pH-controlled fermentation in mild alkaline conditions enhances bioactive compounds and functional features of lentil to ameliorate metabolic disturbances

Running title: Alkaline fermentation enhances lentil biofunctionality

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ABSTRACT

Lentil fermentation has a promising potential as a strategy for development of multifunctional ingredients targeting metabolic syndrome (MetS). Response surface methodology was applied to optimize lentil fermentation and study their effects on generation of peptides, soluble phenolics and bioactivities. Fermentation using *Lactobacillus plantarum* and Savinase® 16 L was carried out at different pHs (6.5-8.5) and times (5.5-30 h). Analysis of variance was performed to evaluate linear, quadratic and interaction effects between fermentation parameters. pH positively affected peptides, soluble phenolic compounds and antioxidant activity whereas a negative impact in lipase inhibitory activity was observed ($p<0.0001$). Time showed positive effect on proteolysis and negatively affected angiotensin I-converting enzyme inhibitory activity of fermented lentil ($p<0.0001$). Multivariate optimization led to high levels of peptides, soluble phenolics and bioactivity of fermented lentil at pH 8.5 and 11.6 h. In conclusion, this study might contribute to the development of functional ingredients from lentil for MetS management.

Keywords: Lentil, pH-controlled fermentation, peptides, phenolic compounds, bioactivity, metabolic syndrome

Abbreviations

ACE: angiotensin I converting enzyme; ANOVA: analysis of variance; DOE: Design of experiment; GAE: gallic acid equivalents; LAB: lactic acid bacteria; LPS: lipopolysaccharide; MetS: metabolic syndrome; MRS: Man Rogosa Sharpe; MW: molecular weight; NO: nitric oxide; ORAC: oxygen radical absorbance capacity; RSM: response surface methodology; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TE: Trolox equivalents

26 1. Introduction

27 The metabolic syndrome (MetS) affects approximately 25% of the world's population
28 (Pistollato & Battino, 2014). It is characterized by the combination of several risk factors for
29 cardiovascular disease and type-2 diabetes, such as hyperglycemia, central obesity, hypertension,
30 insulin resistance and dyslipidemia (Pistollato & Battino, 2014). Moreover, oxidative stress
31 generated by an overproduction of reactive oxygen species has been linked to the promotion of a
32 chronic inflammatory state and the development of MetS (Greene, Huang, Serhan, & Panigrahy,
33 2011). Therefore, development of multifunctional ingredients targeting all of these metabolic
34 dysfunctions would be desirable for early prevention.

35 There is an inverse relationship between a diet rich in legumes and the appearance of factors
36 implicated in MetS such as obesity, hypertension, type-2 diabetes mellitus and dyslipidemia
37 (Martínez et al., 2016). These beneficial effects of legumes may be attributed to the presence of
38 bioactive compounds that may act as physiological modulators of metabolism, given that they
39 exhibit antihypertensive, antioxidant, hypoglycemic, hypocholesterolemic and anti-inflammatory
40 properties (Martínez et al., 2016). Therefore, legumes have a promising potential for industrial
41 application as functional ingredients to tackle the main risk factors associated to MetS.

42 Lentil (*Lens culinaris* Medik.) is an important pulse crop worldwide, grown in over 70
43 countries, consumed in more than 100. Lentil is a dietary source of proteins, starch, fiber and
44 micronutrients important to human nutrition (Campos-Vega, Loarca-Piña, & Dave-Oomah, 2010).
45 Lentil proteins are precursors of bioactive peptides with antioxidant activity that may also act as
46 inhibitors of angiotensin I converting enzyme (ACE) involved in rising blood pressure (Garcia-
47 Mora et al., 2017). This legume is also a dietary source of phenolic compounds, a group of
48 phytochemicals including flavan-3-ols, proanthocyanidins, flavones, flavonols, stilbenes, phenolic
49 acids, and anthocyanidins (Mirali, Purves, & Vandenberg, 2016). Proanthocyanidins make up the
50 largest proportion of phenolic compounds in lentil and they are mainly bound to polysaccharides

51 and proteins. Recent studies point out the potential role of lentil phenolic compounds in weight
52 management and blood glucose control as these compounds are able to inhibit gastrointestinal
53 enzymes involved in carbohydrate (α -glucosidase) and lipid digestion (pancreatic lipase) (Zhang et
54 al., 2015).

55 Previous studies have demonstrated that lactic acid fermentation with selected starters have
56 potential application for enhancing legume functional features (Kapravelou et al., 2015). Many
57 studies have used *Lactobacillus plantarum* as starter in legume fermentation as it produces esterases
58 and a wide range of carbohydrases that result in an increased content of free phenolic compounds
59 (Gan, Shah, Wang, Lui, & Corke, 2016) and improved antioxidant, hypolipidemic and
60 antihypertensive activities of the fermented product (Kapravelou et al., 2015; Limón et al., 2015;
61 Torino et al., 2013). Despite of these advantages, there are still some challenges that need to be
62 faced for the efficient production of bioactive compounds by legume fermentation. Proteolytic
63 activity of lactic acid bacteria (LAB) is low compared to other microorganisms what limits the
64 release of bioactive peptides from legume proteins (Jakubczyk, Karaś, Baraniak, & Pietrzak, 2013).
65 Moreover, it is important to notice that in uncontrolled pH fermentation, acidification by LAB
66 causes protein precipitation and enzymatic inactivation (Liu, Bayjanov, Renckens, Nauta, & Siezen,
67 2010; Rui et al., 2016) hindering the mobilization of bound phenolics and the production of
68 bioactive peptides. To address these challenges, the aim of this study was to develop an innovative
69 approach that consists of pH-controlled fermentation of lentil flour using *L. plantarum* along with a
70 commercial food grade alkaline protease from *Bacillus subtilis* (Savinase® 16 L). The hypothesis of
71 this study is that pH-controlled fermentation of lentil flour using *L. plantarum* and Savinase® 16 L
72 enhances proteolysis, production of bioactive peptides, solubilization of phenolic compounds and
73 biological activity of fermented lentil. Response surface methodology (RSM) was used to study the
74 individual and interaction effects of operating parameters (fermentation pH and time) on
75 proteolysis, release of peptides and phenolic compounds as well as the potential health benefits
76 (antihypertensive, hypoglucemic, hypolipidemic, antioxidant and antiinflammatory activities) of the

