Bioactive phenolic compounds of soybean (*Glycine max* cv. Merit): modifications by different microbial fermentations

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Running title: Changes in phenolic compounds of soybeans by microbial fermentations

Abstract

Phenolic compounds of soybeans (*Glycine max*) are partially responsible of their beneficial health effects. The most abundant phenolics in soybeans are isoflavones, mainly daidzein, glycitein, and genistein and several derivatives of them. Other phenolic compounds are also present but they have been minimally studied. In this work, the soybean seeds were fermented by the microbiota present on the seeds or by inoculation with *Aspergillus oryzae, Rhizopus oryzae* or *Bacillus subtilis*. The effect of microbial fermentation on the phenolic composition of soybeans was studied. All the assayed microorganisms brought out variations in phenolics. An increase in the concentration of phenolic acids was observed. Flavonoids were differently metabolized depending on the microorganism used. In all samples the most important changes were produced in the isoflavones content, observing an increase on the aglycones, glycitein, daidzein and genistein.

Key words: soybean, fermentation, phenolic compounds, isoflavones

1. Introduction

Soybean (*Glycine max* cv. Merit*)* is a good source of proteins as well as of bioactive compounds, such as vitamins, carotenoids and phenolics. The benefits of soybean based foods for human health are well known, and nowadays the demand for soybean products has increased because the renewed interest in functional food. Soybeans contain phenolic compounds, non flavonoids and flavonoids, among them the most abundant are isoflavones, which health benefits are well recognized in the world. Isoflavones are present mainly as glucosides, acetylglucosides and malonylglucosides of genistein, daidzein and glycitein. Isoflavone conjugates content vary considerably in soybean depending on different factors, such as cultivars, cultivation year, geographical and environmental conditions, maturity, etc. (Otieno & Shah, 2007a; Ribeiro et al., 2007). The conjugate glycosides are not bioavailable on this form (Setchell et al., 2002) and they need to be hydrolysed releasing isoflavone aglycones, which are more bioactive forms as they could be absorbed by the intestinal microbiota (Setchell et al., 2003). The initial content in the glucosides, daidzin, glycitin and genistin of soybeans are not affected by temperature bellow 135 ºC, in the absence of other factors, but the presence of glycosidase enzymes, releases the corresponding aglycones (Park, Alenscar, Aguiar, Mascrenhas & Scamparini, 2002; Xu, Wu & Godber, 2002).

There are several forms to consume soybeans such soybean sprouts, soymilk, tofu and as ingredients in cultural cuisine (Kim, Kim, Chung, Chi, Kim, Chung, 2006a). These food products are obtained by different soybean process which could modify the isoflavone conjugated content. The knowledge that conjugated forms have less biological effect that aglycones has stimulated the developing of technological processes to obtain soybean products rich in isoflavone aglycones by different treatments such as addition of enzymes (Park, Lui, & Aguiar, 2003) or by the action of different microorganims (Tsangalis, Ashton, McGill & Shah, 2002; Cho et al., 2009).

57 Fermentation is an ancient technology that remains one of the most practical methods for
58 enhancing the nutritional and organoleptic qualities of foods. In the case of legumes, fermentation
59 reports a general improvement in their nutritional value, by increasing nutrient concentrations
60 (Frías, Vidal-Valverde, Kolowska, Tabera, Honke & Hedley, 1996; Granito, Torres, Frías, Guerra
61 & Vidal-Valverde, 2005) and decreasing non nutritive factors (Doblado, Frías, Muñoz & Vidal-
62 Valverde, 2003; Alonso, Aguirre & Marzo, 2002). Fermentation of legumes is also a good
63 process to increase the DPPH radical scavenging effect, which depend of the starter organism
64 used (Randhir, Vattem & Shetty, 2004; Dueñas, Fernández, Hernández, Estrella & Muñoz, 2005;
65 Kim et al., 2006b).
66 Many researches have demonstrated that microbial fermented soybean products showed higher
67 antioxidant capacities than unfermented soybean. Fermentation of soybeans enhanced the
68 capacity to scavenge DPPH and ABTS radicals (Fernández-Orozco et al., 2007) as consequence
69 of increasing the vitamin E activity. Several microorganisms have been used to ferment soybeans,
70 mainly filamentosus fungi, *Bacillus* sp. and lactic acid bacteria. Lin, Wei & Chou, (2006)
71 demonstrated that soybeans fermented with filamentous fungi enhanced the phenolic content and
72 scavenging DPPH effect. Tempeh made from soybean fermented by *Rhizopus oligosporus,*
73 showed great antioxidant activity, determined by DPPH method (Chang, Hsu, Chou, Chen,
74 Huang & Chung, 2009). Wardhani, Vazquez & Pandiella (2009) described that solid state
75 fermentation of soybeans with *Aspergillus oryzae* for five days provided the best conditions for a
76 maximum antioxidant activity production. Choi, Kin & Suh (2007) described the characteristics
77 of *Bacillus* sp. fermentation and bioavailability of isoflavones in Korean soybean paste.
78 Fermentation of soybeans by *Bacillus pumilus* (Cho et al., 2009) produced changes in the esterase
79 activity and the phenolic content, increasing the level of isoflavone aglycones and decreasing
80 their glycosides and phenolic acids; an increase in the radical scavenging activity was also
81 observed in these samples.

