



Antioxidant and antihypertensive properties of liquid and solid state fermented lentils

Maria Inés Torino^a, Rocío I. Limón^b, Cristina Martínez-Villaluenga^b, Sari Mäkinen^c, Anne Pihlanto^c, Concepción Vidal-Valverde^b, Juana Frias^{b,*}

^a CCT CERELA-CONICET, Chacabuco 145, 4000 SM Tucumán, Argentina

^b Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

^c MTT Biotechnology and Food Research, FIN-31600 Joikionen, Finland

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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 48 h 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) characterised by the highest GABA content (10.42 mg/g extract), ACE-inhibitory potency (expressed as IC₅₀) 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.

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1. Introduction

Cardiovascular diseases (CVD) remain the biggest cause of deaths worldwide. More than 17 million people died from CVDs in 2008 (WHO, 2011). In terms of attributable deaths, the leading cardiovascular risk factor globally is raised blood pressure (to which 13% of global deaths are attributed) (WHO, 2009). Implementing population-wide interventions such as promoting physical activity with a healthy diet has been estimated to be a low-cost and highly feasible option to prevent and control CVD. Frequent consumption of legumes, as part of a healthy diet, has been inversely associated with CVD (Bazzano et al., 2001; Flight & Clifton, 2006). Human studies have shown that legume consumption attenuate oxidative stress, improves serum antioxidant capacity and reduces serum concentration of total and low-density lipoprotein-cholesterol, triglycerides, adhesion molecules and inflammatory biomarkers, all of which are risk factors for the development of CVD (Azadbakht et al., 2007; Crujeiras, Parra, Abete, & Martinez, 2007; Esmailzadeh & Azadbakht, 2012; Taku et al., 2007). These protective effects of legumes against CVD have been related to their nutritional composition (Campos-Vega,

Loarca-Piña, & Oomah, 2010). Legumes, besides its high protein, dietary fibre and slow-digesting carbohydrates content are good sources of phenolic compounds such as flavonoids, isoflavones and phenolic acids. Several studies demonstrated that legume proteins and fibre have lipid-lowering effects (Sirtori et al., 2012). Additionally, legume proteins are sources of hypotensive peptides with angiotensin converting-enzyme (ACE) inhibitory activity (Boye & Maltais, 2011). Flavonoids have been reported as dietary modulators of cardiovascular function by regulation of blood pressure (Galleano, Pechanova, & Fraga, 2010), oxidative stress (Cordova, Sumpio, & Sumpio, 2012; Siow & Mann, 2010) and inflammation (Pan, Lai, Dushenkov, & Ho, 2009) in the cardiovascular system.

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

* Corresponding author. Tel.: +34 912587510; fax: +34 915644853.

E-mail address: frias@ictan.csic.es (J. Frias).

Fermentation is an ancient technology for enhancing the shelf-life, nutritional and organoleptic quality of food (Doblado, Frias, Muñoz, & Vidal-Valverde, 2003). Recently, this bioprocess has been applied for the production and extraction of bioactive compounds in the food, chemical and pharmaceutical industries (Martins et al., 2011). In the last years, fermentation has been performed to increase the content of bioactive phenolic compounds in legumes, thus enhancing their antioxidant activity (Fernandez-Orozco et al., 2007; Lee, Hung, & Chou, 2008). Additionally, bioconversion of conjugate forms of phenolic compounds to their free forms during fermentation improves their health-link functionality. For instance, microbial biotransformation of isoflavones to aglycones and equol improved the antiosteoporotic and anti-inflammatory effect of fermented soymilk (Chiang & Pan, 2011; Di Cagno et al., 2010). Moreover, lactic acid bacteria have been employed to produce ACE-inhibitory peptides and γ -aminobutyric acid (GABA) in foods, both useful in the prevention and treatment of hypertension (Kono & Himeno, 2000; Matheson, Freed, & Tunnicliff, 1986; Ricci, Artacho, & Olalla, 2010). In contrast, fermentation has not been extensively applied for production of antihypertensive compounds in legumes, with the exception of soybean (Juan & Chou, 2010).

