Vitamin E activity, however, decreased notably (around 60%) in natural fermentations and in *L. plantarum* fermentations carried out either with cracked or with milled soybeans, mainly due to the drastic reduction that occurred in α-tocopherol. Comparing the vitamin E data of liquid-state fermentation between flour and cracked seeds, no significant ($P \leq 0.05$) differences were found.

Table 2. Effect of Fermentation on the Vitamin C and Vitamin E Content of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	vitamin C (mg/100 g d.m.)	tocopherols (mg/100 g d.m.)				Vitamin E activity[b]
		α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	(α-TE/100 g d.m.)
raw	ND	1.00 ± 0.05[e]	0.25 ± 0.02[b]	4.10 ± 0.02[b]	1.65 ± 0.05[a]	1.59 ± 0.02[b]
Solid-State Fermentation						
Aspergillus oryzae (cracked seed)	ND	0.16 ± 0.00[b]	0.32 ± 0.01[c]	12.79 ± 0.33[d]	15.82 ± 0.28[d]	2.08 ± 0.05[c]
Rhizopus oryzae (cracked seed)	ND	0.32 ± 0.01[c]	0.35 ± 0.02[d]	10.89 ± 0.21[c]	16.24 ± 0.35[e]	2.07 ± 0.04[c]
Bacillus subtilis (cracked seed)	ND	0.79 ± 0.02[d]	0.57 ± 0.02[e]	15.37 ± 0.13[e]	13.35 ± 0.20[c]	3.01 ± 0.02[d]
Liquid-State Fermentation						
Lactobacillus plantarum (cracked seed)	ND	0.01 ± 0.00[a]	0.22 ± 0.01[a]	3.34 ± 0.05[a]	5.93 ± 0.26[b]	0.63 ± 0.02[a]
Lactobacillus plantarum (flour)	ND	0.01 ± 0.00[a]	0.22 ± 0.01[a]	3.33 ± 0.10[a]	6.17 ± 0.24[b]	0.63 ± 0.02[a]
natural fermentation (cracked seed)	ND	0.01 ± 0.00[a]	0.21 ± 0.01[a]	3.41 ± 0.05[a]	6.07 ± 0.10[b]	0.63 ± 0.01[a]
natural fermentation (flour)	ND	0.01 ± 0.00[a]	0.21 ± 0.01[a]	3.43 ± 0.07[a]	6.17 ± 0.05[b]	0.64 ± 0.01[a]

[a] Mean value ± standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$). ND = not detected. [b] Vitamin E activity (mg of αTE/100 g d.m.) = (mg of α-tocopherol × 1.0) + (mg of β-tocopherol × 0.5) + (mg of γ-tocopherol × 0.1) + (mg of δ-tocopherol × 0.03).

Table 3. Effect of Fermentation on the Total Phenolic Compounds (TPC) Content of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	TPC (mg of catechin/g d.m.)
raw	2.98 ± 0.06[a]
Solid-State Fermentation	
Aspergillus oryzae (cracked seed)	3.54 ± 0.12[b]
Rhizopus oryzae (cracked seed)	3.43 ± 0.08[b]
Bacillus subtilis (cracked seed)	12.50 ± 0.26[f]
Liquid-State Fermentation	
Lactobacillus plantarum (cracked seed)	5.40 ± 0.03[d]
Lactobacillus plantarum (flour)	5.51 ± 0.08[de]
natural fermentation (cracked seed)	4.98 ± 0.19[c]
natural fermentation (flour)	5.68 ± 0.07[e]

[a] Mean value ± standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$).

Vitamin E, a major biological antioxidant, quenches free radicals and acts as a terminator of lipid peroxidation, especially in membranes that contain highly unsaturated fatty acids (*39*). The results in tocopherols obtained in the present study for raw seeds are higher than those found by Zielinski (*38*) in *G. max* cv. Mazovia seeds. In relation to the vitamin E activity of other legumes, data found show a wide range of values, from 0.09 mg of α-TE/100 g d.m. in *Phaseolus vulgaris* (*40*) to 11.5 mg of α-TE/100 g d.m. in *Lupinus angustifolius* cv. Emir (*41*).