77 bioaccessible (soluble) fraction of fermented lentil. Moreover, RSM was applied to determine the
78 optimum operating conditions (fermentation pH and time) to improve the content of bioactive
79 compounds and biological activities. The second order polynomial equations (regression models)
80 provided an excellent explanation of the relationship between the response variables and operating
81 parameters.

82 2. Materials and Methods

83 2.1. Materials

84 Lentil (*Lens culinaris* Medik. var. castellana) seeds were purchased from Semillas Iglesias
85 S.A. (Salamanca, Spain) and stored at 4 °C until use. Lentil seeds were milled in a coffee grinder
86 (Moulinex, Allenoçon, France) and passed through a 60-mesh sieve with 0.5 mm pore size. The
87 commercial food grade protease Savinase® 16 L (16 KNU/g) was provided by Novozyme
88 (Bagsvaerd, Denmark). *Lactobacillus plantarum* CECT 748 was purchased from the Spanish Type
89 Culture Collection (CECT, Valencia, Spain). Bacterial cultures were propagated using Man Rogosa
90 Sharpe (MRS) broth (Pronadisa, Madrid, Spain) as described previously (Torino et al., 2013) and
91 stored at -80 °C. Enzymes used in biochemical assays including ACE from rabbit lung (EC
92 3.4.15.1), rat intestine α -glucosidase (EC 3.2.1.20) and porcine pancreatic lipase type II (EC
93 3.1.1.3) as well as other chemicals were purchased from Sigma-Aldrich (Madrid, Spain) unless
94 otherwise stated.

95 2.2. Fermentation of lentil flour

96 Cells of *L. plantarum* grown in MRS broth for 5 h at 37 °C were harvested by centrifugation
97 at $8,000 \times g$ for 10 min, washed twice, and suspended in sterile water. Fermentation was carried out
98 in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) mixing 1.5 L of sterile
99 tap water with lentil flour (143 g/L final concentration), Savinase (365 mg/L final concentration)
100 and *L. plantarum* (2×10^8 CFU/L final cell density). Fermentation experiments were performed
101 under stirring conditions (300 rpm) at 37 °C, at different pH values and times according to the

experimental design described in Section 2.10. Control of pH is maintained by a proportional and integral controller which operates peristaltic pumps, assigned to perform acid or base addition. The pH is sensed by a gel-filled pH probe placed in the bioreactor.

Three independent experiments were conducted for each fermentation condition. Flour suspension without enzyme and bacterial culture was used as control. Soluble fractions were obtained by centrifugation at $7,500 \times g$ at 5 °C for 15 min and they were further heated at 70 °C during 10 min for enzyme inactivation. Finally, samples were freeze-dried and stored under vacuum at -20 °C until further analysis.

2.3. Determination of soluble protein, peptide and phenolic content

Soluble protein concentration of samples was quantified by the Detergent Compatible Protein Assay kit (Bio-Rad, Madrid, Spain) using bovine serum albumin as standard (0.1-1.6 mg/mL). Results were expressed as mg of soluble protein/g of soluble fraction.

Peptide concentration of soluble fractions was measured by Pierce Quantitative Colorimetric Peptide Assay (Fisher Scientific, Madrid, Spain) in sample permeates obtained by ultrafiltration through cellulose membranes of 10 kDa pore size (Millipore, Madrid, Spain). Results were expressed as mg of peptides/g of soluble fraction.

Total phenolic content of lentil soluble fractions was determined using Folin-Ciocalteu's reagent (Singleton, Orthofer, & Lamuela-Raventós, 1999). An aliquot of 100 µL of diluted sample was mixed with 625 µL of distilled water, 250 µL of 7.5% (w/v) sodium carbonate and 25 µL of 2 N Folin-Ciocalteu's phenol reagent. Reaction mixtures were incubated at room temperature for 2 h in darkness. The absorbance was measured at 739 nm using a microplate reader Synergy HT (BioTek Instruments, Winooski, VT, USA) controlled by the Gen 5™ Version 1.1 software (BioTek Instruments, Winooski, VT, USA). A gallic acid standard curve with a linear range (0-225 µg/mL) was prepared from a freshly made 1 mg/mL stock solution. Results were expressed as mg of gallic acid equivalents (GAE)/g of soluble fraction.