In spite that the isoflavone content and antioxidant activity of microbial fermented soybean has been extensively studied, however, the variation on the composition of other phenolic compounds during microbial fermentation has been scarcely reported. Therefore the aim of this work was to study the effect of solid-substrate fermentation on the phenolic composition of cracked soybean seeds inoculated with *Aspergillus oryzae*, *Rhizopus oryzae*, *Bacillus subtilis* or fermented by the natural seed microbiota. This study will lead to the selection of an adequate microorganism that could be used as starter to obtain soybean seeds with high increased levels of bioactive phenolic compounds.

2. Materials and Methods

2. 1 Seeds

Soybeans (*Glycine max* cv. Merit) were obtained from Mang Fong Pacific Trading S. A. Seeds were cleaned and stored in darkness at 4º C until use.

2.2 Preparation of cultures

Aspergillus oryzae CECT 2094^T (ATCC 1011), *Rhizopus oryzae* CECT 2340 (ATCC 24563) and *Bacillus subtilis* CECT 39^T (ATCC 6051) were purchased from the Spanish Type Culture Collection (CETC) and used as inocula. Stock cultures were grown and maintained as follows. *A. oryzae* and *R. oryzae* were grown for seven days on potato dextrose agar (Difco Laboratories, Detroit, MI) at 30º C, and the spores were collected and washed 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 30º C. The pelleted cells were washed twice in sterile solution and used as inocula.

2.3 Processed soybeans

Solid-substrate fermentations were carried out using 100 g of cracked soybeans suspended in sterile distilled water (1:2 w/v) for 16 hours and autoclaved at 121º C for 15 min. The sterilized

cracked seeds were inoculated with 5% (v/w) of the above cultures containing 10^5 spores/g of each one microorganism: *A. oryzae* (AF), *R. oryzae* (RF) or *B. subtilis* (BF). The inoculated cracked seeds (30g) were aseptically distributed over Petri dishes and placed in a climatic incubator (Memmert, Germany) at 30 ºC and 90 % relative humidity for 48 h. After fermentation, fermented cracked seeds were autoclaved at 121 º C for 15 min and freeze-dried. Solid-substrate fermentations were performed in duplicate.

A cracked seeds sample was spontaneously fermented by the microbiota present on the seed (NF). A control of raw soybeans was also analyzed (C).

2.4 Chemical and solvents

Methanol, ethanol and acetonitrile used were HPLC grade. The HPLC grade standards, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, sinapic, *trans p*-coumaric, *p*-hydroxyphenylacetic and *trans*-ferulic acids, *p*-hydroxybenzoic aldehyde, the flavonoids genistein, genistein glucoside, daidzein, daidzein glucoside, eriodictyol rutinoside, eriodictyol glucoside, hesperetin rutinoside, naringenin, naringenin neohesperidoside, kaempferol rutinoside, kaempferol glucoside, and *trans*-resveratrol were purchased from Extrasynthèse (France). Glycitein and glycitein glucoside were from PhytoLab (Germany).

2.5 Preparation of samples and extraction of phenolic compounds

Soybeans (10 g) from the different samples C, NF, AF, RF, and BF were macerated with 3 x 80 ml of a solution of methanol-HCl ($1^0/_{00}$)/water (80:20 v/v), following the method of Dueñas et al. (2005). An aliquot of this methanol solution (200 ml) was extracted three times with diethyl ether and three times with ethyl acetate, and organic solutions were combined and dried with anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The residue, dissolved in methanol/water (1:1, v/v), was analysed by high-performance liquid chromatography (HPLC). All samples were filtered through a 0.45 μm cellulose acetate filter (Millipore) before HPLC analysis. The samples were prepared and extracted in duplícate.

2.6 HPLC-PAD and HPLC-ESI/MS Analysis

Analysis was carried out on a HPLC-PAD Waters system (Milford. Mass, USA), comprising an autoinjector, a quaternary pump, a photodiode-array detector 2001 and Millennium 32 chromatography manager software (Waters, Milford, Mass, USA). Separation of phenolic compounds was achieved on a reverse phase C18 column Nova-Pak (300 x 3.9 mm, 4 μm)**.**

The analytical conditions were based on those described by Dueñas et al. (2005) with some modifications. Two mobile phases were employed for elution, solvent A: water/acetic acid (98:2 v/v) and solvent B: water/acetonitrile/acetic acid (78:20:2 v/v/v). The gradient profile was 0-55 min, 100%-20% A; 55-70 min, 20%-10% A; 70-80 min, 10%-5% A; 80-110 min, 100% B. The flow rate was 1 ml/min from the beginning to 55 min and 1.2 ml/min from this point to the end. The column was reequilibrated between injections with 10 ml of acetonitrile and 25 ml of the initial mobile phase. Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1s. A volume of 25 μl was injected. The samples were analyzed in duplicate.