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

2. Materials and methods

2.1. Seeds

Lentil seeds (*Lens culinaris* var. *castellana*) were provided by Legumbres Iglesias (Salamanca, Spain). Seeds were cleaned and stored in darkness in polyethylene containers at 4–8 °C.

2.2. Selection criteria and preparation of cultures

Bacillus subtilis CECT 39^T (ATCC 6051) and *Lactobacillus plantarum* CECT 748^T (ATCC 14917) from the Spanish Type Culture Collection (CECT) were selected for SSF and LSF, respectively, based on their GRAS (Generally Recognized As Safe) status and different physiology. *L. plantarum* grows well in the conditions established in LSF (microaerophilic atmosphere, diluted medium) while *B. subtilis* performs well in the conditions established in SSF (aerobic atmosphere, concentrated medium, low water activity). Cultures were stored at –20 °C in 10% (w/v) sterile reconstituted skimmed milk containing 0.5% (w/v) yeast extract (Scharlau Chemie S.A., Barcelona, Spain), 1.0% (w/v) glucose (Sigma, St. Louis, MO) and 10% (v/v) glycerol (Sigma). *B. subtilis* was grown in Brain Heart Infusion (BHI) broth (Conda S.A. Laboratories, Torrejón de Ardoz, Madrid, Spain) for 16 h at 30 °C. *L. plantarum* was grown in De Man, Rogosa and Sharpe (MRS) broth (Conda S.A. Laboratories) for 16 h at 37 °C.

Bacterial cells were propagated twice (2%, v/v) prior experimental use, recovered by centrifugation (8000 rpm for 5 min at 6 °C) and washed twice in sterile saline solution (0.90% NaCl, w/v). Ob-

tained suspensions were used as inocula for solid or liquid fermentations.

2.3. Lentil fermentation

2.3.1. Liquid state fermentation (LSF)

LSFs were carried out in a New Brunswick 3 L BioFlo/Celligen 115 Fermentor (Eppendorf Iberica, Madrid, Spain) using lentil flour (sieved at 0.5 mm) suspended in sterile distilled water in a proportion of 200 g/L. Fermentations were carried out either spontaneously with the only microorganisms present on the seeds (natural fermentation, NF) or by inoculation of *L. plantarum* suspension (10⁸ CFU/ml) at 1–2% (v/v) (LP). LSF were run for 96 h at 37 °C and 350 rpm. Samples were aseptically collected at 0, 48 and 96 h to determine changes in bacterial populations and pH. Afterwards, samples were centrifuged (10,000 rpm for 15 min at 6 °C) and supernatants were freeze-dried for further analysis. LSF was performed in triplicate. Non-fermented samples collected at 0 h were used as negative control.

2.3.2. Solid state fermentation (SSF)

SSF was carried out using cracked lentils (100 g) suspended in sterile distilled water (1:2 w/v) for 16 h at 6 °C, and subsequently autoclaved at 121 °C for 15 min. Sterile cracked seeds were homogeneously inoculated with 5% (v/w) of *B. subtilis* (10⁵ CFU/g) saline suspension, vigorously mixed and aseptically distributed over Petri dishes at a ratio of 30 g, as in Fernandez-Orozco et al. (2007). A climatic chamber (Snijders-Scientific, Tiburg, Netherlands) was used to incubate the dishes for 96 h at 30 °C and 90% humidity. SSF was monitored by withdrawing samples at 0, 48 and 96 h to determine changes in bacterial populations and pH. Afterwards, the samples were autoclaved at 121 °C for 15 min and freeze-dried for further analysis. SSF was performed in triplicate. Non-fermented samples collected at 0 h were used as negative control.

2.4. Microbiological analysis

Plate counts method in appropriate agarised media was used to determine viable cells of the following microorganisms: Lactic acid bacteria (LAB) were counted in MRS agar plates after incubation in an 5% CO₂ atmosphere during 72 h; aerobic mesophilic bacteria were grown in Plate-Count Agar containing (w/v) 0.5% tryptone (Conda S.A. Laboratories), 0.25% yeast extract (Scharlau Chemie S.A.), 0.1% glucose (Sigma) and 1.5% agar (Conda S.A. Laboratories), after incubation at 30 °C during 72 h; yeasts and moulds were enumerated on sabouraud chloramphenicol agar (Scharlau Chemie S.A.) after incubation at 25 °C for 5 days; *Enterobacteriaceae* were counted in violet red bile glucose agar (VRBG, Conda S.A. Laboratories) plates incubated at 30 °C for 24 h. Coliforms were determined in violet red bile lactose agar (VRBA, Scharlau Chemie S.A.) plates incubated at 37 °C for 24 h. *B. subtilis* was enumerated in BHI broth supplemented with 1.5% (w/v) agar, plates incubated at 30 °C for 48 h. Cell counts were expressed as log₁₀ CFU/ml.