127 2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

128 Protein profiles of **lentil** soluble fractions were analyzed by SDS-PAGE. Electrophoresis
129 was carried out loading 20 µg of protein/well on NuPAGE® Novex 4-12% Bis-Tris Gels
130 (Invitrogen, Madrid, Spain). Gels were placed in XCell-sure lock Mini-Cell (**Invitrogen, Madrid,**
131 **Spain**) and run under reducing conditions at 200 V. NuPAGE® MES-SDS and NuPAGE® LDS
132 (**Invitrogen, Madrid, Spain**) were used as running and sample buffers, respectively. Gels were
133 stained with SimplyBlue SafeStain (Invitrogen, **Madrid, Spain**) for 1 h and destained in deionized
134 water for 2 h. The molecular weight of poly- and oligopeptides was determined by comparison with
135 the molecular weight marker Novex® Sharp Prestained Protein Standard (Invitrogen, **Madrid,**
136 **Spain**) containing proteins from 3.5 to 260 kDa.

137 2.5. Determination of radical scavenging activity

138 Radical scavenging activity of lentil soluble fractions was evaluated by fluorescence using
139 the oxygen radical absorbance capacity (ORAC) method as described previously (García-Mora et
140 al., 2017). Fluorescence was measured in a microplate reader Synergy HT (BioTek Instruments,
141 **Winooski, VT, USA**) at emission and excitation wavelengths of 520 nm and 485 nm, respectively.
142 A Trolox standard curve with a linear concentration range (0-160 µM) was prepared from a freshly
143 made 1 mM stock solution. Results were expressed as mM of Trolox equivalents (TE)/g of soluble
144 fraction.

145 2.6. ACE inhibitory activity

146 For ACE inhibitory activity, 3 kDa permeates (0.5 mg/mL final concentration) were assayed
147 following a previously described method (Garcia-Mora, Peñas, Frias, & Martínez-Villaluenga,
148 2014). Permeates were obtained by ultrafiltration of samples through cellulose membranes of 3 kDa
149 pore size (Millipore, Billerica, MA, **USA**). Fluorescence was read every minute for 30 min at
150 emission and excitation wavelengths of 335 and 405 nm, respectively, in a microplate reader
151 Synergy HT (BioTek Instruments, **Winooski, VT, USA**). The results are presented as percent

152 inhibition relative to the negative control having 100% enzymatic activity. IC₅₀ values
153 (concentration of sample in mg/mL that inhibits 50% of the ACE activity) were calculated for the
154 most active fermented samples plotting the non-linear regression sigmoidal dose-response curves in
155 GraphPad Prism 4.00 (GraphPad Software Inc., San Diego).

156 2.7. *α-glucosidase inhibitory activity*

157 For α -glucosidase inhibition assay (Vilcacundo, Martínez-Villaluenga, Hernandez-Ledesma,
158 2017), 100 μ L of soluble fraction from fermented lentil (1 mg/mL), positive control (1 mM
159 acarbose) or negative control (distilled water) were added to 50 μ L of rat intestine α -glucosidase (1
160 U/mL in 0.1 M maleate buffer pH 6.9). Test tubes were incubated at 37 °C for 5 min. After pre-
161 incubation, 50 μ L of substrate (20 mM sucrose) was added to each tube. The reaction mixtures were
162 incubated using a thermomixer (Eppendorf Iberica, Madrid, Spain) at 37 °C and a shaking speed of
163 1,000 rpm for 30 min. Finally, reaction was stopped placing the tubes in a 100 °C water bath for 5
164 min. Supernatants were collected by centrifugation at 20 °C, 12,000 x g for 5 min and stored at -20
165 °C until glucose quantification. Glucose concentration of reaction mixtures was determined using
166 the Amplex[®] Red glucose/glucose oxidase assay kit (Invitrogen, Madrid, Spain). Absorbance was
167 measured using a Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA) at 560 nm.
168 Glucose concentration was calculated using a linear standard curve (0-200 μ M) from a freshly
169 prepared 400 mM stock solution. Percent inhibition was calculated relative to the negative control
170 having 100% enzymatic activity.

171 2.8. *Lipase inhibitory activity*

172 The ability of the fractions to inhibit porcine pancreatic lipase was evaluated using a
173 previously reported method (Kim et al., 2010) with minor modifications. Briefly, 20 μ L of
174 fermented lentil (2 mg/mL), positive control (10 mM orlistat), or negative control (distilled water)
175 were mixed with 20 μ L of pancreatic lipase solution (20 mg in 1 mL of 10 mM 3-
176 morpholinopropane-1-sulfonic acid and 1 mM 2,2',2'',2'''-(ethane-1,2-diyl)dinitrilo)tetraacetic acid,

177 pH 6.8) and 195 μ L Tris buffer (100 mM Tris-HCl and 5 mM CaCl₂, pH 7.0). Test tubes were
178 placed in a thermomixer (Eppendorf Iberica, Madrid, Spain) and incubated at 37 °C for 15 min.
179 The enzymatic reactions were started by addition of 5 μ L of the substrate solution (10 mM *p*-
180 nitrophenyl butyrate in dimethylsulfoxide) and were incubated at 37 °C for 30 min. Lipase activity
181 was determined by measuring the hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol at 405 nm
182 using a microplate reader Synergy HT (BioTek Instruments, Winooski, VT, USA). Percent
183 inhibition was calculated relative to the negative control having 100% enzymatic activity.

184 2.9. Anti-inflammatory activity

185 2.9.1 Cell culture

186 The murine macrophage cell line RAW 264.7 was obtained from the American Type
187 Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagles's medium
188 (Lonza, Madrid, Spain) supplemented with 10% fetal bovine serum (Lonza, Madrid, Spain) and 1%
189 penicillin/streptomycin (5,000 U/mL, Lonza, Madrid, Spain). Cells were plated at densities of
190 1×10^6 cells in 75 cm² culture flasks and maintained at 37 °C under 5% CO₂ in a humidified
191 incubator until 90% confluence. The culture medium was changed every 2 days. Before a confluent
192 monolayer appeared, subculturing cell process was carried out.