In okara koji, a product from soybean fermented with *A. oryzae*, γ- and δ- tocopherol were found, but α-tocopherol was not detected (*42*). Esaki et al. (*43*) reported that in miso, a product from soya fermented with *A. oryzae*, α-tocopherol content decreased, whereas it was not modified with *B. subtilis* (natto) or with *R. oligosporus* (tempeh). Denter et al. (*44*) observed that in tempeh the tocopherols content slightly decreased as a consequence of fermentation with 14 varieties of *Rhizopus* studied, while the vitamin E activity was kept almost constant. Frias et al. (*29*) reported an increase in α-tocopherol and reductions in γ- and δ-tocopherol when *Lp. albus* var. Multolupa seeds were fermented naturally or with *L. plantarum*. These authors also reported sharp reductions in vitamin E activity. Doblado et al. (*21*) found that natural or *L. plantarum* fermentations of *Vigna sinensis* var. Carilla seeds produced a sharp decrease in γ- and δ-tocopherol contents, and they also observed that fermentation originated β-tocopherol, and because of the contribution of β-tocopherol, the vitamin E activity increased notably (60%) in *L. plantarum* fermentation.

Table 3 shows the content of TPC in raw and fermented soybean products. Solid-state fermentation carried out with *B. subtilis* caused an increase of 319% in TPC, although for those carried out with *A. oryzae* and *R. oryzae* the TPC levels only increased by 19 and 15%, respectively. When liquid-state fermentation was performed naturally or with *L. plantarum* the increase observed in TPC ranged from 67 to 91% (**Table 3**). The particle size in natural fermentation affected significantly ($P \leq 0.05$) the TPC content, and higher values were found when it was carried out with flour. The TPC content in *G. max* cv. Merit seeds is in agreement with the range (1.9–5.7 mg/g d.m.) obtained from the bibliography for different legumes (*37*, *38*, *45–47*). It is interesting to consider that the TPC content appears to be closely related to the color of the seeds. Takahashi et al. (*48*) and Lin and Lai (*49*) showed that dark seeds of *G. max, V. radiata*, and *V. angularis* provided a higher amount of TPC than seeds with a lighter hull.

The results concerning the effect of fermentation on the TPC content of *G. max* cv. Merit match up with results published by other authors. Chiou and Cheng (*50*) observed an increase in daidzein and genistein content when *G. max* was fermented with *A. oryzae* to prepare koji. This is in agreement with results reported by other authors (*51, 52*). Tabera et al. (*53*) and Bartolomé et al. (*54*) reported an increase in phenolic compounds after fermentation of *Lens culinaris*. Dueñas et al. (*55*) studied the effect of *L. plantarum* fermentation on the content of phenolic compounds in *V. sinensis* flours, and they suggest fermentation as an adequate and effective process for increasing nutritional and biological quality owing to the improvement in the concentration of phenolic compounds. These results could occur because fermentation hydrolyzes complexes of polyphenols to other simpler and biologically more active ones.

The contents of GSH and GSSG in raw and fermented *G. max* cv. Merit are recorded in **Table 4**. In soybean seeds, these values were 3.5 and 0.6 μmol/g d.m., respectively, and the GSH/GSSG ratio was 5.7. After soybean fermentation, the GSH content decreased by 34, 50, and 36% when fermentation was carried out with *A. oryzae*, *R. oryzae*, and *B. subtilis* inoculum, respectively, and by 25% when cracked seeds were fermented naturally. Nevertheless, the GSSG content increased by 39% with *A. oryzae* fermentation, 61–62% with natural fermentation, 75–85% with *L. plantarum* fermentation, 107% with *R. oryzae* and 136% with *B. subtilis*. Natural fermentation of soybean flour showed higher GSH than the natural fermentation of cracked seeds ($P \leq 0.05$). The GSH/GSSG ratio dropped as a consequence of the processing.