2.5. Extracts preparation

LSF extracts corresponded to the recovered freeze-dried supernatants after fermentation of lentil flour. The yield after freeze-drying was ~3.5 g extract per 100 ml of supernatant. For LSF extracts, 20 mg were dissolved in 1 ml of distilled water just before analysis. For SSF, 500 mg of freeze-dried SSF-lentils were suspended in 10 ml of cold distilled water and kept overnight in continuous agitation at 4 °C. Afterwards, samples were centrifuged at 15,000 rpm for 20 min at 4 °C and supernatant was collected. The residue was then suspended in 2 ml of cold distilled water, vortexed and centrifuged in the same conditions. The supernatants were collected, fil-

tered through Whatman n° 1 paper and freeze-dried. Five mg of freeze-dried sample were dissolved in 1 ml of distilled water just before analysis.

2.6. Chemical analysis

2.6.1. GABA content

The quantification of GABA in fermented lentil extracts was conducted by high-performance liquid chromatography as described in Rozan, Kuo, and Lambein (2000), with some modifications. Briefly, 50 µl of fermented lentil extracts were derivatised with phenylisothiocyanate (PITC 99%, Sigma–Aldrich). Allyl-L-glycine (Sigma–Aldrich) was used as internal standard. The chromatographic system consisted of an Alliance Separation Module 2695 (Waters, Milford, USA), a Photodiode Array detector 2996 set at 242 nm (Waters) and a personal computer running the Empower 2 for Microsoft Windows chromatographic software (Waters). Sample (20 µl) was injected onto a C18 reversed phase Alltima 250 × 4.6 mm i.d., 5 µm size column (Alltech) equipped with a guard column (Alltech) at a constant temperature of 43 °C. The chromatogram was developed at a flow rate of 1.0 ml/min by eluting in a linear gradient mobile phase A (0.1 M ammonium acetate pH 6.5) and mobile phase B (0.1 M ammonium acetate, acetonitrile, methanol, 44/46/10, v/v/v, pH 6.5) as follows: 100% A for 15 min, 100% B for 25 min, 100% B for 7 min and finally, column was equilibrated with 100% A for 5 min. The content of GABA in the extracts was quantified from a calibration curve built with standard GABA (Sigma–Aldrich) and with the response factor relative to the internal standard. Regression coefficients were always >0.99. Results were expressed in mg/g of extract.

2.6.2. Total phenolic content (TPC)

TPC was determined in fermented lentil extracts using the Folin–Ciocalteu reagent as described by Singleton, Orthofer, & Lamu-ela-Raventos (1999). Briefly, 100 µl of diluted extract was mixed with 625 µl of distilled water, 250 µl 7.5% (w/v) Na₂CO₃ and 25 µl of 2 N Folin–Ciocalteu reagent (Sigma–Aldrich). Samples were vortexed and incubated for 2 h at room temperature in darkness. The absorbance was measured at 739 nm using a microplate reader (Synergy HT microplate reader, BioTek Instruments). Total phenolics were quantified by external calibration using gallic acid (Sigma–Aldrich) as standard. Samples were independently analysed in triplicate and results were expressed as mg of gallic acid equivalents (GAE) per g of extract (mg GAE/g).