193 2.9.2. Cell viability

194 Cell viability was determined by using the Cell Titer 96® AQueous One Solution
195 Proliferation Assay kit. RAW264.7 cells were seeded in 96-well plates (5×10^4 cells/well) and
196 allowed to adhere for 24 h. Cells were exposed to serum-free medium containing 0.5 mg/mL of
197 fermented lentil soluble fraction and incubated at 37 °C for 24 h. Control cells (non-treated) were
198 incubated in serum-free medium. After incubation period, the medium was replaced by 100 μ L of
199 serum-free medium and 20 μ L of Cell Titer 96®. After 1 h of incubation, absorbance was read at
200 515 nm in a microplate reader Synergy HT (BioTek Instruments, Winooski, VT, USA). The
201 viability was calculated considering the viability of control cells as 100% .

202 2.9.3. Quantification of nitric oxide (NO)

203 For inflammatory experiments, 5×10^4 cells/well were seeded in a 96-well plate. Nitrite
204 accumulation, and indicator of NO synthesis, was measured in the culture medium of treated and
205 control cells by the Griess reaction (Green, Wagner, Glogowski, Skipper, Wishnok, Tannenbaum,
206 1982). After 24 h of incubation, the medium was removed, cells were washed with 200 μ L
207 Dulbecco's phosphate buffer saline and treated with 0.5 mg/mL soluble fractions of fermented lentil
208 and 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 for 24 h. Control cells
209 were incubated in serum-free medium for 24 h. Finally, cell culture medium was collected and
210 stored at -20 °C for NO quantification. Briefly, 100 μ L of cell culture medium was plated in 96-well
211 plate and an equal amount of Griess reagent constituted by 1% (w/v) sulfanilamide and 0.1% (w/v)
212 N-1-(naphthyl) ethylenediamine-diHCl in 2.5% (v/v) H_3PO_4 , was added. The plate was incubated
213 for 5 min and the absorbance measured at 550 nm in a Synergy HT microplate reader (BioTek
214 Instruments, Winooski, VT, USA). The amount of NO was calculated using a sodium nitrite
215 standard curve (0-10 μ g/mL). Percent inhibition was calculated relative to control cells stimulated
216 with 100 ng/mL LPS.

217 2.10. Experimental design and statistical analysis

218 A face-centered composite rotational design was employed to investigate the effects of two
219 independent variables (pH and fermentation time) on eight response variables (protein and peptide
220 content, total phenolic content, antioxidant activity, ACE-, α -glucosidase- and lipase-inhibitory
221 activities, and inhibition of NO production in RAW 264.7 macrophages). RSM was also used to
222 determine the optimum conditions for pH-controlled fermentation of lentil flour. Design of
223 experiment (DOE) function in Statistica v7.0 (Statsoft, CA, USA) was used for experimental
224 design. A two-level-two factor design was applied resulting in 10 experimental runs. The lowest,
225 low, center point, high and highest levels of each variable were coded as $-\alpha$, -1, 0, +1 and $+\alpha$,
226 respectively (Table 1). In order to correlate the response variable to the independent variables,

multiple regression analysis was performed on the data to fit the coefficient of the second-order polynomial model of the response. The correlation between response and the selected factors can be expressed by a quadratic equation [Eq. 1]:

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i \leq j}^2 \beta_{ij} X_i X_j \quad (1)$$

where Y represents the response variable, β_0 is the intercept, and X_i and X_j are the independent variables. The terms β_i , β_{ii} and β_{ij} are the regression coefficients of the linear, quadratic and interaction parameters. The adequacy of the models was determined by the coefficient of determination (R^2) and adjusted R^2 , the F-test value (F-value) and the significance level at $p < 0.05$ obtained from the analysis of variance (ANOVA).

A simultaneous optimization of all responses was performed to achieve a minimum content of protein and maximum content of peptides, soluble phenolics and biological activities of fermented lentil. The simultaneous optimization was performed by the desirability approach. For these purpose, the models for all responses were combined and condensed to overall Desirability Function using Statistica v.7.0. For verification of the calculated optimal fermentation conditions predicted values were compared with experimental values.

Experimental data shown in **Table 1 and 3** represent the mean and standard deviation of three independent experiments. Values in **Table 1** and **Table 3** were subjected to one-way ANOVA to compare mean values between fermented and non-fermented lentil using Statgraphics 5.0 (Statistical Graphics Corporation, Rockville, MD, USA). Comparison between groups was performed using a Duncan's multiple-range test, and differences were considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Response surface models data fitting and statistical analysis

In order to know whether fermentation pH and time were factors significantly affecting protein, peptides, phenolic compounds and biological activities (antioxidant, ACE-, α -glucosidase-,

pancreatic lipase-inhibitory and anti-inflammatory activities) of lentil soluble fraction, the experimental design shown in **Table 1** was performed.

Table 1 shows a wide variability among the average response values as function of the parameter settings of the experiments (245.8-813.9 mg protein/g, 162.5-378.5 mg peptide/g, 40.1-74.7 mg GAE/g, 338.5-582.2 mM TE/g, 46.6-95.2% inhibition of ACE, non detected-89.4% inhibition of intestinal α -glucosidase, 35.8-57.4% inhibition of pancreatic lipase and 33.6-50.3% inhibition of NO production in LPS-induced macrophages). These results indicated that fermentation conditions have a noticeable impact on the composition and biological activities of the soluble fraction of fermented lentil.

