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1 **Antioxidant and antihypertensive properties of liquid and solid state fermented**
2 **lentils**
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Abstract

The effect of liquid (LSF) and solid state fermentation (SSF) of lentils for production of water-soluble fractions with antioxidant and antihypertensive properties was studied. LSF was performed either spontaneously (NF) or by *Lactobacillus plantarum* (LP) while SSF was performed by *Bacillus subtilis* (BS). Native lactic flora in NF adapted better than *L. plantarum* to fermentative broth and BS counts increased 4.0 log CFU/g up to 48h of SSF. LSF water-soluble fractions had higher ($P \leq 0.05$) free amino groups, GABA content, antioxidant and angiotensin I-converting enzyme inhibitory (ACEI) activities than SSF. In addition, GABA and ACEI activity of LSF increased in a time-dependent manner. Proteolysis by BS was limited, with slight changes in free amino groups, while GABA, total phenolic compounds and antioxidant capacity increased throughout fermentation. Higher antihypertensive potential was observed in NF (96 h) characterized by the highest GABA content (10.42 mg/g extract), ACE-inhibitory potency (expressed as IC_{50}) of 0.18 mg protein/mL and antioxidant capacity of 0.26 mmol Trolox equivalents/g extract. Therefore, water-soluble fermented lentil extracts obtained by LSF are particularly promising as functional ingredients in preventing hypertension.

Keywords: Lentils, fermentation, functional ingredients, antioxidant capacity, antihypertensive compounds.

48 **1. Introduction**

49 Cardiovascular diseases (CVD) remain the biggest cause of deaths worldwide.
50 More than 17 million people died from CVDs in 2008 (WHO, 2011). In terms of
51 attributable deaths, the leading cardiovascular risk factor globally is raised blood
52 pressure (to which 13% of global deaths are attributed) (WHO, 2009). Implementing
53 population-wide interventions such as promoting physical activity with a healthy diet
54 has been estimated to be a low-cost and highly feasible option to prevent and control
55 CVD. Frequent consumption of legumes, as part of a healthy diet, has been inversely
56 associated with CVD (Bazzano et al., 2001; Flight & Clifton, 2006). Human studies
57 have shown that legume consumption attenuate oxidative stress, improves serum
58 antioxidant capacity and reduces serum concentration of total and low-density
59 lipoprotein- cholesterol, triglycerides, adhesion molecules and inflammatory
60 biomarkers, all of them risk factors for the development of CVD (Azadbakht,
61 Kimiagar, Mehrabi, Esmaillzadeh, Hu, & Willett, 2007; Taku, Umegaki, Sato, Taki,
62 Endoh, & Watanabe, 2007; Crujeiras, Parra, Abete, & Martinez, 2007; Esmaillzadeh
63 & Azadbakht, 2011). These protective effects of legumes against CVD have been
64 related to their nutritional composition (Campos-Vega, Loarca-Piña, & Oomah, 2010).
65 Legumes, besides its high protein, dietary fiber and slow-digesting carbohydrates
66 content are good sources of phenolic compounds such as flavonoids, isoflavones
67 and phenolic acids. Several studies demonstrated that legume proteins and fiber
68 have lipid-lowering effects (Sirtori et al., 2012). Additionally, legume proteins are
69 sources of hypotensive peptides with angiotensin converting-enzyme (ACE) inhibitory
70 activity (Boye & Maltais, 2011). Flavonoids have been reported as dietary modulators
71 of cardiovascular function by regulation of blood pressure (Galleano, Pechanova, &
72 Fraga, 2010), oxidative stress (Cordova, Sumpio, & Sumpio, 2012; Siow & Mann,

73 2010) and inflammation (Pan, Lai, Dushenkov, & Ho, 2009) in the cardiovascular
74 system.

75 Lentil (*Lens culinaris*, L.) is among the oldest commodities cultivated by
76 humans with a global consumption steadily increasing. The annual production has
77 increased from 4 million tons (MT) in 2009 to more than 5 MT in 2010 (FAO, 2012).
78 Unlike other legumes, lentil contains higher amounts of total phenolic compounds,
79 saponins and condensed tannins (Campos-Vega et al., 2010). Moreover, recent
80 studies have shown the potential application of lentil protein hydrolysates as
81 hypotensive ingredients containing angiotensin I-converting enzyme inhibitory
82 peptides (Boye, Roufik, Pesta & Barbana, 2010; Barbana & Boye, 2011). Therefore,
83 lentil could be considered as a valuable source of cardioprotective compounds.