2.6.3. Antioxidant capacity

Oxygen Radical Absorbance Capacity (ORAC) was determined in fermented lentil water-soluble fractions by fluorescence (ORAC-FL) as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004). The reaction was carried out at 37 °C in 75 mM phosphate buffer at pH 7.4, and the final assay mixture (200 µl) contained 70 nM fluorescein (Sigma–Aldrich), 12 mM 2,2'-azobis(2-methylpropionamidine) dihydrochloride (Sigma–Aldrich), and Trolox (Sigma–Aldrich) [concentration range 1–8 µM] or different dilutions of sample. 2,2'-Azobis(2-methylpropionamidine) dihydrochloride and Trolox solutions were prepared daily, and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer at pH 7.4. Fluorescence measurements were carried out on a Synergy HT microplate reader (BioTek Instruments) equipped with a fluorescent filter ($\lambda_{\text{excitation}}$ 485 nm and $\lambda_{\text{emission}}$ 520 nm) using a black 96 well plate (Fisher Scientific, Spain). The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 150 min. The equipment was controlled by Gene5™ software (version 1.1.). All reaction mixtures were prepared in triplicate, and at least two independent analyses were performed for each sample. The areas under the fluorescence

decay curve (AUC), based on relative fluorescence values to the initial reading were recorded and the AUC of blanks subtracted. Results were expressed as mmol of Trolox equivalents (TE)/g of extract.

2.6.4. Free amino groups content

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

2.6.5. Soluble protein content

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

2.6.6. ACE inhibitory activity

ACE-inhibitory activity was determined following the method described by Hyun and Shin (2000). In the assay, 90 µl of hippuryl-L-histidyl-L-leucine solution (5 mM in 0.1 M borate buffer pH 8.3, containing 0.4 M NaCl) were incubated with 10 µl of sample (1 mg protein/ml) at 37 °C for 5 min at 250 rpm, after which 30 µl of ACE solution (60 mU/ml) were added and incubated for 1 h with the above condition. The hippuric acid (HA) liberated by ACE was measured by RP-HPLC on a Novapak C8 (3.9 × 150 mm, 4 µm, Waters, Milford, USA) column. The injection volume was 10 µl, the flow rate was 1 ml/min with a linear gradient (0–88% in 29 min) of acetonitrile in 0.1% TFA, and the effluent was monitored at 228 nm. All determinations were carried out in duplicate. ACE-inhibitory activity was calculated according to the following equation: Inhibitory activity (%) = [(HA_{control} × HA_{sample})/HA_{control}] × 100. The IC₅₀ value was defined as the concentration of extract (in mg protein/ml) required to reduce 50% the height of the HA peak (50% ACE inhibition). IC₅₀ values were determined by the non-linear regression sigmoidal dose–response curves in which the range of protein concentrations was transformed to logarithmic scale using the curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.7. Statistical analysis

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

3. Results and discussion

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

Table 1 shows pH values and major microbial populations during LSF of lentils by indigenous LAB (NF) and *L. plantarum* (LP). The pH fell from 6.63 to 3.72 and 3.53 up to 48 h for NF and LP, respectively, and no further changes were observed thereafter. Regarding microbial population, enterobacteria was the predominant group of total mesophiles (56.6% in average) followed by LAB (Table 1) at the beginning of NF. Native LAB was the predominant bacterial group at 48 h showing higher growth ($\Delta\log 8.9$) in NF than *L. plantarum* ($\Delta\log 2.0$) in LP at the same fermentation time. LAB viability was maintained for 96 h in NF while *L. plantarum* viability fell 3.0 log units in LP (Table 1). The sanitary quality of lentil flour was improved by *L. plantarum* inoculation. Indeed, enterobacteria, coliforms, yeasts and moulds were not detected in LP after 48 h. On the contrary, yeasts and moulds reached 51 CFU/ml in NF after 48 h. pH gradually decreased as lentil fermentation progressed, and the presence of LAB was expected to be responsible for it, as shown in other legumes (Fernandez-Orozco et al., 2007, 2008). This decline in pH during fermentation is known to act as a preservative factor against bacteria associated with spoilage and against non-desirable and pathogenic microorganisms which are not able to

proliferate under these conditions (Nout, Rombouts, & Havelaar, 1989; Wang & Hesseltine, 1981).