Mass spectra were obtained using a Hewlet Pakard 1100MS (Palo Alto, CA) chromatograph equipped with an API source, using an ESI interface. The solvents, gradient conditions and column used were the same as for HPLC-PAD, with a flow rate of 0.7 ml/min. ESI conditions were as follows: negative mode, nitrogen was used as the nebulizing pressure, 40 psi, drying gas, 10 L/min at 340ºC; voltage at capillary entrance, 4000V; and variable fragmentation voltage, 100 V (m/z < 200), 200 V (m/z 200-1000), 250 V (m/z 1000-2500). Mass spectra were recorded from m/z 100 to m/z 2500.

2.7 Identification and quantification of the phenolic compounds

Chromatographic peaks were identified by comparison of retention times, UV spectra and data of UV spectral parameters with those of standards and confirmed by HPLC-ESI/MS. Other compounds, for which no standards were available, were identified and confirmed by HPLC-PAD and HPLC-ESI/MS.

Quantification was made using the external standard method, with commercial standards. The calibration curves were obtained by injection of different volumes of the stock solution over the range of concentration observed for each of the compounds, using a linear regression for the relationship of area sum versus concentration under the same conditions as for the samples analysed. The unknown compounds were quantified by the calibration curves of the more similar compounds.

3. Results and Discussion

3.1 Phenolic compounds in soybeans

Phenolic compounds were identified in soybean samples by means of HPLC analysis, which correspond to non-flavonoid compounds, hydroxybenzoics and hydroxycinnamics, and to flavonoid compounds, among which were flavanones, flavonols, and primarily, isoflavones. The most abundant compounds identified were isoflavones, mainly the aglycones daidzein, genistein and glycitein along with some of their derivatives, glucosides, malonylglycosides and acetylglycosides The phenolic compounds were identified by comparing retention times and UV spectra with regard to those of standards and confirmed by HPLC-ESI/MS analysis (Table 1, 2). For those which standards were not available, analysis of UV spectra recorded for each peak and retention times, together with MS-MS, led to their identification from the chromatographic conditions.

In the control soybean sample, which corresponded to raw soybean, the following compounds were identified and quantified; hydroxybenzoic acids, such as protocatechuic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, vanillic and syringic acids and *p*-hydroxybenzoic aldehyde, and hydroxycinnamic acids, as *trans p*-coumaric, *cis p*-coumaric, sinapic and *trans*-ferulic (Table 3). The total content of these compounds, obtained via HPLC analysis in the control sample was 10.7 μg/g for the hydroxybenzoics, where *p*-hydroxybenzoic and vanillic acids, were in a higher concentration; for the hydroxycinnamics the content was 3.3 μg/g, and *trans p*-coumaric acid was

185 the most abundant (Table 3). The *trans*-resveratrol glucoside was identified in small
186 concentrations, only in the sample control.
187 Various authors have reported the presence of these compounds in a variety of soybean cultivars
188 as well as in derived products, both from fermentation and also from germination. Kim et al.,
189 (2006a) has collected data regarding the presence of some of these compounds in various soybean
190 cultivars, in concentrations similar to those stated this work, and vary with the germination process
191 and light conditions. Xu & Chang, (2008) detected a large number of hydroxybenzoics and
192 hydroxycinnamics in soybeans, with the latter ones generally having higher concentrations, but
193 whose presence and concentration depended on soybean cultivars, the year and location of
194 production, and the colour of the seed coat. Generally speaking, the concentration of these
195 compounds varied in the studies that were carried out, also due to the fact that the soybean samples
196 from different origins were being studied.
197 In this sample, flavanones such as eriodictyol rutinoside, hesperidin, naringenin neohesperidoside
198 (naringin), naringenin, a derivative of eriodictyol and two flavanones (flavanone 1, flavanone 2)
199 were identified (Table 1); these two flavanones have been identified as such by their UV spectrum,
200 but it was not possible to assign them a molecular ion using HPLC-MS. The greatest flavanone
201 content in the control sample corresponded to eriodictyol rutinoside (7.8 μg/g) and hesperidin (5.3
202 μg/g) (Table 3). Kaempferol rutinoside was also identified in the control sample at low
203 concentration (Table 3). In the control sample the total content of these flavonoid compounds
204 obtained as a sum of the calculated concentrations starting with the HPLC analysis was 19.1 μg/g,
205 of which almost 96% corresponded to flavanones eriodictyol rutinoside, hesperidin and
206 naringenin.
207 The presence of some of these compounds in the whole seed, such as flavanones and flavonols,
208 has been described in different soybeans cultivars in varying concentrations. This depends on
209 several factors among which are the size of seeds, which suggests that the synthesis and
210 accumulation of these compounds are related to embryo and cotyledon size (Arora, Muraleedharan