84 Fermentation is an ancient technology for enhancing the shelf-life, nutritional
85 and organoleptic quality of food (Doblado, Frias, Muñoz & Vidal-Valverde, 2003).
86 Recently, this bioprocess has been applied for the production and extraction of
87 bioactive compounds in the food, chemical and pharmaceutical industries (Martins
88 Mussatto, Martinez-Avila, Montañez-Saenz, Aguilar & Texeira, 2011). In the last
89 years, fermentation has been performed to increase the content of bioactive phenolic
90 compounds in legumes, thus enhancing their antioxidant activity (Fernandez-Orozco
91 et al., 2007; Lee, Hung & Chou, 2008). Additionally, bioconversion of conjugate forms
92 of phenolic compounds to their free forms during fermentation improves their health-
93 link functionality. For instance, microbial biotransformation of isoflavones to
94 aglycones and equol improved the antiosteoporotic and anti-inflammatory effect of
95 fermented soymilk (Chiang & Pan, 2011; Di Cagno et al., 2010). Moreover, lactic acid
96 bacteria have been employed to produce ACE-inhibitory peptides and γ-aminobutyric
97 acid (GABA) in foods, both useful in the prevention and treatment of hypertension

98 (Ricci, Artacho, & Olalla, 2010; Matheson, Freed & Tunnicliff, 1986; Kono & Himeno,
99 2000). In contrast, fermentation has not been extensively applied for production of
100 antihypertensive compounds in legumes, with the exception of soybean (Juan &
101 Chou, 2010).

102 The objective of the present work was to study the efficiency of liquid (LSF)
103 and solid state fermentation (SSF) of lentil for production of water-soluble fractions
104 with antioxidant and antihypertensive properties. This study has addressed the use of
105 the liquid-fraction that results from LSF, which is generally a by-product in the food
106 industry. This fraction can be collected and concentrated as a source of soluble-
107 containing bioactive products overcoming, at the same time, the environmental
108 problems connected with the dumping. In addition, SSF is an economically
109 favourable fermentation system due to its lower impact on the environment, smaller
110 fermenter-size and, reduced downstream processing and stirring as well as lower
111 sterilization costs (Hölker & Lenz, 2005; Raghavarao, Ranganathan & Karanth,
112 2003).

113

114 **2. Materials and methods**

115 *2.1. Seeds*

116 Lentil seeds (*Lens culinaris* var. *castellana*) were provided by Legumbres
117 Iglesias (Salamanca, Spain). Seeds were cleaned and stored in darkness in
118 polyethylene containers at 4-8 ºC.

119

120 *2.2. Selection criteria and preparation of cultures*

121 *Bacillus subtilis* CECT 39^T (ATCC 6051) and *Lactobacillus plantarum* CECT
122 748^T (ATCC 14917) from the Spanish Type Culture Collection (CECT) were selected

123 for SSF and LSF, respectively, based on their GRAS (Generally Recognized As
124 Safe) status and different physiology. *L. plantarum* grows well in the conditions
125 established in LSF (microaerophilic atmosphere, diluted medium) while *B. subtilis*
126 performs well in the conditions established in SSF (aerobic atmosphere,
127 concentrated medium, low water activity). Cultures were stored at − 20 °C in 10%
128 (w/v) sterile reconstituted skim milk containing 0.5% (w/v) yeast extract (Scharlau
129 Chemie S.A., Barcelona, Spain), 1.0% (w/v) glucose (Sigma, St. Louis, MO) and 10%
130 (v/v) glycerol (Sigma). *B. subtilis* was grown in Brain Hearth Infusion (BHI) broth
131 (Conda S.A. Laboratories, Torrejón de Ardoz, Madrid, Spain) for 16 h at 30 ºC. *L.*
132 *plantarum* was grown in De Man, Rogosa and Sharpe (MRS) broth (Conda S.A.
133 Laboratories) for 16 h at 37 ºC.

134 Bacterial cells were propagated twice (2%, v/v) prior experimental use,
135 recovered by centrifugation (8000 rpm for 5 min at 6 ºC) and washed twice in sterile
136 saline solution (0.90% NaCl, w/v). Obtained suspensions were used as inocula for
137 solid or liquid fermentations.