Little information has been found about the glutathione contents in raw and processed legumes. Zielinski (*38*) determined GSH and GSSG levels in raw *G. max* var. Mazovia, and he found values of 3.3 and 0.2 μmol/g d.m., respectively. Fernandez-Orozco et al. (*37*) determined GSH content in four

Table 4. Effect of Fermentation on the Reduced and Oxidized Glutathione Content of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	GSH (μmol/g d.m.)	GSSG (μmol/g d.m.)	GSH/GSSG
raw	3.51 ± 0.09^{cd}	0.61 ± 0.01^{a}	5.71 ± 0.26^{d}
Solid-State Fermentation			
Aspergillus oryzae (cracked seed)	2.33 ± 0.35^{ab}	0.85 ± 0.10^{b}	2.76 ± 0.27^{b}
Rhizopus oryzae (cracked seed)	1.76 ± 0.28^{a}	1.26 ± 0.06^{f}	1.40 ± 0.25^{a}
Bacillus subtilis (cracked seed)	2.24 ± 0.24^{a}	1.44 ± 0.12^{g}	1.56 ± 0.14^{a}
Liquid-State Fermentation			
Lactobacillus plantarum (cracked seed)	3.30 ± 0.21^{cd}	1.13 ± 0.02^{e}	2.92 ± 0.16^{b}
Lactobacillus plantarum (flour)	3.60 ± 0.19^{d}	1.07 ± 0.02^{de}	3.36 ± 0.12^{c}
natural fermentation (cracked seed)	2.64 ± 0.21^{b}	0.98 ± 0.02^{c}	2.70 ± 0.23^{b}
natural fermentation (flour)	3.20 ± 0.30^{c}	0.99 ± 0.03^{cd}	3.24 ± 0.22^{c}

[a] Mean value $\pm$ standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$).

Table 5. Effect of Fermentation on the SOD-like Activity of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	SOD-like activity (U SOD/g d.m.)
raw	556.6 ± 5.7^{f}
Solid-State Fermentation	
Aspergillus oryzae (cracked seed)	ND[a]
Rhizopus oryzae (cracked seed)	2.1 ± 0.2^{b}
Bacillus subtilis (cracked seed)	ND[a]
Liquid-State Fermentation	
Lactobacillus plantarum (cracked seed)	13.2 ± 0.2^{d}
Lactobacillus plantarum (flour)	15.5 ± 1.0^{e}
natural fermentation (cracked seed)	8.8 ± 0.4^{c}
natural fermentation (flour)	7.8 ± 1.5^{c}

[a] Mean value $\pm$ standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$). ND = not detected.

Table 6. Effect of Fermentation on the Peroxyl-Radical Trapping Capacity (PRTC) of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	PRTC (μmol of Trolox/g d.m.)
raw	2.54 ± 0.62^{a}
Solid-State Fermentation	
Aspergillus oryzae (cracked seed)	4.59 ± 0.03^{d}
Rhizopus oryzae (cracked seed)	6.77 ± 0.39^{e}
Bacillus subtilis (cracked seed)	9.23 ± 0.20^{f}
Liquid-State Fermentation	
Lactobacillus plantarum (cracked seed)	3.60 ± 0.04^{b}
Lactobacillus plantarum (flour)	4.25 ± 0.09^{c}
natural fermentation (cracked seed)	2.22 ± 0.11^{a}
natural fermentation (flour)	3.40 ± 0.08^{b}

[a] Mean value $\pm$ standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$).

lentil varieties, and the range obtained was 3.2–4.8 μmol/g d.m. Doblado et al. (*21*) reported for raw *V. sinensis* var. Carilla contents of 3.3 μmol/g d.m. for GSH and 0.6 μmol/g d.m. for GSSG. Fernandez-Orozco et al. (*31*) presented values of 4.5 μmol/g d.m. for GSH and 1.2 μmol/g d.m. for GSSG. The bibliography shows that the GSH content (antioxidant agent) is significantly higher (4–8 times) than the GSSG content, in agreement with our data.

Very few references have been found about the effect of legume fermentation on the glutathione content. Doblado et al. (*21*) reported that natural and *L. plantarum* fermentation carried out in *V. sinensis* conducted caused a decrease in the GSH, whereas levels of GSSG increased and the GSH/GSSG ratio decreased. GSH is a tripeptide which, apart from taking part in oxidative processes, can be metabolized by proteases belonging to the microorganisms that intervene in the fermentation processes. Thus, the GSH content after fermentation depends on the type of fermentation and legume utilized.