The pH values and microbial population during SSF of lentils with *B. subtilis* (BS) are shown in Table 1. An increase of 0.63 pH units was observed after 96 h of fermentation which suggests limited release of ammonia. *B. subtilis* is able to grow over a wide pH range producing proteases responsible of raising the pH up to 8.5 by the release of peptides and, in last instance, ammonium (Sarkar & Tamang, 1995). The pH reached during BS seems to be dependent on temperature as it has been shown in *natto*-like products obtained by cultivation of 38 *B. subtilis* strains (Chantawannakul, Oncharoen, Klanbut, Chukeatirote, & Lumyong, 2002). High temperatures (45 °C) resulted in higher activity of *B. subtilis* proteases which has been associated with higher ammonia concentration. Lentil fermentation with *B. subtilis* at 37 °C resulted in lower pH values (6.9), possibly associated to lower protease activity.

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

Table 1

Cell counts and acidification of microbial populations during fermentation of lentil (*Lens culinaris* var. *castellana*).

Fermentation	Time (h)	pH	Microbial Population (log CFU/mL or log CFU/g)				
			Aerobic mesophilic bacteria	Lactic acid bacteria	<i>B. subtilis</i>	Enterobacteria ^a	Yeast and moulds ^a
NF	0	6.63	3.34 ± 0.15	1.45 ± 0.12	<1	2.58 ± 0.10	<1
	48	3.72	8.85 ± 0.10	8.85 ± 0.23	<1	<1	<1
	96	3.67	8.23 ± 0.19	8.82 ± 0.26	<1	<1	<1
LP	0	6.63	7.45 ± 0.09	7.48 ± 0.04	<1	2.56 ± 0.08	<1
	48	3.53	8.82 ± 0.04	8.65 ± 0.19	<1	<1	<1
	96	3.46	5.74 ± 0.12	5.62 ± 0.21	<1	<1	<1
BS	0	6.27	5.42 ± 0.17	ND	5.53 ± 0.05	<1	<1
	48	6.36	9.05 ± 0.45	ND	9.14 ± 0.25	<1	<1
	96	6.90	9.06 ± 0.17	ND	8.82 ± 0.28	<1	<1

Values are means of three independent fermentations ± standard deviation.

NF, natural fermentation; LP, *L. plantarum*; BS, *B. subtilis*.

^a The lowest dilution tested was 1 CFU/mL.

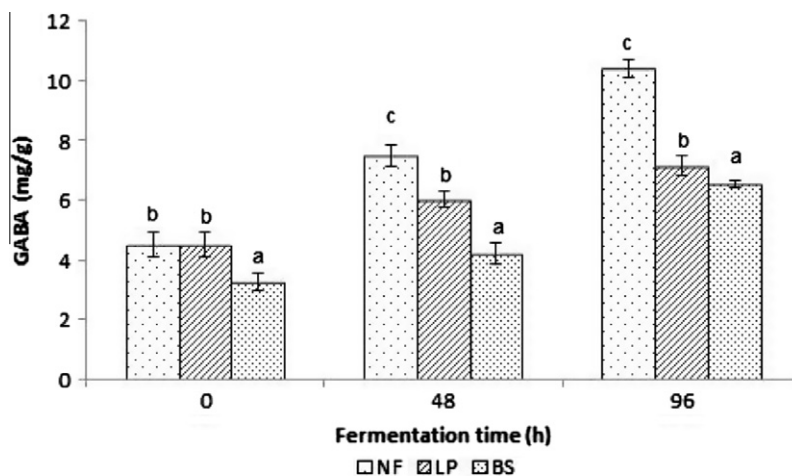


Fig. 1. GABA content in fermented lentil water-soluble extracts obtained from liquid and solid state fermentation. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the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

3.2. GABA production during lentil fermentation

GABA content of lentil increased significantly ($P \leq 0.05$) in a time-dependent manner, regardless the fermentation system employed (Fig. 1). The highest GABA content was observed in lentil extracts obtained by NF (10.42 mg/g extract) followed by LP (7.16 mg/g extract) and BS (6.54 mg/g extract) after 96 h. The development of GABA-enriched foods is of special interest in the treatment of hypertension. This study shows for first time GABA production in lentil by two different fermentation systems: liquid and solid-state fermentation. Soybean has been the only legume seed used as raw material for the development of GABA-enriched products so far. Aoki et al. (2003) developed a fermentation process for the preparation of GABA-enriched tempeh-like product that exhibited hypotensive effect on spontaneously hypertensive rats (Aoki, Furuya, Endo, & Fujimoto, 2003). Those GABA-tempeh products contained 1 g of GABA per 100 g of product, a concentration quite similar to that observed in water-soluble extracts from naturally fermented (NF) lentil.