138

139 *2.3. Lentil fermentation*

140 *2.3.1. Liquid state fermentation (LSF)*

141 LSFs were carried out in a New Brunswick 3 L BioFlo/Celligen 115 Fermentor
142 (Eppendorf Iberica, Madrid, Spain) using lentil flour (sieved at 0.5 mm) suspended in
143 sterile distilled water in a proportion of 200 g/L. Fermentations were carried out either
144 spontaneously with the only microorganisms present on the seeds (natural
145 fermentation, NF) or by inoculation of *L. plantarum* suspension (10^8 CFU/ml) at 1-2%
146 (v/v) (LP). LSF were run for 96 h at 37 ºC and 350 rpm. Samples were aseptically
147 collected at 0, 48 and 96 h to determine changes in bacterial populations and pH.

148 Afterwards, samples were also centrifuged (10,000 rpm for 15 min at 6 ºC) and
149 supernatants were freeze-dried for further analysis. LSF was performed in triplicate.
150 Non-fermented samples collected at 0 h were used as negative control.
151
152 *2.3.2. Solid state fermentation (SSF)*
153 SSF was carried out using cracked lentils (100 g) suspended in sterile distilled
154 water (1:2 w/v) for 16 h at 6 ºC, and subsequently autoclaved at 121 ºC for 15 min.
155 Sterile cracked seeds were homogeneously inoculated with 5% (v/w) of *B. subtilis*
156 (10^5 CFU/g) saline suspension, vigorously mixed and aseptically distributed over
157 Petri dishes at a ratio of 30 g, as in Fernandez-Orozco et al. (2007). A climatic
158 chamber (Snijders-Scientific, Tiburg, Netherlands) was used to incubate the dishes
159 for 96 h at 30 ºC and 90% humidity. SSF was monitored by withdrawing samples at
160 0, 48 and 96 h to determine changes in bacterial populations and pH. Afterwards, the
161 samples were autoclaved at 121 ºC for 15 min and freeze-dried for further analysis.
162 SSF was performed in triplicate. Non-fermented samples collected at 0 h were used
163 as negative control.
164
165 *2.4. Microbiological analysis*
166 Plate counts method in appropriate agarised media was used to determine
167 viable cells of the following microorganisms: Lactic acid bacteria (LAB) were counted
168 in MRS agar plates after incubation in an 5% CO_2 atmosphere during 72 h; aerobic
169 mesophilic bacteria were grown in Plate-Count Agar containing (w/v) 0.5% tryptone
170 (Conda S.A. Laboratories), 0.25% yeast extract (Scharlau Chemie S.A.), 0.1%
171 glucose (Sigma) and 1.5 % agar (Conda S.A. Laboratories), after incubation at 30 ºC
172 during 72 h; yeasts and moulds were enumerated on sabouraud chloramphenicol

173 agar (Scharlau Chemie S.A.) after incubation at 25 ºC for 5 days; *Enterobacteriaceae*
174 were counted in violet red bile glucose agar (VRBG, Conda S.A. Laboratories) plates
175 incubated at 30 ºC for 24 h. Coliforms were determined in violet red bile lactose agar
176 (VRBA, Scharlau Chemie S.A.) plates incubated at 37 ºC for 24 h. *B. subtilis* was
177 enumerated in BHI broth supplemented with 1.5% (w/v) agar, plates incubated at 30
178 ºC for 48 h. Cell counts were expressed as $\log_{10}$ CFU/ml.
179
180 *2.5. Extracts preparation*
181 LSF extracts corresponded to the recovered freeze-dried supernatants after
182 fermentation of lentil flour. The yield after freeze-drying was ~3.5 g extract per 100
183 mL of supernatant. For LSF extracts, 20 mg were dissolved in 1 mL of distilled water
184 just before analysis. For SSF, 500 mg of freeze-dried SSF-lentils were suspended in
185 10 mL of cold distilled water and kept overnight in continuous agitation at 4 ºC.
186 Afterwards, sample was centrifuged at 15,000 rpm for 20 min at 4 ºC and
187 supernatant was collected. The residue was then suspended in 2 mL of cold distilled
188 water, vortexed and centrifuged in the same conditions. The supernatants were
189 collected, filtered through Whatman nº 1 paper and freeze-dried. Five mg of freeze-
190 dried sample were dissolved in 1 mL of distilled water just before analysis.
191
192 *2.6. Chemical analysis*
193 *2.6.1. GABA content*
194 The quantification of GABA in fermented lentil extracts was conducted by high-
195 performance liquid chromatography as described in Rozan, Kuo and Lambein (2000),
196 with some modifications. Briefly, 50μL of fermented lentil extracts were derivatised
197 with phenylisothiocyanate (PITC 99 %, Sigma-Aldrich). Allyl-L-glycine (Sigma-