Table 5 shows the effect of fermentation on the SOD-like activity of soybean products. Raw seeds showed levels of 556.6 U SOD/g d.m., and all the types of fermentation carried out caused a drastic decrease in SOD-like activity (97–99%), which was even not detected when cracked seeds were inoculated with *A. oryzae* and *B. subtilis*. The particle size affected SOD-like activity in liquid-state fermentations only when fermentation was carried out with *L. plantarum*, and it was higher when the process was carried out with flour. Little information has been published about SOD-like activity in raw soybeans, and our results coincide with those obtained by Bamforth (*56*). In other legumes, Fernandez-Orozco et al. (*57*), Doblado et al. (*21*), and Fernandez-Orozco et al. (*31*) reported values of SOD-like activity of different varieties of *Lens culinaris*, *V. sinensis*, and *Lupinus angustifolious* ranging from 3.3 to 5.8 U SOD/mg of protein.

The results obtained for SOD-like activity in our work for fermented soybean are in agreement with those reported by Doblado et al. (*21*) for fermented cowpea. However, Wang et al. (*58*) observed that fermentation of soybean milk caused an increase of 43–65% in SOD-like activity, depending on the inoculum used (*L. acidophilus* CCRC 14079, *Streptococcus thermophilus* CCRC 14085, *Bifidobacterium infantis* CRC 14633 or *Bf. longum* CCRC B6). These results suggest that during fermentation microorganisms involved in the process present different proteolitic activities that are responsible for the SOD-like activity of the final fermented product.

Table 6 shows the PRTC values for raw and processed seed. The PRTC of raw seeds (2.5 μmol of Trolox/g d.m.) increased considerably after induced fermentation, and higher values were obtained with *R. oryzae* and *B. subtilis* (6.8 and 9.2 μmol of Trolox/g d.m., respectively) than when samples were inoculated with *A. oryzae* or *L. plantarum* (4.6, 3.6–4.2 μmol of Trolox/g d.m., respectively). In naturally fermented soybean products, only when this was carried out with soybean flour was a 34% increase ($P \leq 0.05$) in PRTC observed. Liquid-state fermentation performed with soybean flour conducted to higher PRTC values that those carried out with cracked seeds. Zielinski (*25*) showed values of PRTC from 2.9 μmol of Trolox/g d.m. in seeds of *G. max* to 7.3 μmol of Trolox/g d.m. in raw *Vicia sativa*. In different varieties of raw lentils, Fernandez-Orozco et al. (*36*) and Zielinski (*25*) reported values from 5.0 to 16.1 μmol of Trolox/g d.m. Doblado et al. (*21*) and Fernandez-Orozco et al. (*31*) found PRTC values of raw V*igna sinensis* var. Carilla and *Lupinus angustifolious* var. Zapaton of 6.6 and 2.2 μmol of Trolox/g d.m., respectively. Concerning fermentation, different results were reported by Doblado et al. (*21*), and they found a

Table 7. Effect of Fermentation on the Trolox Equivalent Antioxidant Capacity (TEAC) of *Glycine max* cv. Merit[a]

Glycine max cv. Merit	TEAC (μmol of Trolox/g d.m.)
raw	63.03 ± 0.36^{e}
Solid-State Fermentation	
Aspergillus oryzae (cracked seed)	38.03 ± 1.41^{a}
Rhizopus oryzae (cracked seed)	46.70 ± 1.24^{b}
Bacillus subtilis (cracked seed)	131.15 ± 3.89^{f}
Liquid-State Fermentation	
Lactobacillus plantarum (cracked seed)	57.43 ± 1.51^{d}
Lactobacillus plantarum (flour)	54.54 ± 1.22^{c}
natural fermentation (cracked seed)	44.58 ± 1.14^{b}
natural fermentation (flour)	57.63 ± 1.34^{d}

[a] Mean value ± standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$).

decrease in PRTC values after cowpea fermentation with *L. plantarum* or naturally.