211 & Gale, 1998). Some of these compounds are mainly located in the seed coat whose concentration
212 in this part of the seed was on the order of 1 to 15 μg/g (Kim et al., 2006b).
213 The largest quantity of flavonoid compounds identified was isoflavones, compounds which are
214 amply described in the seeds and various soybean products. The following have been identified in
215 the raw seed, daidzein and daidzein 7-glucoside (daidzin), glycitein 7-glucoside (glycitin),
216 genistein 7-glucoside (genistin), genistein malonylglycoside, genistein acetylglycoside, whiles
217 other isoflavonoids were only identified as genistein and daidzein derivatives (Table 2). The total
218 isoflavone content was 472 μg/g, and the highest concentrations corresponded to daidzin (186.8
219 μg/g), genistin (107 μg/g) and the aglycone daidzein (121.2 μg/g) (Table 4).
220 In the control sample, the sum of concentrations of the daidzein compounds was 315.2 μg/g, of
221 which 121.2 μg/g, corresponded to free daidzein; the concentration of genistein compounds was
222 143.3 μg/g and no free genistein was detected; the glycitein derivatives were those that were
223 found in smaller concentrations, 13.7 μg/g, and no free glycitein was detected.
224 Various authors have commented on the presence of malonyl and acetyl derivatives of daidzein,
225 genistein and glycitein, in the raw soybean seed in diverse concentrations (Rostagno, Palma &
226 Barroso, 2004; Ribeiro et al., 2007), but in most cases the majority presence of the glycosides
227 stood out. The soybean content in isoflavones depends on various factors, fundamentally on
228 cultivation, the degree of maturation, and the environmental culture conditions.

229 ***3.2 Changes in phenolic compounds during microbial fermentation***

230 Variations in the phenolic compounds were observed after microbial fermentation, (Table 3, 4),
231 occurring mainly in the isoflavone concentration (Table 4). There were qualitative and quantitative
232 differences in the analyzed soybean samples; some of the compounds were only present as a
233 consequence of fermentation, and concentration differences were also observed.

234 *Hydroxybenzoics and hydroxycinnamics*. The microorganisms used in this work have modified
235 the content of these compounds (Table 3). In general an increase in the types of compounds is
236 observed, which is more obvious for hydroxybenzoics, such as 93% for *A. oryzae* (AF), 83% for

237 *R. oryzae* (RF) or 73% for *B. subtilis* (BF). The sample that fermented with its own microbiota
238 (NF) underwent a smaller increase (64%) of these compounds. Hydroxycinnamics showed more
239 differences depending of the microorganism, but in all the fermentations, a slight increase was
240 also observed (Table 3).
241 The *p*-hydroxybenzoic, vanillic and syringic acids increased their concentration as a consequence
242 of the microorganism action. The *p*-hydroxyphenylacetic acid and the *p*-hydroxybenzoic
243 aldehyde were detected in the samples that underwent microorganism action. Aldehydes
244 formation was also found in other grain-legumes that underwent a germination process or an
245 addition of enzymes (López-Amorós, Hernández & Estrella, 2006; Dueñas, Hernández &
246 Estrella, 2007a, 2007b).
247 Hydroxycinnamic acids underwent modifications that depended on the type of microorganism
248 used (Table 3); a general increase after fermentation was observed, corresponding the greater one
249 (64.2%) to AF sample and the lower (6.4%) to RF. The higher content correspond to *trans p*-
250 coumaric and sinapic acids that reached the maxima concentration as consequence of
251 fermentation, in AF (74.4%) and RF (51.7%), respectively. The *trans*-ferulic acid was only
252 detected in the control sample.
253 *Flavonoids.* In relation to flavonoids, the changes also depended on the microorganism which
254 was used as inocula (Table 3). Two kaempferol hexosides, which were not detected in the control
255 sample, were detected in the samples that were spontaneously fermented with natural microbiota
256 (NF), and in *A. oryzae* (AF) and *R. oryzae* (RF) samples.
257 The content in flavanones was also modified due to the effect of fermentation. Only eriodictyol
258 rutinoside and naringenin remained in the majority of samples, although they underwent
259 quantitative changes that depended on the microorganism used; an increase in their concentration
260 (26%) was observed only in samples NF, but in the rest of fermented samples different behaviours
261 were found; only BF suffered a general decrease (Table 3). Hesperidin was detected only in the
262 samples fermented with *R. oryzae* (RF) and *B. subtilis* (BF).

263 *Isoflavones*. The samples that have undergone a fermentation process showed qualitative and
264 quantitative variations in their isoflavone content; an increase in the total concentration of these
265 compounds in the NF and AF, which corresponded to spontaneous fermentation and in the
266 presence of *A. oryzae*, respectively was observed. The microorganism action releases isoflavone
267 aglycones, daidzein, genistein and glycitein (Table 2), which appear in free form in the fermented
268 samples (Table 4); a great increase in these compounds in all the samples was observed with the
269 exception of *R. oryzae* fermentation (RF) which produced a smaller content in genistin and
270 daidzein. In the different samples obtained after fermentation under varying conditions, a large
271 number of isoflavone derivatives were detected, fundamentally derived from genistein, that were
272 only identified as derivatives, by their UV spectrum and the molecular ion related to the
273 corresponding aglycone. Daidzein malonylglycoside, daidzein acetylglycoside, genistein
274 acetylglycoside and glycitein malonylglycoside were identified (Table 2), along with other
275 derivatives, only identified as derivatives of daidzein, genistein and glycitein, compounds that
276 were not present in the control sample (Table 4).
277 The action of the various microorganisms seemed to be accompanied by glucosidase activity,
278 which gave rise to the liberation of aglycones. The increase in aglycones content varied
279 depending on the microorganism and the aglycon (Figure 1). In the NF samples, an increase was
280 observed in daidzein by 75.8%, in genistein by 321.5% and in glycitein by 36.3%. In the AF
281 samples, these values were: 49.4% for daidzein, 154% for genistein and 20.4% for glycitein. The
282 RF sample showed a 30% decrease in daidzein and increases in genistein and glycitein of 108.5%
283 and 23%, respectively. The BF sample showed an increase in the three aglycones, 43% for
284 daidzein, 60.8% for genistein and 18.8% for glycitein.
285 Fermentation by the seed microbiota (NF) brought out the most important increases in the
286 identified phenolic compounds (Tables 3 and 4); the isoflavones showing the greatest increases
287 due to *Aspergillus oryzae* action (AF) and also the spontaneously fermented by the seed