198 Aldrich) was used as internal standard. The chromatographic system consisted of an
199 Alliance Separation Module 2695 (Waters, Milford, USA), a Photodiode Array
200 detector 2996 set at 242 nm (Waters) and a personal computer running the Empower
201 2 for Microsoft Windows chromatographic software (Waters). 20 µL of sample was
202 injected onto a C18 reversed phase Alltima 250 x 4.6 mm i.d., 5 µm size column
203 (Alltech) equipped with a guard column (Alltech) at a constant temperature of 43 ºC.
204 The chromatogram was developed at a flow rate of 1.0 mL/min by eluting in a linear
205 gradient mobile phase A (0.1 M ammonium acetate pH 6.5) and mobile phase B (0.1
206 M ammonium acetate, acetonitrile, methanol, 44/46/10, v/v/v, pH 6.5) as follows:
207 100% A for 15 min, 100% B for 25 min, 100% B for 7 min and finally, column was
208 equilibrated with 100% A for 5 min. The content of GABA in the extracts was
209 quantified from a calibration curve built with standard GABA (Sigma-Aldrich) and with
210 the response factor relative to the internal standard. Regression coefficients were
211 always > 0.99. Results were expressed in mg/g of extract.
212
213 *2.6.2. Total phenolic content (TPC)*
214 TPC was determined in fermented lentil extracts using the Folin-Ciocalteu
215 reagent as described by Singleton, Orthofer, & Lamuela-Raventos (1999). Briefly,
216 100 µL of diluted extract was mixed with 625 µL of distilled water, 250 mL 7.5% (w/v)
217 Na_2CO_3 and 25 µL of 2 N Folin-Ciocalteu reagent (Sigma-Aldrich). Samples were
218 vortexed and incubated for 2h at room temperature in darkness. The absorbance
219 was measured at 739 nm using a microplate reader (Synergy HT microplate reader,
220 BioTek Instruments). Total phenolics were quantified by external calibration using
221 gallic acid (Sigma-Aldrich) as standard. Samples were independently analyzed in

222 triplicate and results were expressed as mg of gallic acid equivalents (GAE) per g of
223 extract (mg GAE/g).
224
225 *2.6.3. Antioxidant capacity*
226 Oxygen Radical Absorbance Capacity (ORAC) was determined in fermented
227 lentil water-soluble fractions by fluorescence (ORAC-FL) as described by Dávalos,
228 Gómez-Cordovés and Bartolomé (2004). The reaction was carried out at 37 °C in 75
229 mM phosphate buffer at pH 7.4, and the final assay mixture (200 µL) contained 70
230 nM fluorescein (Sigma-Aldrich), 12 mM 2,2′-azobis(2-methylpropionamidine)
231 dihydrochloride (Sigma-Aldrich), and Trolox (Sigma-Aldrich) [concentration range 1−8
232 µM] or different dilutions of sample. 2,2′-Azobis(2-methylpropionamidine)
233 dihydrochloride and Trolox solutions were prepared daily, and fluorescein was diluted
234 from a stock solution (1.17 mM) in 75 mM phosphate buffer at pH 7.4. Fluorescence
235 measurements were carried out on a Synergy HT microplate reader (BioTek
236 Instruments) equipped with a fluorescent filter ($\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 520 nm)
237 using a black 96 well plate (Fisher Scientific, Spain). The plate was automatically
238 shaken before the first reading, and the fluorescence was recorded every minute for
239 150 min. The equipment was controlled by Gene5^{TM} software (version 1.1.). All
240 reaction mixtures were prepared in triplicate, and at least two independent analyses
241 were performed for each sample. The areas under the fluorescence decay curve
242 (AUC), based on relative fluorescence values to the initial reading were recorded and
243 the AUC of blanks subtracted. Results were expressed as mmol of Trolox
244 equivalents (TE)/g of extract.
245
246

247 *2.6.4. Free amino groups content*

248 The free amino groups content was measured with 2,4,6-
249 trinitrobenzenesulphonic acid (TNBS) using the method described by Adler-Nissen
250 (1979) adapted to a microplate reader. Twenty-five μL of sample extracts were added
251 to 200 μL 0.2125 M phosphate buffer at pH 8.2. Then, 200 μL of 0.01% TNBS
252 (Sigma-Aldrich) were added. Microtubes were shaken and placed in a water bath at
253 50 °C in darkness for 60 min. Reaction was stopped by adding 400 μl 0.1N HCl, and
254 microtubes were allowed to stand at room temperature for 30 min. After that period,
255 absorbance was read at 340 nm in a Synergy HT multimode microplate reader
256 (BioTek Instruments, IncVermont, USA). The blank was carried out by replacing the
257 sample with water. Calibration curve was plotted using L-Leucine as standard
258 (Sigma-Aldrich) dissolved in 1% sodium-dodecyl sulfate (Sigma-Aldrich) at a
259 concentration range from 0 to 1.5 mM. Results were expressed as mmol Leu/g of
260 extract.