To study the effect of fermentation parameters on the soluble fraction composition and biological activities of fermented lentil, experimental data of each dependent variable (**Table 1**) were submitted to ANOVA and regression analysis. The estimated linear, quadratic and interaction coefficients of equations are shown in **Table 2**. Experimental values for intestinal α -glucosidase inhibition were insufficient to get a regression model, therefore this response variable is not shown in **Table 2**. Linear, quadratic and interaction coefficients with significant probability value ($p \leq 0.05$) were included in the regression equations. According to ANOVA results (**Table 2**), the empirical polynomial regression models [Eqs. 2-8] for all response variables used to fit the data are shown below:

$$\text{Protein (mg/g)} = 6537.76 - 1538.18 X_1 + 106.62 X_1^2 - 92.40 X_2 + 1.19 X_2^2 + 4.49 X_1 X_2 \quad (2)$$

$$\text{Peptides (mg/g)} = 3659.45 + 990.35 X_1 + 70.51 X_1^2 + 9.29 X_2 - 0.22 X_2^2 \quad (3)$$

$$\text{Phenolic compounds (mg GAE/g)} = 648.08 + 167.71 X_1 + 12.06 X_1^2 - 2.80 X_2 + 0.05 X_2^2 \quad (4)$$

$$\text{ORAC (mM TE/g)} = 9975.33 + 2613.08 X_1 + 176.64 X_1^2 - 0.13 X_2^2 \quad (5)$$

$$\text{ACE inhibition (\%)} = 1159.02 - 291.61 X_1 + 19.42 X_1^2 - 0.10 X_2^2 \quad (6)$$

$$\text{Lipase inhibition (\%)} = 404.22 - 103.61 X_1 + 7.41 X_1^2 - 4.78 X_2 - 0.05 X_2^2 - 0.82 X_1 X_2 \quad (7)$$

$$\text{NO inhibition (\%)} = 368.60 - 92.64 X_1 + 6.49 X_1^2 + 3.42 X_2 - 0.49 X_1 X_2 \quad (8)$$

where X_1 and X_2 were pH and time (h), respectively. The negative and positive coefficients for the model components indicate the favorable and unfavorable effects on each dependent variable, respectively.

To avoid poor response surface results, the adequacy and fitness of the models were checked using regression analysis and ANOVA. In general, ANOVA results confirmed that the equations can efficiently depict the relationship between independent and dependent variables (**Table 2**). The F-value, ratio of the regression mean square and real error mean, indicate the effect of the individual controlled factor on the model. F-values with a very low probability value ($p < 0.0001$) show that the deviation in the responses could be explained by the regression equation, indicating that all models were significant ($p \leq 0.05$), with the exception of the model for antiinflammatory activity ($p = 0.014$).

The adequacy and suitability of the models were checked by the coefficient of determination values. Acceptable R^2 were obtained for most of the models including protein (0.96), peptide (0.85), phenolic compounds (0.91), antioxidant activity (0.89), ACE inhibition (0.88), and lipase inhibition (0.79) (**Table 2**). These values indicated that at least 80% of experimental data suit the model. The R^2 adjusted-values for protein (0.95), peptides (0.83), phenolic compounds (0.91), **ORAC** (0.89), ACE inhibition (0.87) and lipase inhibition (0.76) denoted high correlation of the observed and predicted values. The quadratic model for NO inhibition (R^2 and R^2 adjusted values of 0.37 and 0.26, respectively) was poor, therefore, this model was not used to interpret the impact of pH and time on the anti-inflammatory activity of fermented lentil soluble fraction nor in computing predicted values at optimal fermentation conditions.

3.2. *Effect of fermentation time and pH on soluble protein, peptides and phenolic compounds*

The significant effects of fermentation pH and time (linear and quadratic) and their interactions on response variables can be observed from the Pareto charts (**Fig. 1A-F**). Three

301 dimensional surface graphs of the significant mutual interaction of pH and time and their optimum
302 levels are shown in **Fig. 2A-F**.

303 Soluble protein content was mainly affected by linear effects of time and pH although
304 quadratic and interaction effects were also significant ($p < 0.0001$, **Fig. 1A**). The protein content was
305 reduced by increasing time (**Fig. 2A**), which is attributed to proteolysis and generation of peptides
306 and free amino acids by the action of Savinase (Garcia-Mora et al., 2014) and *L. plantarum*
307 proteolytic system (De Angelis, Calasso, Cavallo, Di Cagno, & Gobbetti, 2016). Conversely,
308 soluble protein content increased with higher pH values (**Fig. 2A**) due to the increased solubility of
309 lentil proteins at pH 8-12 (Limón, Peñas, Martínez-Villaluenga, & Frias, 2014).

310 Fermentation pH was the most dominant factor affecting the peptide content of fermented
311 lentil (**Fig. 1B**). Linear and quadratic effects of pH had a significant positive influence in peptide
312 content while the quadratic effect of time have a negative impact ($p < 0.0001$, **Fig. 1B**). Peptide
313 content increased significantly at higher pH values. This result suggests a higher proteolytic
314 efficiency at alkaline pH which is consistent with the optimum protease activity of Savinase at pH 8
315 (Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015; Garcia-Mora et al., 2014).
316 Peptide content increased along with fermentation time reaching a maximum at 20 h, after which
317 peptide content decreased (**Fig. 2B**).