3.3. Total phenolic content during lentil fermentation

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

SSF of lentils performed with *B. subtilis* led to a significant ($P \leq 0.05$) rise in TPC, from 24 mg GAE/g to 34–35 mg GAE/g after

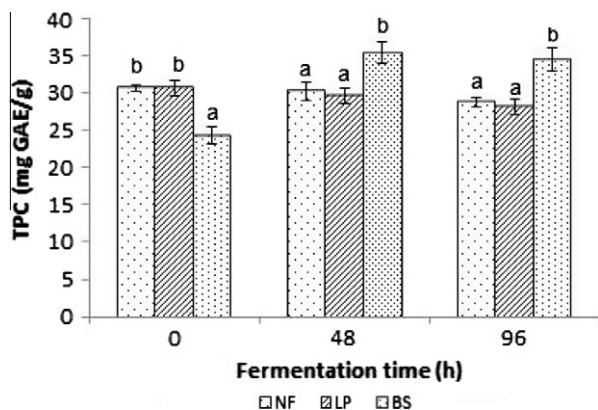


Fig. 2. Total phenolic content in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples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

48 and 96 h of fermentation (Fig. 2). These results are in agreement with those reported in soybean fermented by *Bacillus* strains (Fernandez-Orozco et al., 2007; Juan & Chou, 2010).

3.4. Antioxidant activity of fermented lentil extracts

Oxidative stress has been assigned as a causing factor of hypertension by decreasing nitric oxide availability for smooth muscle relaxation (Berry, Brosnan, Fennell, Hamilton, & Dominiczak, 2001). Therefore, production of foods providing antioxidant capacity is of special interest in the field of functional food research. In this context, antioxidant capacity of fermented lentil extracts was measured using ORAC-FL assay, one of the most used methods for antioxidant capacity determination. NF and LP fermentations for 48 h increased significantly ($P \leq 0.05$) the antioxidant capacity of water-soluble extracts. Longer fermentation time up to 96 h led to either non-significant changes ($P \geq 0.05$) or slight ($P \leq 0.05$) rises in NF and LP extracts, respectively (Fig. 3). These results are in accordance with those reported by Fernandez-Orozco et al. (2007) who found that soybean fermentation by *L. plantarum* provided higher antioxidant capacity than natural fermentation.

On the other hand, SSF showed lower antioxidant activity than LSF (Fig. 3). Lentil fermentation by *B. subtilis* raised ORAC levels from 0.17 to 0.22 and 0.24 mmol TE/g up to 48 and 96 h, respectively. These values were lower ($P \leq 0.05$) than those found in LSF lentil extracts. These results differ substantially from those found in *B. subtilis* fermented soybean flours that presented higher antioxidant capacity than liquid-state fermented flours (Fernandez-Orozco et al., 2007). These results indicate that during legume fermentation different changes take place in antioxidant components that depend on the type of legume, the fermentation process, the microorganism involved and the extract preparation. In addition, no correlation was found between TPC and ORAC values. Although it has been suggested that the phenolic content of plant materials is usually correlated with their antioxidant capacity (Velioglu, Mazza, Gao, & Oomah, 1998), the results found in fermented water-soluble lentil extracts show that TPC and the extent of antioxidant capacity are not necessarily correlated, possibly due to antioxidant compounds do not usually act alone. Niki and Noguchi (2000) postulated that the interaction among antioxidants can affect total antioxidant capacity, producing synergistic or antagonistic effects. Results obtained from lentil flour (Dueñas et al., 2005) and black soybeans (Kim, Son, & Oh, 2009) fermentations support this statement.