261

262 *2.6.5. Soluble protein content*

263 Protein concentration of water-soluble fractions was determined using the DC
264 Protein Assay (Bio-Rad Laboratories, Hercules, CA) following the manufacturer´s
265 instructions and bovine serum albumin (Sigma-Aldrich) was used as the standard at
266 a concentration range from 0 to 1 mg/mL. Results were expressed in mg protein/g of
267 extract.

268

269 *2.6.6. ACE inhibitory activity*

270 ACE-inhibitory activity was determined following the method described by
271 Hyun and Shin (2000). In the assay, 90 μL of hippuryl-L-histidyl-L-leucine solution (5

272 mM in 0.1 M borate buffer pH 8.3, containing 0.4 M NaCl) were incubated with 10 μL
273 of sample (1 mg protein/mL) at 37 ºC for 5 min at 250 rpm, after which 30 μL of ACE
274 solution (60 mU/mL) were added and incubated for 1 h with the above condition. The
275 hippuric acid (HA) liberated by ACE was measured by RP-HPLC on a Novapak C8
276 (3.9 x 150 mm, 4 μm, Waters, Milford, USA) column. The injection volume was 10 μl,
277 the flow rate was 1 ml/min with a linear gradient (0–88 % in 29 min) of acetonitrile in
278 0.1 % TFA, and the effluent was monitored at 228 nm. All determinations were
279 carried out in duplicate. ACE-inhibitory activity was calculated according to the
280 following equation: Inhibitory activity (%) = [(HAcontrol x HAsample)/HAcontrol] x
281 100. The IC_{50} value was defined as the concentration of extract (in mg protein/mL)
282 required to reduce 50% the height of the HA peak (50% ACE inhibition). IC_{50} values
283 were determined by the non-linear regression sigmoidal dose–response curves in
284 which the range of protein concentrations was transformed to logarithmic scale using
285 the curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego,
286 CA, USA).
287

288 *2.7. Statistical analysis*

289 Each fermentation experiment was carried out in triplicate and water-soluble
290 lentil fractions were analysed in duplicate. Data were expressed as means of the
291 three independent replicates. One-way analysis of variance (ANOVA) using the least
292 significant difference test was conducted to determine differences among samples at
293 the same fermentation time and among time within the same fermentation type with the
294 Statgraphic 4.0 software (Statistical Graphics Corporation, Rockville, MD, USA).
295
296

297 **3. Results and discussion**

298 *3.1. Growth and acidification activity of microbial populations during lentil*
299 *fermentation*

300 Table 1 shows pH values and major microbial populations during LSF of lentils
301 by indigenous LAB (NF) and *L. plantarum* (LP). The pH fell from 6.63 to 3.72 and
302 3.53 up to 48 h for NF and LP, respectively, and no further changes were observed
303 thereafter. Regarding microbial population, enterobacteria was the predominant
304 group of total mesophiles (56.6% in average) followed by LAB (Table 1) at the
305 beginning of NF. Native LAB was the predominant bacterial group at 48 h showing
306 higher growth (Δ log 8.9) in NF than *L. plantarum* (Δ log 2.0) in LP at the same
307 fermentation time. LAB viability was maintained for 96 h in NF while *L. plantarum*
308 viability fell 3.0 log units in LP (Table 1). The sanitary quality of lentil flour was
309 improved by *L. plantarum* inoculation. Indeed, enterobacteria, coliforms, yeasts and
310 moulds were not detected in LP after 48 h. On the contrary, yeasts and moulds
311 reached 51 CFU/mL in NF after 48 h. pH gradually decreased as lentil fermentation
312 progressed, and the presence of LAB was expected to be responsible for it, as
313 shown in other legumes (Fernandez-Orozco et al., 2007 & 2008). This decline in pH
314 during fermentation is known to act as a preservative factor against bacteria
315 associated with spoilage and against nondesirable and pathogenic microorganisms
316 which are not able to proliferate under these conditions (Wang & Hesseltine, 1981;
317 Nout, Rombouts & Havelaar, 1989).