318 Proteolytic efficiency at experimental fermentation conditions was further confirmed by
319 SDS-PAGE. **Figure 3** shows the protein profiles of untreated lentil flour and fermented lentil by *L.*
320 *plantarum* and Savinase at different pH and time. Untreated lentil flour (**Figure 3**, lane 1) showed a
321 similar protein profile to that previously reported (Garcia-Mora et al., 2014) composed of nine
322 predominant polypeptides (bands A-J) with estimated molecular weights (MW) ranging from 14 to
323 90 kDa likely corresponding to lipoxygenase isoforms (bands A and B, lane 1) and subunits of 7S
324 (bands C, D, E and F) and 11S globulins (bands G, H, I and J). In general, pH-controlled
325 fermentation using *L. plantarum* and Savinase (lanes 2-9) led to an extensive proteolysis and

326 protein degradation, which was reflected in the disappearance or loss of intensity of the main
327 protein bands, along with the appearance of low MW protein fragments (3.5-15 kDa). Protein
328 degradation increased as function of time regardless the pH in which fermentation was performed.
329 Moreover, pH affected proteolytic efficiency of enzymes during fermentation. Lentil fermentation
330 at pH above 8 (lanes 7, 8 and 9) showed a faster protein degradation and generation of low MW
331 protein fragments compared to fermentation at pH 6 (lanes 2, 3 and 4) and pH 7 (lanes 5 and 6).
332 These results confirmed a higher proteolytic efficiency during lentil fermentation at pH 8-8.5.

333 Total phenolic content in the soluble fraction was positively affected by linear and quadratic
334 effects of pH ($p < 0.05$, **Figure 1C**). Similar to peptide content, soluble fraction had a higher
335 concentration of phenolic compounds when fermentation was carried out at higher pH (**Figure 2C**).
336 The increase of phenolic compounds in the soluble fraction of fermented lentil could be related to
337 the enzymatic and non-enzymatic release of bound phenolic compounds (Chandrasekara & Shahidi,
338 2010). Limon et al. (2014) observed that phenolic compounds solubility is pH-dependent, being
339 higher at pH 8-12. Alkaline conditions favor the hydrolysis of linkages between phenolic
340 compounds and polysaccharides of the cell wall (Acosta-Estrada, Gutiérrez-Urbe, & Serna-
341 Saldívar, 2014). On the other hand, *L. plantarum* produces carbohydrases, β -glucosidases and a
342 broad range of esterases which are highly specific and effective in releasing bound phenolic
343 compounds that are cross-linked with cellulose and hemicelluloses in plant cell wall matrix, linked
344 to glucosides or esterified to arabinoxylans and pectin, respectively (Esteban-Torres et al., 2016).
345 Activity of these enzymes is similar at neutral and mild alkaline conditions (Esteban-Torres et al.,
346 2015; Sestelo, Poza, & Villa, 2004). In addition, Savinase was characterized to have esterase
347 activity at pH 8 (Garcia-Mora et al., 2015), therefore, Savinase could contribute to the increased
348 soluble phenolic content of fermented lentil in mild alkaline conditions.

349 3.3. Effect of fermentation time and pH on biological activity of lentil

350 Oxidative stress generated by an overproduction of reactive oxygen species (ROS) has been
351 linked to the promotion of inflammation processes and MetS (Greene et al., 2011). Fermentation pH
352 was the main factor affecting the antioxidant activity of lentil soluble fraction. **Fig. 1D** shows that
353 pH had positive linear and quadratic effects on antioxidant activity ($p < 0.0001$) while quadratic
354 effect of time showed a negative influence ($p < 0.05$). Antioxidant activity decayed up to pH 7.5
355 from which it increased gradually to reach a maximum value at pH 8.5 (**Fig. 2D**). As shown in **Fig.**
356 **2D**, time had no linear effect on antioxidant activity in contrast with previous findings showing that
357 antioxidant activity of casein hydrolysates is higher at longer hydrolysis time (Nongonierma, Maux,
358 Esteveny, & FitzGerald, 2017). The increased antioxidant activity of fermented lentil at alkaline
359 conditions might be attributed to the higher content of soluble phenolics and peptides in the soluble
360 fraction. Lentil proteolysis using Savinase at pH 8.5 produces peptides with the ability to scavenge
361 peroxy radicals (García-Mora et al., 2017). In addition, alkaline pH favors the non-enzymatic
362 release of bound phenolic compounds, fact that is related to higher soluble phenolic content and
363 antioxidant activity in fermented lentil. Both peptides and phenolic compounds have shown to elicit
364 synergistic antioxidant activity as recently reported by Cao & Xiong (2017) for whey protein
365 hydrolysate, gallic acid and epigallocatechin gallate mixtures. On the other hand, pH could
366 influence the antioxidant activity during lentil fermentation by changing the structure of the
367 phenolic compounds. Catechins, one of the predominant phenolic compounds in lentils, enhance its
368 electron-donating ability upon deprotonation with increasing pH values (Muzolf, Szymusiak,
369 Gliszczynska-Swiglo, Rietjens, & Tyrakowska, 2008).

370 ACE indirectly increases blood pressure by causing blood vessels to constrict. The inhibition
371 of ACE is considered to be one of the most effective strategies for managing hypertension and
372 attenuate the progression of MetS. Peptides and phenolic compounds are considered to be the main
373 compounds in lentil displaying ACE-inhibitory activity (García-Mora et al., 2017; Mamilla &
374 Mishra, 2017). Fermentation time had a negative quadratic influence on ACE-inhibitory activity of
375 soluble fraction ($p < 0.0001$, **Fig. 1E**). ACE-inhibitory activity increased up to 16 h, time after

376 which begins to decay (**Fig. 2E**). This trend is consistent with a previous study in which longer
377 hydrolysis times caused the degradation of ACE-inhibitory peptides in lentil hydrolysates (Garcia-
378 Mora et al., 2014). In contrast, pH had a significant positive impact on the antihypertensive
379 potential of fermented lentil which **reached a maximum value** when fermentation was performed at
380 pH 8.5 (**Fig. 2E**). This effect could be explained by the higher hydrolytic efficiency on proteins and
381 bound phenolics that take place at alkaline pH.