288 microbiota (NF). Fermentations by *Bacillus subtilis* (BF) and *Rhyzopus oryzae* (RF) produced the
289 lowest concentrations of isoflavones, overall, in genistein derivatives.
290 Studies carried out using a variety of microorganisms reflected that the liberation of isoflavone
291 aglycones depends on the glucosidase activity inherent in each of them, although factors such as
292 temperature, pressure, action time, etc., influences on this reaction (Pham & Shah, 2009). Thus the
293 *Bifidobacterium animalis, Lactobacillus casei* and *Lactobacillus acidophilus* actions in soymilk
294 result in an increase in the aglycone concentration by 5.87, 6.07 and 5.94 fold respectively, after 4
295 hours of fermentation at 37 º C (Otieno & Shah, 2007b).
296 Cho et al., (2009) found that the fermentation of *cheonggukjang* by *Bacillus pumilius* considerably
297 increased the content in isoflavone aglycones and diminished the content in isoflavone glycosides,
298 as a result of the *β*-glucosidase and esterase activities. They also observed a noticeable increase in
299 the radical DPPH scavenging activity after the fermentation.
300 Various authors associated the increase in antioxidant activity, as a result of soybean fermentation,
301 with the increase in phenolic compound concentration. Lin et al., (2006) found that soybean
302 fermented with filamentous fungi possess enhanced DPPH radical scavenging effect, and total
303 phenolic content. Malencic, Maksimovic, Popovic & Niladinovic, (2007) has established a
304 positive linear correlation between the antioxidant activity evaluation based on DPPH method, and
305 the phenolic compound content in soybean seeds.
306 From our results we can conclude that in the conditions of the study all the assayed
307 microorganisms produced an increase not only in some isoflavones, but also in the concentration
308 of phenolic acids and flavonols; the spontaneous fermentation produce the major increase in the
309 phenolic compounds of soybeans, mainly in isoflavone aglycones, following by the fermentation
310 with *Aspergillus oryzae.* It is well known that phenolic acids and most flavonols contribute to the
311 antioxidant capacity, together with isoflavones, so the fermentation processes in the conditions of
312 this work bring out a product with greater health benefits.
313 4.-**References**

-Alonso, R., Aguirre, A., & Marzo, F. (2002). Effects of extrusion and traditional processing methods on antinutrients and in vitro digestivility of protein and starch in faba and kidney beans. *Food Chemistry,* 68, 159-165.

-Arora, A., Muraleedharan, G. N., & Gale, M. S. (1998). Antioxidant Activities of Isoflavones and their Biological Metabolites in a Liposomal System. *Archives of Biochemistry and Biophysics*, 356, 133-141.

-Chang, C. T., Hsu, C. K., Chou, S. T., Chen, Y. C., Huang, F. S., & Chung, Y. C. (2009). Effect of fermentation time on the antioxidant activities of tempeh prepared from fermented soybean using *Rhizopus oligosporus*. *International Journal of Food Science and Technology*, 44, 799-806.

-Cho, K. M., Hong, S. Y., Math, R. K., Lee, J. H., Kambiranda D. M., Kim J. M., et al. (2009). Biotransformation of phenolics (isoflavones, flavanols and phenolic acids) during the fermentation of *cheonggukjang* by *Bacillus pumilus* HY1. *Food Chemistry* 114, 413-419.

-Choi, Y.M., Kin, Y. S., & Suh, Y. J. (2007). Characteriztics of fermentation and bioavailability of isoflavones in Korean soybean paste (*doenjang*) with application of *Bacillus* sp. KH-15. *International Journal of Food Science and Technology*, 42, 1497-1503.

-Doblado, R., Frías, J., Muñoz, R., & Vidal-Valverde C., (2003). Fermentation of *Vigna sinensis* var. carilla flours by natural microflora and *Lactobacillus*. *Journal of Food Protection,* 65, 2313-2320.

-Dueñas, M., Fernández, M. L., Hernández, T., Estrella, I., & Muñoz, R. (2005). Bioactive phenolic compounds of cowpeas (*Vigna sinensis* L.). Modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *Journal of the Sciences of Food and Agriculture,* 85, 297-304.

-Dueñas, M., Hernández, T., & Estrella, I. (2007a). Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity. *Food Chemistry*, 101, 90-97.