318 The pH values and microbial population during SSF of lentils with *B. subtilis*
319 (BS) are shown in Table 1. An increase of 0.63 pH units was observed after 96h of
320 fermentation which suggests limited release of ammonia. *B. subtilis* is able to grow
321 over a wide pH range producing proteases responsible of raising the pH up to 8.5 by

322 the release of peptides and, in last instance, amonium (Sakar & Tamang, 1995). The
323 pH reached during BS seems to be dependent on temperature as it has been shown
324 in *natto*-like products obtained by cultivation of 38 *B. subtilis* strains
325 (Chantawannakula, Oncharoen, Klanbut, Chukeatirote and Lumyong, 2002). High
326 temperatures (45 °C) resulted in higher activity of *B. subtilis* proteases which has
327 been associated with higher ammonia concentration. Lentil fermentation with *B.*
328 *subtilis* at 37ºC resulted in lower pH values (6.9), possibly associated to lower
329 protease activity.

330 Total mesophile counts were similar to *B. subtilis* counts after 48 and 96 h of
331 fermentation. This indicates that only *B. subtilis* grew throughout SSF of lentil. *B.*
332 *subtilis* counts increased about 4.0 log units from the beginning of incubation to 48 h,
333 and no further changes were observed thereafter. Enterobacteria, yeasts and moulds
334 were not detected throughout BS. In steamed black soybean, Wu & Chou (2009)
335 found that *B. subtilis* BCRC 14715 grew rapidly at 30-50 ºC reaching 9.5 logs
336 CFU/mL after 18 h, regardless the cultivation temperature.

337

338 *3.2. GABA production during lentil fermentation*

339 GABA content of lentil increased significantly (P≤0.05) in a time-dependent
340 manner, regardless the fermentation system employed (Fig. 1). The highest GABA
341 content was observed in lentil extracts obtained by NF (10.42 mg/g extract) followed
342 by LP (7.16 mg/g extract) and BS (6.54 mg/g extract) after 96 h. The development of
343 GABA-enriched foods is of special interest in the treatment of hypertension. This
344 study shows for first time GABA production in lentil by two different fermentation
345 systems: liquid and solid-state fermentation. Soybean has been the only legume
346 seed used as raw material for the development of GABA-enriched products so far.

347 Aoki, Uda, Tagami, Furuya, Endo & Fujimoto (2003) developed a fermentation
348 process for the preparation of GABA-enriched tempeh-like product that exhibited
349 hypotensive effect on spontaneously hypertensive rats (Aoki, Furuya, Endo &
350 Fujimoto, 2003). Those GABA-tempeh products contained 1 g of GABA per 100 g of
351 product, a concentration quite similar to that observed in water-soluble extracts from
352 naturally fermented (NF) lentil.

353

354 *3.3. Total phenolic content during lentil fermentation*

355 Lentil seeds are an interesting source of polyphenols which are in part
356 responsible for their antioxidant activity (Velioglu, Mazza, Gao & Oomah, 1998;
357 Dueñas, Hernandez & Estrella, 2009). Non-fermented water-soluble extracts showed
358 30 mg GAE/g extract, indicating that water is an efficient solvent for extracting
359 phenolic compounds (Sulaiman, Sajak, & Ooi, 2011). No significant differences ($P \geq$
360 0.05) in TPC were found between NF and LP, and a slight down tendency was
361 observed after 96 h (28 mg GAE/g extract for both fermentations) (Fig. 2).
362 Fernandez-Orozco et al. (2007) observed an increase in TPC after 48 h of natural
363 and induced fermentation by *L. plantarum* of soybean flours. These results are in
364 agreement with those reported by Tabera, Frias, Estrella, Villa and Vidal-Valverde
365 (1995) and Bartolome, Hernández & Estrella (1997) in different fermented lentil
366 varieties. Dueñas, Fernández, Hernández, Estrella & Muñoz (2005) suggested that
367 natural and induced fermentation by *L. plantarum* is an adequate process for
368 improving the concentration of phenolic compounds in fermented cowpea (*Vigna*
369 *sinensis*) flour. In addition, they reported that complex polyphenols are hydrolysed to
370 other simpler and biologically more active compounds during fermentation.