382 The inhibition of α -glucosidase reduces intestinal digestion of disaccharides and further
383 absorption of glucose, consequently controlling the postprandial glycemic response (Tan, Chang, &
384 Zhang, 2017). Differences in terms of the α -glucosidase inhibitory activity of fermented lentil were
385 observed (**Table 1**). The α -glucosidase inhibitory activity of 0.5 mg/mL of fermented lentil ranged
386 from 0 to 89% for sucrose hydrolysis. Experimental data did not fit the regression model; therefore,
387 differences on α -glucosidase inhibitory activity could not be explained as function of fermentation
388 pH and time. Additional parameters (e.g. temperature, enzyme/flour ratio, inoculum size) may be
389 studied in order to further identify those that have a significant impact in α -glucosidase activity.
390 Zhang et al. (2015) evaluated the α -glucosidase inhibitory activity of phenolic extracts from 20
391 Canadian lentil cultivars. Results from this study demonstrated that only flavonols were able to
392 inhibit intestinal α -glucosidase. On the basis of the study of Zhang et al. (2015), differences on the
393 α -glucosidase inhibitory activity observed in our study among fermentation conditions could be due
394 to variations on the flavonol content.

395 Pancreatic lipase, a key enzyme responsible for triglyceride absorption in the small intestine,
396 is secreted from the pancreas and hydrolyses triglyceride into glycerol and fatty acids (Buchholz &
397 Melzig, 2015). Suppression and delay of triglyceride digestion and absorption through inhibition of
398 lipase is a key approach to the control of hyperlipidaemia and obesity, both components of the
399 MetS. Lipase-inhibitory activity of lentil varied depending on fermentation conditions. The linear,
400 quadratic and interaction effects of fermentation pH and time were significant ($p<0.05$, **Fig. 1F**).
401 The parameter showing the highest impact was pH, which negatively influenced the lipase-

inhibitory activity of fermented lentil (**Fig. 1F**). Similarly, interaction of both fermentation parameters had a negative impact on pancreatic lipase inhibition. In contrast, linear and quadratic terms of time and quadratic term of pH showed a positive effect. The inhibitory effects on pancreatic lipase activity of polyphenol-rich extracts have been reported in previous studies (Buchholz & Melzig, 2015). Lipase inhibitory activity of lentil has been associated to flavonols such as kaempferol and quercetin aglycones and glycosides (Zhang et al., 2015). Considering that flavonols become unstable under neutral and alkaline conditions (Li et al., 2016), it was consistent to reach the maximum lipase inhibitory activity (80%) at longer fermentation times (above 30 h) and lower pH values (6.4) (**Fig. 2F**).

MetS is characterized by a chronic inflammation grade below the threshold of pain, which raises the concentration of inflammatory markers in the systemic circulation (Martínez-Larrad et al., 2016). Because NO is an important mediator involved in multiple inflammatory pathways, inhibition of its production by bioactive components is a clear indication of antiinflammatory activity (Sharma, Al-Omran, & Parvathy, 2007). Cell treatments with soluble fraction of fermented lentil (0.5 mg/mL) did not affect RAW264.7 cells viability (data not shown) and reduced significantly the secretion of NO by LPS-stimulated RAW267.4 macrophages (33.5-50.2% inhibition) compared to control cells (**Table 1**). Statistical differences in the antiinflammatory potential of fermented lentil produced at different pH and time were observed. It is relevant to notice that maximum antiinflammatory activity in fermented lentil was reached at pH 6.5 for 15 h. Considering that the regression model for anti-inflammatory activity [Eq. 8] was not statistically significant (**Table 2**) relationships between fermentation parameters (pH and time) and antiinflammatory activity of fermented lentil could not be established. Based on these results, additional processing parameters should be further studied. There is evidence showing that flavonols (kaempferol, quercetin and their derivatives) are the most potent antiinflammatory compounds in lentil soluble extracts. These flavonoids inhibit the mRNA expression of proinflammatory cytokines including TNF- α , IL6 and IL1 β *in vivo* (Zhang et al., 2017) and hinder

428 NO production induced by LPS in RAW264.7 cells, thereby lowering inflammatory response (Devi
429 et al., 2015). Based on the literature, the highest antiinflammatory activity found in fermented lentil
430 at pH 6.5 and 15 h (50% inhibition) could be related to the higher stability of flavonols at those
431 fermentation conditions (Li et al., 2016). Previous studies have attributed the antiinflammatory
432 activity of protein hydrolysates to specific bioactive peptides (López-Barrios, Antunes-Ricardo, &
433 Gutiérrez-Urbe, 2016); however, anti-inflammatory peptides derived from lentil proteins have not
434 been identified so far.