-Dueñas, M., Hernández, T., & Estrella, I. (2007b). Influence of the action of exogenous enzymes on the polyphenolic composition of pea (P*isum sativum*, variety Esla). Effect on the antioxidant activity. *European Food Research and Technology*. 225, 493-500.

-Fernández-Orozco, R., Frías, J., Muñoz, R., Zielinski, H., Piskula, M.K., Kozlowska, H., et al. (2007). Fermentation as a bio-process to obtain functional soybean flours. *Journal of Agricultural and Food Chemistry,* 55, 8972-8979.

-Frías, J., Vidal-Valverde, C., Kolowska, H., Tabera, J., Honke, J., & Hedley, C. L. (1996). Natural fermentation of lentils. Influence of time, flour concentration and temperature on the kinetics of monosaccharides, disaccharides and α-galatosides. *Journal of Agricultural and Food Chemistry* 44, 579-584.

-Granito, M., Torres, A., Frías, J., Guerra, M., & Vidal-Valverde, C. (2005). Influence of fermentation on the nutritional value of two varieties of *Vigna sinensis*. *European Food Research and Technology,* 220, 176-181.

-Kim, E. H., Kim, S. H., Chung, J. I., Chi, H. Y., Kim, J. A., & Chung, I. M. (2006a). Analysis of phenolic compounds and isoflavones in soybean seeds (*Glycine max* (L.) Merill) and sprouts grown under different conditions. *European Food Research and Technology*, 222, 201-208.

-Kim, J. A., Jung, W. S., Chun, S. C., Yu, C. Y., Ma, K. H., Gwag J. G. et al. (2006b). A correlation between the level of phenolic compounds and the antioxidant capacity in cooked-with-rice and vegetables soybean (*Glycine max* L.) varieties. *European Food Research and Technology*, 224, 259-270.

-Lin, C.-H., Wei, Y.-T., & Chou C.-C. (2006). Enhanced antioxidant activity of soyben koji prepared with various filamentous fungi. *Food Microbiology*, 23, 628-633.

-López-Amorós, M.L., Hernández, T., & Estrella, I. (2006). Effect of germination on legume phenolic compounds and their antioxidant activity. *Journal of Food Composition and Analysis,* 19, 277-283.

-Malencic, D., Maksimovic, Z., Popovic, M., & Niladinovic, J. (2007). Polyphenol content and antioxidant activity of soybean seed extracts. *Bioresource Technology* 99, 6688-6691.

-Otieno, D. O. & Shah, N.P. (2007a). Endogenous *β*-glucosidase and *β*-galactosidase activities from selected probiotic micro-organisms and their role in isoflavone biotransformation in soymilk. *Journal of Applied Microbiology*, 103, 601-612.

-Otieno D. O., & Shah N. P. (2007b). A comparison of changes in the transformation of isoflavones in soymilk using varying concentrations of exogenous and probiotic-derived endogenous β-glucosidases. *Journal of Applied Microbiology*, 103, 601-612.

-Pham, T. T., & Shah, N.P. (2009). Hydrolysis of isoflavone glycosides in soy milk by *β*-galactosidase and *β*-glucosidase. *Journal of Food Biochemistry*, 33, (1) 38-60.

-Park, Y. H., Alenscar, S. M., Aguiar, C. L., Mascrenhas, H. A. A., & Scamparini, A. R. P. (2002). Conversion of malonyl β-glucoside isoflavones found in some cultivars of Brazilian soybeans. *Ciênce. Tecnol. Alimen.* 22, 130-135.

-Park, Y. K., Lui, M. C. Y., & Aguiar, C. L. (2003). Production of enriched isoflavone aglycones during processing of soy protein isolates and soy protein concentrates (Abstract). In *2003 IFT Annual Meeting Book of Abstracts* (pp. 215-221). Chicago II; Institute of Food Technologists.

-Randhir, R., Vattem, D., & Shetty, K. (2004). Solid-state bioconversion of fava bean by *Rhizopus oligosporus* for enrichment of phenolic antioxidants and L-DOPA. *Innovative Food Science and Emerging Technologies*, 5, 235-244.

-Ribeiro, M. L. L., Mandarino, J. M. G., Carrao-Panizzi, M. C., de Oliveira, M. C. N., Campo, C. B. H., Nepomuceno A. L. et al. (2007). Isoflavone content and β-glucosidase activity in soybean cultivars of different maturity groups. *Journal of Food Composition and Analysis*, 20, 19-24.

-Rostagno, M. A., Palma, M., & Barroso, C. G. (2004). Pressurized liquid of isoflavones from soybeans. *Analytica Chimica Acta*, 522, 169-177.

-Setchell, K. D. R., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S. et al. (2002). Soy isoflavone glycosides do not cross the enterocyte intact: Quantitative importance of intestinal metabolism in their bioavailavility. *Journal of Nutrition*, 132, 593S-

-Setchell, K. D. R., Brown, N. M., Desai, P. B., Zimmer-Nechemias, L., Wolfe, B. E., Jakate, A. S. et al. (2003). Bioavailability, disposition and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *Journal of Nutrition*, 133, 1027-1035.