371 SSF of lentils performed with *B. subtilis* led to a significant (P ≤ 0.05) rise in
372 TPC, from 24 mg GAE/g to 34-35 mg GAE/g after 48 and 96 h of fermentation (Fig.
373 2). These results are in agreement with those reported in soybean fermented by
374 *Bacillus* strains (Fernandez-Orozco et al., 2007; Juan & Chou, 2010).
375
376 *3.4. Antioxidant activity of fermented lentil extracts*
377 Oxidative stress has been assigned as a causing factor of hypertension by
378 decreasing nitric oxide availability for smooth muscle relaxation (Berry et al., 2001).
379 Therefore, production of foods providing antioxidant capacity is of special interest in
380 the field of functional food research. In this context, antioxidant capacity of fermented
381 lentil extracts was measured using ORAC-FL assay, one of the most used methods
382 for antioxidant capacity determination. NF and LP fermentations for 48 h increased
383 significantly (P≤0.05) the antioxidant capacity of water-soluble extracts. Longer
384 fermentation time up to 96 h led to either non-significant changes (P≥0.05) or slight
385 (P≤0.05) rises in NF and LP extracts, respectively (Fig. 3). These results are in
386 accordance with those reported by Fernandez-Orozco et al. (2007) who found that
387 soybean fermentation by *L. plantarum* provided higher antioxidant capacity than
388 natural fermentation.
389 On the other hand, SSF showed lower antioxidant activity than LSF (Fig. 3).
390 Lentil fermentation by *B. subtilis* raised ORAC levels from 0.17 to 0.22 and 0.24
391 mmol TE/g up to 48 and 96 h, respectively. These values were lower (P≤0.05) than
392 those found in LSF lentil extracts. These results differ substantially from those found
393 in *B. subtilis* fermented soybean flours that presented higher antioxidant capacity
394 than liquid-state fermented flours (Fernandez-Orozco et al., 2007). These results
395 indicate that during legume fermentation different changes take place in antioxidant

396 components that depend on the type of legume, the fermentation process, the
397 microorganism involved and the extract preparation. In addition, no correlation was
398 found between TPC and ORAC values. Although it has been suggested that the
399 phenolic content of plant materials is usually correlated with their antioxidant capacity
400 (Velioglu et al., 1998), the results found in fermented water-soluble lentil extracts
401 show that TPC and the extent of antioxidant capacity are not necessarily correlated,
402 possibly due to antioxidant compounds do not usually act alone. Niki and Noguchi
403 (2000) postulated that the interaction among antioxidants can affect total antioxidant
404 capacity, producing synergistic or antagonistic effects. Results obtained from lentil
405 flour (Dueñas et al., 2005) and black soybeans (Kim, Son & Oh, 2009) fermentations
406 support this statement.
407
408 *3.5. Proteolytic activity of microbial populations during lentil fermentation*
409 Bioactive peptides can be produced during fermentation by microbial
410 proteases (Ricci et al., 2010), therefore, proteolytic activity expressed as free amino-
411 groups released during fermentation was measured. Free amino groups released
412 during LSF of lentil were measured on water-soluble extracts as mmol Leu
413 equivalents/g of dry extract (Fig. 4). Free amino groups increased ($P \leq 0.05$) from 0.6
414 mmol Leu/g to 1.5 mmol Leu/g in NF after 48 h and no larger changes were observed
415 thereafter. LP released lower free amino groups than NF which reached 1.1 and 1.0
416 mmol Leu/g after 48 h and 96 h, respectively (Fig. 4). Our results indicate that natural
417 microbial population exhibited higher proteolytic activity than *L. plantarum*. In the
418 case of fermentation with *B. subtilis,* free amino groups slightly increased ($P \leq 0.05$)
419 from 0.42 mmol Leu/g extract to 0.46 mmol Leu/g after 48h and 0.55 mmol Leu/g
420 after 96 h of fermentation, (Fig. 4). These results suggest that SSF of lentil released

421 lower free amino groups than LSF, despite the high proteolytic activity associated to
422 *B. subtilis* (Sarkar et al., 1993; Chantawannakul, Oncharoen, Klanbut, Chukeatirole,
423 & Lumyong, 2002). These results might be linked to the initial steaming process
424 carried out with the cracked seeds before SSF (section 2.3.1.). This thermal
425 treatment could cause protein denaturalization and further insolubilization, as it has
426 been previously found in faba bean, lentil and chickpea over a pH range from 2.0 to
427 10.0 (Carbonaro et al., 1997). In fact, albumins are more susceptible to heat than
428 other protein fractions, as it has been observed in cooked karkade seeds and lentils
429 (Yagoub, Mohamed, Ahmed & El Tinay, 2004; Sulieman et al., 2008).