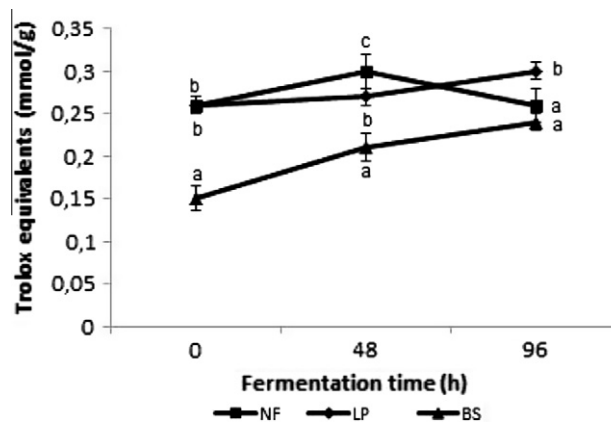


Fig. 3. Antioxidant capacity in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples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

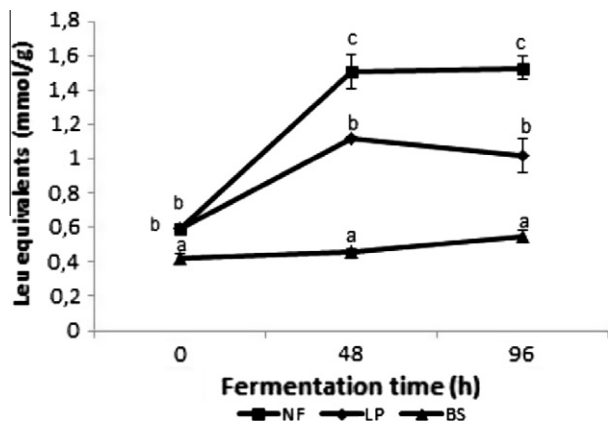


Fig. 4. Free amino groups content in fermented lentil water-soluble extracts obtained from liquid and solid-state fermentation. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among samples at the same fermentation time ($P \leq 0.05$ in one way ANOVA analysis). NF, natural fermentation; LP, *L. plantarum*; BS, *B. subtilis*.

3.5. Proteolytic activity of microbial populations during lentil fermentation

Bioactive peptides can be produced during fermentation by microbial proteases (Ricci, Artacho, & Olalla, 2010), therefore, proteolytic activity expressed as free amino-groups released during fermentation was measured. Free amino groups released during LSF of lentil were measured on water-soluble extracts as mmol Leu equivalents/g of dry extract (Fig. 4). Free amino groups increased ($P \leq 0.05$) from 0.6 mmol Leu/g to 1.5 mmol Leu/g in NF after 48 h and no larger changes were observed thereafter. LP released lower free amino groups than NF which reached 1.1 and 1.0 mmol Leu/g after 48 h and 96 h, respectively (Fig. 4). Our results indicate that natural microbial population exhibited higher proteolytic activity than *L. plantarum*. In the case of fermentation with *B. subtilis*, free amino groups slightly increased ($P \leq 0.05$) from 0.42 mmol Leu/g extract to 0.46 mmol Leu/g after 48 h and 0.55 mmol Leu/g after 96 h of fermentation (Fig. 4). These results suggest that SSF of lentil released lower free amino groups than LSF, despite the high proteolytic activity associated to *B. subtilis* (Chantawannakul et al., 2002; Sarkar, Cook, & Owens, 1993). These results might be linked to the initial steaming process carried out with the cracked seeds before SSF (Section 2.3.1.). This thermal

treatment could cause protein denaturalisation and further insolubilisation, as it has been previously found in faba bean, lentil and chickpea over a pH range from 2.0 to 10.0 (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). In fact, albumins are more susceptible to heat than other protein fractions, as it has been observed in cooked karkade seeds and lentils (Suliman et al., 2008; Yagoub, Mohamed, Ahmed, & El Tinay, 2004).