-Tsangalis, D., Ashton, J. F., McGill, A. E. J., & Shah, N. P. (2002). Enzymatic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase producing bifidobacteria. *Journal of Food Science*, 67, 3104-3113.

-Wardhani D. H., Vázquez J. A., & Pandiella S. S. (2009). Mathematical modelling of the development of antioxidant activity in soybeans fermented with *Aspergillus oryzae* and *Aspergillus awamori* in the solid state. *Journal of Agricultural and Food Chemistry*, 57, 540-544.

-Xu, Z., Wu, Q., & Godber, J. S. (2002). Stabilities of Daidzin, Glycitin, Genistin, and Generation of Derivatives during Heating. *Journal of Agricultural Food and Chemistry,* 50, 7402-7406.

-Xu, B., & Chang, S. K. C. (2008). Characterization of Phenolic Substances and Antioxidant Propeerties of Food Soybeans Grown in the North Dakota-Minnesota Region. *Journal of Agricultural and Food Chemistry*, 56, 9102-9113.

Acknowledgements.–This work was supported by the financed project AGL2002-02905ALI, through the Spanish "Comisión Interministerial de Ciencia y Tecnología".

Table(s)

Table 1.-Wavelength of maximum UV absorption and molecular ions of identified hydroxybenzoics, hydroxycinnamics, flavanones and flavonols in the soybean samples

compounds	**λ (nm)**	$[M-H]^-$	fragments
hydroxybenzoics and hydroxycinnamics			
protocatechuic acid	297	153	
p-hydroxybenzoic acid	255	137	
p-hydroxyphenyl acetic acid	229, 274	151	
p-hydroxybenzoic aldehyde	286	121	
vanillic acid	261, 293	167	
syringic acid	275	197	
trans p-coumaric acid	310	163	
cis p-coumaric acid	295	163	
sinapic acid	237, 323	223	
trans ferulic acid	323	193	
flavanones and flavonols			
flavanone 1	283		
flavanone 2	291		
eriodictyol rutinoside	284, 325	595	287
hesperidin	284, 330	609	301
kaempferol rutinoside	265, 347	593	
kaempferol glycoside 1	266, 349	447	285
eriodictyol glycoside	287, 327sh	449	287
naringenin neohesperidoside	284, 330	579	271
kaempferol glycoside 2	266, 348		285
naringenin	289, 326	271	
stilbene			
trans resveratrol glucoside	318	389	

Table(s)

Table 2.-Wavelength of maximum UV absorption and molecular ions of identified isoflavones in the soybean samples

compounds	**λ (nm)**	$[M-H]^-$	fragments
daidzein derivative 1	256		253
daidzein 7-glucoside (daidzin)	256, 313 sh	415	253
glycitein 7-glucoside (glycitin)		445	283
daidzein derivative 2	258		253
genistein derivative 1	260		269
genistein derivative 2	260		269
genistein 7-glucoside (genistin)	260, 327	431	269
daidzein derivative 3	252		253
daidzein derivative 4	253		253
daidzein derivative 5	255		253
daidzein malonylglycoside		501	253, 457
genistein derivative 3	259, 326		269
glycitein derivative			283
genistein derivative 4	259, 326		269
daidzein acetylglicoside		457	253
glycitein malonylglycoside		531	283
daidzein	250, 298	253	
genistein derivative 5	259	473	269
genistein malonylglycoside		518	269, 473
glycitein		283	
genistein acetylglycoside	253	473	431, 269
naringenin	289, 326	271	
daidzein derivative 6	258		253
genistein	260	269	
genistein derivative 6	258		269

Table(s)

Table 3.–Concentration (*μg/g)* of hydroxybenzoics, hydroxycinnamics, flavanones and flavonols in soybean samples

Compounds	control	NF	AF	RF	BF
		hydroxybenzoics			
protocatechuic acid	1.37±0.03	nd	2.92±0.26	2.07±0.14	nd
p–hydroxybenzoic acid	3.21±0.15	6.27±1.24	7.87±0.77	9.45±1.04	9.35±0.69
p–hydroxyphenylacetic acid	nd	nd	29.05±1.55	13.18±0.83	21.75±2.75
p–hydroxybenzaldehyde	nd	1.88±0.11	1.20±0.15	1.11±0.13	1.63±0.09
vanillic acid	3.92±0.14	4.71±1.01	14.05±0.67	15.41±1.06	2.80±0.09
syringic acid	2.22±0.01	4.76±0.28	11.32±1.05	17.51±4.23	5.18±0.65
		hydroxycinnamics			
trans p–coumaric acid	1.94±0.21	4.19±0.39	7.55±0.26	2.35±0.93	6.37±0.44
cis p–coumaric acid	0.21±0.05	0.70±0.02	0.74±0.06	nd	nd
sinapic acid	0.58±0.01	0.27±0.01	1.01±0.05	1.20±0.07	nd
trans ferulic acid	0.61±0.02	nd	nd	nd	nd
		flavanones and flavonols			
flavanone 1	0.85±0.06	nd	nd	nd	nd
flavanone 2	0.39±0.01	nd	nd	nd	nd
eriodictyol rutinoside	7.78±0.25	9.89±0.33	3.27±0.22	nd	0.91±0.05
hesperidin	5.28±0.27	nd	nd	2.32±0.01	2.32±0.67
kaempferol rutinoside	0.79±0.09	nd	4.02±0.81	0.98±0.01	nd
kaempferol glycoside 1	nd	2.40±0.23	2.07±0.82	1.08±0.20	nd
eriodictyol glycoside	1.01±0.06	nd	nd	nd	nd
naringin	0.81±0.02	nd	nd	nd	nd
kaempferol glycoside 2	nd	1.39±0.01	1.49±0.31	2.21±0.74	nd
naringenin	2,22±0.32	13.10±0.47	3.60±0.71	3.27±0.88	nd
		stilbene			
trans resveratrol glucoside	0.23±0.01	nd	nd	nd	nd