Table 7 shows the effect of fermentation on the TEAC. The TEAC content of raw soybean seeds was 63 μmol of Trolox /g d.m. Fermentation with *B. subtilis* caused a sharp rise in TEAC of 108%. However, fermentation produced a notable decrease when performed naturally (9–29%) or with inocula *A. oryzae*, *R. oryzae*, or *L. plantarum* (40, 26, and 9–13%, respectively). When cracked soybeans were fermented with *L. plantarum*, the content of TEAC was higher than those carried out with flour; however, in naturally fermented soybeans, the use of flour caused higher TEAC than cracked seeds. In other raw legume seeds, Fernandez-Orozco et al. (*37*) observed that TEAC from different varieties of *Lens culinaris* ranged between 13 and 23 μmol of Trolox/g d.m. Doblado et al. (*21*), Frias et al. (*29*), Fernandez-Orozco et al. (*31*), and Torres et al. (*59*) found TEAC values of 25.4 μmol of Trolox/g d.m. for *V. sinensis* var. Carilla, of 71.4 μmol of Trolox/g d.m. for *Lupinus albus* var. *multolupa*, of 43.04 μmol of Trolox/g d.m. for *Lupinus angustifolius* var. Zapaton and of 33.2 μmol of Trolox/g d.m. for *Cajanus cajan*, respectively.

Torres et al. (*33*) observed that fermentation of *Cajanus cajan* seeds caused a decrease in TEAC of 4%. Frias et al. (*29*) reported that natural fermentation of whole *Lupinus albus* var. Multolupa seeds produced a reduction in TEAC of 23%, which did not change significantly ($P \leq 0.05$) when this was carried out with lupin flours. However, when lupin flour was inoculated with *L. plantarum* a slight but significant ($P \leq 0.05$) rise (5%) was observed. Pyo et al. (*52*) studied the effect of lactic fermentation of soybean to enhance antioxidant properties, and they concluded that fermentation enhanced the capacity to scavenge DPPH and ABTS radicals. Similar results were observed by Doblado et al. (*21*) in *V. sinensis* var. Carilla. These results indicate that during legume fermentation different changes occur in TEAC that depend on the type of legume, the fermentation process, and the microorganisms involved.

The assay of the inhibition of PC peroxidation was carried out in 80% methanolic extracts of raw soybeans and of the solid-state fermentation inoculated with *B. subtilis* (BS) and of the liquid-state fermentation in soybean flour inoculated with *L. plantarum* (LPF) or naturally (NFF). The results are shown in **Figure 1**. **Table 8** shows the peroxidation inhibition percentage of these extracts after 2 h of incubation with AAPH radical, according to Azuma et al. (*32*). The formation of PC peroxides (PC-OOH) was rather lower for the fermented soybean products than for control (80% methanol), mainly when fermentation was carried out with *B. subtilis* (**Figure 1**). Therefore, the antioxidant activity of these extracts, presented by the inhibition percentage

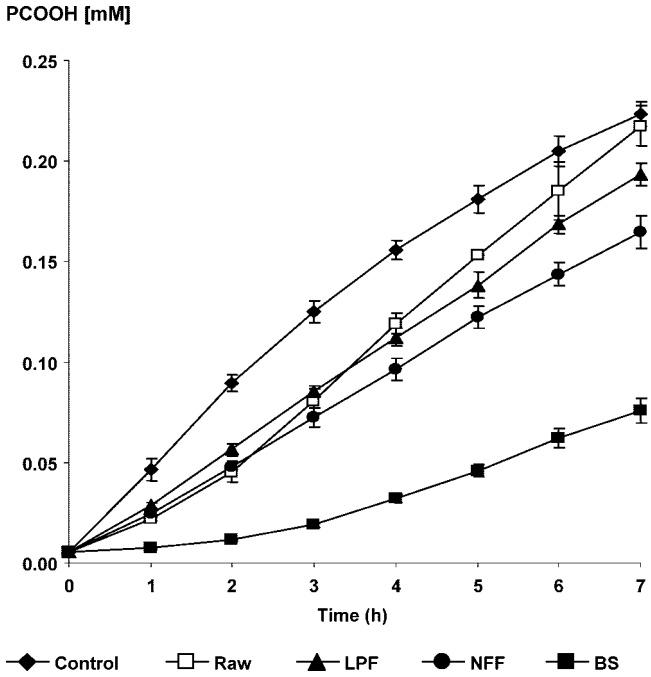

Figure 1. Inhibition by raw and fermented *Glycine max* cv. Merit extracts of AAPH-initiated peroxidation of PC liposomes.