3.6. ACE inhibitory activity of fermented lentil extracts

ACE modulates arterial blood pressure converting angiotensin I, an inactive decapeptide, into angiotensin II, and octapeptide with potent vasoconstrictor action (Skeggs et al., 1957). Moreover, ACE degrades bradykinin which exerts an important vasodilation activity. Inhibition of ACE by natural or synthetic inhibitors has been shown to reduce blood pressure in experimental animals and humans (Hong et al., 2008). Several research studies have shown that protein hydrolysates from lentils contain ACE-inhibitory peptides (Bamdad, Dokhani, Keramat, & Zareie, 2009; Barbana & Boye, 2011; Boye, Roufik, Pesta, & Barbana, 2010). Hydrolysis of proteins occur throughout fermentation and bioactive peptides with ACE inhibitory activity may be released by the action of microbial proteases or proteases from specialised protein bodies found in the organelles of seeds (Müntz, Belozersky, & Dunaevsky, 2001). The ACE inhibitory activity of fermented lentil extracts is shown in Fig. 5. NF improved ACE inhibitory activity of lentil extracts from 67.5% to 90% and 92% inhibition after 48 and 96 h, respectively. Similarly, LP led to even higher ACE inhibition values (93% regardless fermentation time). Contrary to LSF, extracts from lentils fermented by *B. subtilis* showed lower ($P \leq 0.05$) ACE inhibitory activity (24% and 39% after 48 and 96 h, respectively). The lower ACE inhibitory activity observed in SSF may be attributed to variations in the type of ACE-inhibitory peptides released which will depend on enzyme specificity and the specific structure of the parent proteins. Additionally, processing conditions can also greatly influence the release of ACE-inhibitory peptides (Gómez-Ruiz, Ramos, & Recio, 2004). In this way, proteases from native LAB, *L. plantarum* and *B. subtilis* might differ in their activity and/or specificity. In addition, steaming of cracked seeds before SSF may cause protein denaturation and aggregation which can make difficult the access of the enzyme to the substrate and, hence, the low ACE inhibitory activity observed in SSF at 0 h.

IC_{50} values were calculated only in those LSF extracts with higher ACE inhibition, obtained after 96 h fermentation. NF and LP water soluble extracts exhibited similar IC_{50} values (0.18 and

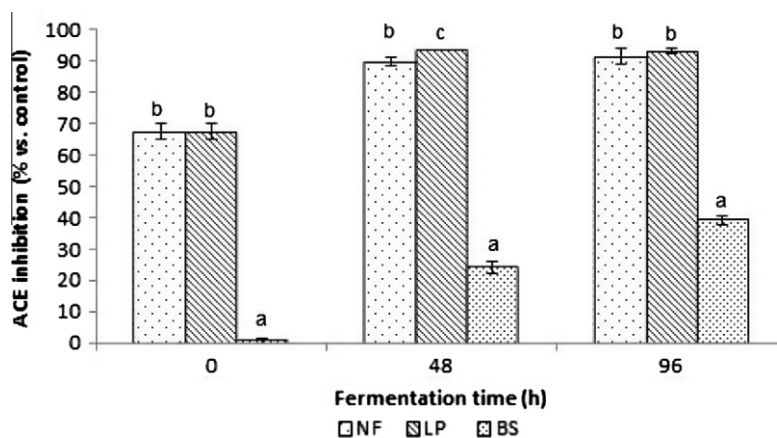


Fig. 5. ACE inhibitory activity (%) of fermented lentil water-soluble extracts obtained by liquid and solid-state fermentations. Each bar corresponds to the mean of three independent replicates with error bars indicating the standard deviations. Different letters indicate significant differences among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

0.20 mg protein/ml, respectively). To our knowledge, this is the first time that ACE inhibitory activity is reported in liquid-state fermented lentil extracts. LSF lentil extracts obtained in the present study exhibited a more potent ACE-inhibitory activity compared to lentil protein hydrolysates ($IC_{50} = 0.44$ mg protein/ml) (Boye et al., 2010) which indicate LSF lentil extracts as a particularly promising potential to be used as functional ingredients.

4. Conclusions

Our results reveal that fermentation of lentils is an eligible process to obtain water soluble extracts with potential antihypertensive compounds (GABA and ACE inhibitors) as well as antioxidant properties. Although LSF and SSF fermentations of lentils provided functional water soluble extracts, those obtained by LSF have higher health-promoting potential against hypertension and could extend the possibilities for value-added applications of fermented lentils. Furthermore, with the growing consumer interest for functional foods, identification of avenues for the use of lentil purified extracts aimed at preventing hypertension could be promising. Thus, our group is currently performing further studies to identify the compounds responsible for ACE inhibitory activity in fermented lentil extracts and to evaluate the bioefficacy of those ingredients in animal models.

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