435 3.4. *Multivariate optimization of fermentation parameters and validation of predictive models*

436 Response and desirability profiling were performed to determine the levels of the
437 independent variables that produce the most desirable predicted values for peptide, phenolic
438 compounds and biological activities of the soluble fraction of fermented lentil by using desirability
439 function (**Fig. 4A**). The optimal condition of lentil fermentation was ascertained to obtain the lowest
440 soluble protein content and the highest peptides and phenolics contents, antioxidant, ACE and
441 pancreatic lipase inhibitory activities. Based on this criterion, lentil fermentation at pH 8.5 for 11.63
442 h with an overall desirability of 0.66 was predicted to produce 693.1 mg protein, 368.3 mg peptides,
443 75.7 mg GAE and 561.1 mM TE per gram of soluble fraction. Moreover, soluble fraction of
444 fermented lentil produced in the optimal conditions inhibited ACE by 95.85% at 0.5 mg/mL (IC_{50} =
445 0.14 mg/mL) and pancreatic lipase by 39.34% inhibition at 2.0 mg/mL. The overall performance of
446 fermented lentil produced in optimal conditions using *L. plantarum* and Savinase were improved
447 compared to our previous studies focused on uncontrolled pH fermentation of lentil using *L.*
448 *plantarum* (30 mg GAE/g, 300 mM TE/g and IC_{50} for ACE inhibition of 0.20 mg/mL) and lentil
449 protein hydrolysis using Savinase (34 mg peptides/g, 224 mM TE/g and IC_{50} value for ACE
450 inhibition of 0.20 mg/mL) (Garcia-Mora et al., 2014; Torino et al., 2013).

451 The suitability of the established regression models for prediction of response values was
452 tested selecting a fermentation experiment within the range of maximum desirability. Fermentation

conditions with the highest desirability value were pH 8.5-8.6 and 10-26 h (**Fig. 4B**). Based on this observation, **Table 3** shows predicted and experimental values at pH 8.5 and 15 h and their comparison to non-fermented lentil soluble fraction at pH 8.5. Experimental values were similar to predicted values, which indicated that empirical models are suitable for the chemical and biological characterization of fermented lentil and optimization of pH-controlled fermentation using *L. plantarum* and Savinase. As compared to water soluble extract obtained from non-fermented lentil at pH 8.5, lentil fermentation at pH 8.5 for 15 h provided 6.0, 1.3, 1.9, 4.8, 53.2, and 2.5-fold higher levels of peptides, soluble phenolic compounds, antioxidant, ACE-inhibitory and α -glucosidase-inhibitory and anti-inflammatory activities (**Table 3**). Moreover, 74% of initial lipase inhibitory activity of lentil soluble fraction was retained after fermentation in mild alkaline conditions for 15 h.

4. Conclusions

This is the first study that have employed a RSM approach during fermentation and enzymatic hydrolysis of lentil proteins with the objective to optimize the generation of peptides and soluble phenolics displaying an array of relevant bioactivities to attenuate the cardiometabolic risk factors of MetS. The two main fermentation parameters (pH and time) had an effect on proteins, peptides and phenolics contents as well as on potential antioxidant, antihypertensive, hypoglycemic, hypolipidemic and antiinflammatory activities of fermented lentil. Fermentation pH is the main factor affecting positively to peptides, soluble phenolic compounds and antioxidant activity whereas it has a negative impact on lipase inhibitory activity. Fermentation time has a dominant positive effect on protein hydrolysis whereas affects negatively ACE inhibitory activity of fermented lentil. In contrast, fermentation parameters having an impact on α -glucosidase inhibitory and antiinflammatory activities were not identified. Optimal fermentation conditions were pH 8.5 and 11.6 h yielding a combination of desirable levels of peptides, soluble phenolic and bioactivities. These findings encourage the implementation of pH-controlled fermentation using *L. plantarum* and

478 Savinase for the production of bioactive compounds and enhancement of lentil functional properties
479 and may serve as a starting point for innovation in the agri-food industry.

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607

608 **FIGURE CAPTIONS**

609 **Fig. 1.** Pareto charts of standardized effect estimates (absolute value) for (A) protein content (mg/g
610 soluble fraction), (B) peptide content (mg/g soluble fraction), (C) phenolic compounds (mg GAE/g
611 soluble fraction), (D) **Oxygen radical absorbance capacity (ORAC)** (mM TE/g soluble fraction), (E)
612 **Angiotensin I-converting enzyme (ACE)** inhibitory activity (% inhibition), (F) pancreatic lipase
613 inhibitory activity (% inhibition). L: linear effect; Q: quadratic effect; 1L by 2L: interaction effect.

614 **Fig. 2.** Response surface 3D contour plots for combined effect of fermentation pH and time on (A)
615 protein content, (B) peptides content, (C) phenolic compounds content, (D) **Oxygen radical**
616 **absorbance capacity (ORAC)**, (E) **Angiotensin I-converting enzyme (ACE)** inhibitory activity, (F)
617 lipase inhibitory activity of the soluble fraction of fermented lentil.

618 **Fig. 3.** **Sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE)** under denaturing
619 conditions of untreated lentil flour (lane 1) and fermented lentil by *L. plantarum* and Savinase at pH
620 6.8 for 5.5 h (lane 2); pH 6.5 for 15 h (lane 3) pH 6.8 for 25.5 h (lane 4); pH 7.5 for 30 h (lane 5);
621 pH 7.5 for 15 h (lane 6); pH 8.2 for 5.5 h (lane 7); pH 8.5 for 15 h (lane 8); pH 8.2 for 25.5 h (lane
622 9). MW: prestained molecular weight marker.

623 **Fig. 4.** (A) Desirability function profile for protein, peptide and phenolic content, **Oxygen radical**
624 **absorbance capacity (ORAC)**, **Angiotensin I-converting enzyme (ACE)-inhibitory**, and lipase
625 inhibitory activities of soluble fraction of fermented lentil. (B) Bidimensional contour plot for
626 desirability as function of fermentation pH and time.

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