Table 8. Antioxidative Activity of Raw and Fermented *Glycine max* cv. Merit in a Liposomal PC Suspension System[a]

Glycine max cv. Merit	antioxidant activity (% inhibition)[b]
raw	46.92 ± 4.54^{b}
Solid-State Fermentation	
Bacillus subtilis (cracked seed)	87.42 ± 0.56^{c}
Liquid-State Fermentation	
Lactobacillus plantarum (flour)	38.44 ± 3.57^{a}
natural fermentation (flour)	48.77 ± 1.00^{b}

[a] Mean value ± standard deviation. Different letters in the same column mean significant difference ($P \leq 0.05$). [b] Against control (extract without sample).

of PC peroxidation after 2 h (**Table 8**), was higher for *B. subtilis* (87.4%), while for raw seeds, LP and NF was 46.9, 38.4, and 48.7%, respectively. The results obtained in the present paper show that fermentation with *B. subtilis* gives rise to antioxidant metabolites that could take part in lipid peroxidation inhibition. It is worth emphasizing that the largest rise in TPC was observed in this kind of fermentation, and there would seem to exist a close relationship between these antioxidant compounds and lipid peroxidation inhibition of PC in vitro, as suggested by Azuma et al. (*32*).

Most of the studies found in the literature about inhibition of PC peroxidation are based on model systems to evaluate antioxidant capacity using pure compounds such as epicatechin, epicatechin gallate, quercetin, or vitamin E (*28*, *60*). Nevertheless, few studies have focused on peroxidation inhibition using food matrix, such as raw and processed legume seeds (*21*, *29*, *31*, *61*).

Little information has been found about lipid peroxidation inhibition in fermented samples. Results reported by Frias et al. (*29*) are in agreement with our data, and these authors observed that natural and *L. plantarum* fermentation in *Lupinus albus* var. Multolupa caused slight changes in the inhibition of lipid peroxidation compared with raw seeds. However, Doblado

et al. (*21*) found that fermented *V. sinensis* var. Carilla flours, naturally or with *L. plantarum*, showed a higher inhibition than raw seeds.

In this work has also been studied the relative contribution of phenolic compounds, tocopherols, and GSH to the antioxidant capacity of raw and processed *G. max* cv. Merit. To do calculations, the following TEAC values of individual compounds were taken: 2.40 μmol/g d.m. for catechin (*62*), 0.97 μmol/g d.m for α-tocopherol, 0.90 μmol/g d.m for GSH, and 0.99 μmol/g d.m. for vitamin C (*63*). The relative contribution of individual studied antioxidant compounds to the total antioxidant capacity showed a marked high contribution of TPC in raw soybeans (89%), whereas GSH and vitamin E activity contributed about 11 and 0.1%, respectively. In fermented soybean flours, the contribution of TPC to the total antioxidant capacity was higher (93–98%), whereas the contribution of GSH decreased (2–7%) and the vitamin E contribution was rather low (0.03–0.2%).

Correlations among the antioxidant compounds analyzed (GSH, vitamin E, and TPC) and the data for antioxidant capacity obtained (SOD-like activity, PRTC, inhibition of PC peroxidation, and TEAC) were performed. Correlations after fermentation were as follows: a negative correlation between GSH content with vitamin E activity and TPC was observed ($r = -0.77$ and $r = -0.90$, respectively) ($P \leq 0.05$), while there was a positive correlation between TPC content and vitamin E activity ($r = 0.67$) ($P \leq 0.05$). GSH content and vitamin E activity of samples correlated with PRTC, inhibition of PC peroxidation, and TEAC ($r = -0.89$ and 0.74; $r = -0.93$ and 0.90; $r = -0.91$ and $r = 0.92$, respectively) ($P \leq 0.05$). Results indicated that there was a negative correlation between PRTC and SOD-like activity ($r = -0.60$) ($P \leq 0.05$) and a positive one between PRTC and inhibition of PC peroxidation ($r = 0.90$) ($P \leq 0.05$). Furthermore, TEAC correlated positively with PRTC and inhibition of PC peroxidation ($r = 0.93$, $r = 0.98$, respectively) ($P \leq 0.05$).

In conclusion, fermentation with *B. subtilis* inoculum gave optimum results and could be recommended to obtain functional soybean flours with a high content of antioxidant compounds and antioxidant capacity. When fermentation was performed with *B. subtilis* inoculum, vitamin E activity, TPC, inhibition of PC peroxidation, PRTC, and TEAC increased 89, 319, 87, 263, and 108%, respectively.

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