430

431 *3.6. ACE inhibitory activity of fermented lentil extracts*

432 ACE modulates arterial blood pressure converting angiotensin I, an inactive
433 decapeptide, into angiotensin II, and octapeptide with potent vasoconstrictor action
434 (Skeggs et al., 1956). Moreover, ACE degrades bradykinin which exerts an important
435 vasodilation activity. Inhibition of ACE by natural or synthetic inhibitors has been
436 shown to reduce blood pressure in experimental animals and humans (Li et al., 2004;
437 Hong et al., 2008). Several research studies have shown that protein hydrolysates
438 from lentils contain ACE-inhibitory peptides (Bamdad, Dokhani, Keramat & Zareie,
439 2009; Barbana & Boye, 2011; Boye et al., 2010). Hydrolysis of proteins occur
440 throughout fermentation and bioactive peptides with ACE inhibitory activity may be
441 released by the action of microbial proteases or proteases from specialized protein
442 bodies found in the organelles of seeds (Müntz, Belozersky & Dunaevsky, 2001). The
443 ACE inhibitory activity of fermented lentil extracts is shown in Fig. 5. NF improved
444 ACE inhibitory activity of lentil extracts from 67.5% to 90% and 92% inhibition after
445 48 and 96 h, respectively. Similarly, LP led to even higher ACE inhibition values

446 (93% regardless fermentation time). Contrary to LSF, extracts from lentils fermented
447 by *B. subtilis* showed lower ($P \leq 0.05$) ACE inhibitory activity (24% and 39% after 48
448 and 96h, respectively). The lower ACE inhibitory activity observed in SSF may be
449 attributed to variations in the type of ACE-inhibitory peptides released which will
450 depend on enzyme especifity and the specific structure of the parent proteins.
451 Additionally, processing conditions can also greatly influence the release of ACE-
452 inhibitory peptides (Gómez-Ruíz, Ramos & Recío, 2004). In this way, proteases from
453 native LAB, *L. plantarum* and *B. subtilis* might differ in their activity and/or specificity.
454 In addition, steaming of cracked seeds before SSF may cause protein denaturation
455 and aggregation which can make difficult the access of the enzyme to the substrate
456 and, hence, the low ACE inhibitory activity observed in SSF at 0 h.
457 IC_{50} values were calculated only in those LSF extracts with higher ACE
458 inhibition, obtained after 96 h fermentation. NF and LP water soluble extracts
459 exhibited similar IC_{50} values (0.18 and 0.20 mg protein/mL, respectively). To our
460 knowledge, this is the first time that ACE inhibitory activity is reported in liquid-state
461 fermented lentil extracts. LSF lentil extracts obtained in the present study exhibited a
462 more potent ACE-inhibitory activity compared to lentil protein hydrolysates (IC_{50} =
463 0.44 mg protein/mL) (Boye et al., 2010) which indicate LSF lentil extracts as a
464 particularly promising potential to be used as functional ingredients.
465
466 **4. Conclusions**
467 Our results reveal that fermentation of lentils is an eligible process to obtain
468 water soluble extracts with potential antihypertensive compounds (GABA and ACE
469 inhibitors) as well as antioxidant properties. Although LSF and SSF fermentations of
470 lentils provided functional water soluble extracts, those obtained by LSF have higher

471 health-promoting potential against hypertension and could extend the possibilities for
472 value-added applications of fermented lentils. Furthermore, with the growing
473 consumer interest for functional foods, identification of avenues for the use of lentil
474 purified extracts aimed at preventing hypertension could be promising. Thus, our
475 group is currently performing further studies to identify the compounds responsible
476 for ACE inhibitory activity in fermented lentil extracts and to evaluate the bioefficacy
477 of those ingredients in animal models.
478

479 **Acknowledgements**

480 This work was supported by AGL2010-16310 projects from the Ministerio de Ciencia
481 e Innovación (Spain). M. I. T. is indebted to CONICET (Argentina) for the Foreign
482 Postdoctoral Fellowship under the Scholarship Program for Young Researchers 2010
483 and R. I. L. to a JAE-pre grant funded by CSIC and FSE-EU.
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689

FIGURE CAPTIONS

Figure 1. GABA content in fermented lentil water-soluble extracts obtained from liquid and solid state fermentation. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF = Natural fermentation; LP= *L. plantarum;* BS = *B. subtilis.*

Figure 2. Total phenolic content in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF = Natural fermentation; LP= *L. plantarum*; BS = *B. subtilis*.

Figure 3. Antioxidant capacity in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF = Natural fermentation; LP= *L. plantarum;* BS = *B. subtilis.*

Figure 4. Free amino groups content in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the

same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF = Natural fermentation; LP= *L. plantarum;* BS = *B. subtilis*.

Figure 5. ACE inhibitory activity (%) of fermented lentil water-soluble extracts obtained by liquid and solid-state fermentations. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF = Natural fermentation; LP= *L. plantarum;* BS = *B. subtilis.*