NF: natural microbiota; AF: *A. oryzae;* RF: *R. oryzae;* BF: *B. subtilis*; nd: no detected; Values are mean ± SD ($n = 2$)

Table(s)

Table 4.–**Concentration** (μg/g) of isoflavones in fermented soybeans

Compounds	control	NF	AF	RF	BF
daidzein derivative 1	2.86±0.22	nd	nd	nd	nd
daidzein 7-glucoside (daidzin)	186.79±9.63	210,38±16,02	143.57±22.3	55.74±4.32	150.11±8.79
glycitein 7-glucoside (glycitin)	13.72±1.03	17.85±1.37	18.07±0.94	11.27±0.09	13.29±0.56
daidzein derivative 2	nd	nd	nd	52.54±1.33	1.18±0.31
genistein derivative 1	nd	nd	18.34±1.01	nd	nd
genistein derivative 2	nd	nd	0.65±0.06	0.09±0.01	nd
genistein 7-glucoside (genistin)	107.04±5.81	183.69±22.54	66.25±0.88	51.96±1.33	62.24±2.19
daidzein derivative 3	nd	0.77±0.11	14.82±1.08	nd	4.43±0.15
daidzein derivative 4	0.94±0.05	95.53±10.61	4.36±0.92	nd	4.84±0.31
daidzein derivative 5	1.38±0.25	nd	14.97±0.31	nd	7.50±0.91
daidzein malonylglycoside	nd	7.09±0.35	9.34±0.63	6.85±1.62	3.64±0.67
genistein derivative 3	nd	5.18±0.68	1.21±0.25	nd	nd
glycitein derivative	nd	2.17±0.11	1.09±0.11	nd	nd
genistein derivative 4	nd	nd	2.50±0.72	nd	nd
daidzein acetylglycoside	nd	20.02±1.71	35.77±6.38	9.37±2.07	nd
glycitein malonylglycoside	nd	3.45±0.08	1.88±0.25	0.62±0.05	0.67±0.04
daidzein	121.19±14.97	213.12±79.57	181.11±19.18	85.65±13.81	173.25±2.61
genistein derivative 5	8.38±0.51	63.61±4.11	20.95±5.27	7.77±1.08	0.19±0.02
genistein malonylglycoside	2.56±0.09	67.06±3.95	1.97±0.06	0.63±0.03	0.78±0.03
glycitein	nd	36.32±4.68	20.37±4.01	23.02±0.29	18.79±0.63
genistein acetylglycoside	1.81±0.23	10.92±0.37	23.62±1.66	9.39±2.15	1.98±0.06
daidzein derivative 6	1.99±0.19	nd	nd	nd	nd
genistein	nd	321.53±58.01	154.73±22.22	108.48±14.09	60.78±5.02
genistein derivative 6	23.53±1.68	27.34±2.09	12.13±0.13	nd	nd

NF: natural microbiota; AF: *A. oryzae;* RF: *R. oryzae;* BF: *B. subtilis* ; nd: no detected; Values are mean ± SD ($n = 2$)

Figure(s)

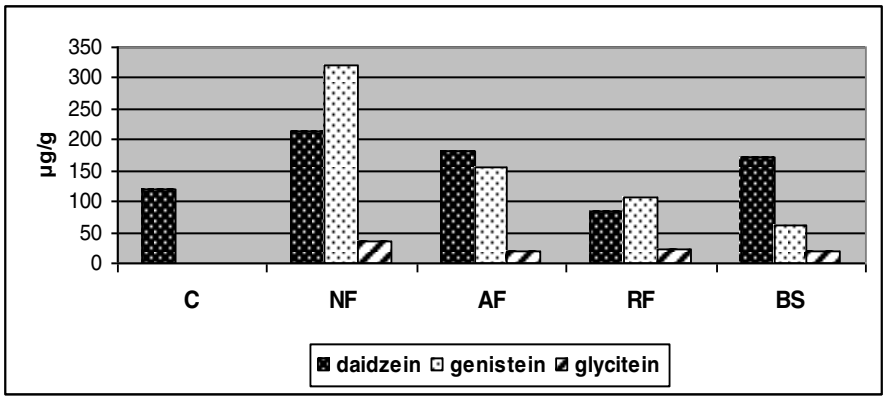

Figure 1