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Enhancing Protein Extraction from Soybean Expeller: Exploring the Impact of Precipitating Agents and Flour-to-Water Ratios on Functional Properties

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Abstract

This study investigates sustainable methods for producing protein from soybean expeller via pH-shifting processes, aiming to reduce water usage in alkaline extraction by adjusting solid-to-liquid ratios per cycle and employing isoelectric precipitants like lactic acid and lactic acid bacteria (*Lactiplantibacillus plantarum and Lactococcus Lactis*) to enhance functional and antioxidant properties over a wide pH range. Results indicate that the most efficient approach involves three 1:10 (w/v) extraction cycles with lactic acid bacteria as precipitants, demonstrating high productivity and low specific water consumption. Protein content and recovery yield showed no significant differences compared to alternatives with higher water consumption or less eco-friendly precipitants. Despite lower solubility, protein products precipitated with lactic acid bacteria formed stable emulsions, exhibiting superior free radical scavenging activity.

Keywords

Soybean expeller, pH-shifting process, protein extraction yield, water consumption, precipitating agents, lactic acid bacteria.

1 Introduction

Soybean (*Glycine max*) is a profitable and suitable option to animal-derived protein sources (Ghumman et al., 2016). Particularly, by-products generated during the solvent oil extraction process, referred to as defatted soybean meals, constitute essential raw materials to produce highly soluble protein components, including soybean protein concentrates (SPC), soybean protein isolates (SPI), and textured soybean proteins (TSP) (Accoroni et al., 2019). On the other hand, the mechanical extraction of soybean oil yields another by-product, namely soybean expeller (EE). This partially defatted by-product, obtained through the extruding-expelling process, exhibits a fat content of 4.5-9 %, protein content of 30-42 %, and notably enhanced digestibility attributed to alterations in protein structures facilitated by extrusion temperatures (Ghumman et al., 2016).

In Argentina, the production of soybean expeller has exhibited almost uninterrupted growth since 2004, according to data provided by the Ministry of Agriculture, Livestock, and Fisheries from Argentina (Calzada & Ferrari, 2021). After experiencing a high average annual growth

51 rate, expeller production reached 936,000 tons processed in 2020. In light of these trends,
52 interest has surged in enhancing the value of Argentinean agricultural supply chains, with a
53 specific focus on advancing the social and economic aspects within the agricultural sector.

54 Numerous studies have reported significant advancements in the protein extraction process
55 from soybean expeller using alkaline extraction and isoelectric precipitation of solubilized
56 proteins, a technique referred to as pH-shifting, Brasil et al. (2016); Das et al. (2022); Jiang et
57 al. (2009); Zhao et al. (2023a), which is a well-established, relatively inexpensive, and efficient
58 method for attaining a high yield of protein. Other approaches have also been explored to
59 improve protein functionality or to achieve more water-efficient processes, such as membrane
60 separation and alternative precipitation methods using salts or selective solvents at the pI
61 (Preece et al., 2017a; Zhao et al., 2023b). Nevertheless, these techniques required larger
62 investment costs (Kim et al.,2015). The pH-shifting method implies extracting and solubilizing
63 proteins in a pH interval from 8 to 11, and acidifying to reach the isoelectric pH, causing around
64 90 % of globular proteins to become insoluble (Nishinari et al., 2018). Different alkalinization
65 pH values in the extraction stage have been tested since high pH values may improve the
66 protein recovery performance (Vioque et al., 2001). However, a notable drawback of the pH-
67 shifting process is its substantial water consumption during the extraction phase, leading to the
68 generation of substantial volumes of wastewater. This aspect requires careful consideration due
69 to environmental and economic concerns (Cheng et al., 2018; Hadnadjev et al., 2017).

70 Moreover, incorporating a precipitant agent generally recognized as safe (GRAS) for food
71 applications (GRAS) during the precipitation phase requires thorough testing to ensure its
72 seamless integration into industrial processes. Then, lactic acid could serve as a viable option,
73 given its GRAS classification, and considering that it is a biotechnologically significant
74 compound extensively employed in the food industry for its roles as an acidulant, pH regulator,
75 and preservative (Ojo et al., 2023). The usage of fermentation has also been reported as a means
76 of regulating the pH during the protein recovery process, particularly using GRAS lactic acid
77 bacteria (LAB). This type of microorganisms has the potential to enhance the nutritional
78 quality, prolong shelf life, and optimize gel product manufacturing in soybean processing
79 (Cheng et al., 2018; Hadnadjev et al., 2017). Lactic acid bacteria have been assessed for their
80 potential in reducing soybean allergens attributed to its protein composition (Liu et al., 2021;
81 Meinlschmidt et al., 2016). Aguirre et al. (2008) explored the utilization of LAB suspensions
82 in producing soybean hydrolysates from defatted soybean meal, noting shifts in HPLC profiles
83 with certain peaks diminishing in intensity while new peaks emerged, although total protein

84 content was not reported. Conversely, the precipitation step in the protein recovery from peas
85 was also investigated by Emkani et al. (2021), where the pH was reduced solely through lactic
86 fermentation with *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and
87 *Bifidobacterium lactis* at 37 °C. This approach increased the albumin fraction by 20-30%,
88 thereby enhancing the pea protein solubility, possibly linked to the proteolytic activity of the
89 bacteria. Therefore, these precipitants can constitute suitable substitutes for hydrochloric acid,
90 are considered to be more environmentally friendly, are derived from natural sources, and do
91 not produce hazardous by-products (Alhamad et al., 2020). Lactic acid fermentation might
92 additionally be employed to enhance the organoleptic properties of legume proteins (i.e.,
93 product taste and texture), by reducing off-flavors and improving the solubility of proteins (Liu
94 et al., 2023; Yang et al., 2020).

95 Within this framework, the aim of this investigation is to evaluate different approaches to
96 produce a protein product from soybean expeller. The primary focus involves decreasing he
97 water usage during the extraction phase of the pH-shifting process by adjusting the solid-to-
98 liquid ratio per cycle, and incorporating a GRAS precipitant agent (such as lactic acid or lactic
99 acid bacteria) in the precipitation stage to also enhance the techno functional attributes of the
100 obtained protein products. For this purpose, a design of experiments was adopted which
101 included 12 experimental runs in duplicate, given by the adoption of four different
102 combinations of solid-to-liquid ratio (1:10 or 1:20 w/v) in each of the 3 cycles of the alkaline
103 extraction stage, where the pH value was adjusted to 8.5, and the usage of three precipitant
104 agents, hydrochloric acid (HCl), lactic acid (LA), or a mixture of two strains of lactic acid
105 bacteria (LAB) in the isoelectric precipitation stage. In addition, 3 experimental runs
106 induplicate were conducted to evaluate the impact of adjusting the pH value of the alkaline
107 extraction stage to 10, for each of the precipitant agents. The performance of the protein
108 recovery process was evaluated by means of the protein recovery yield, productivity, and
109 specific water consumption. Lastly, the functional and antioxidant properties of the obtained
110 spray dried protein product were determined and analyzed.

111

112 2 Materials and methods

113 2.1 Materials

114 Soybean expeller was provided by small scale processing plants from Santa Fe province,
115 Argentina. These plants use the expeller pressing process for producing soybean oil and discard

116 the expeller as a byproduct with little economic value. Samples were kept in airtight bags and
117 maintained at -18 ºC until additional processing. Chemicals here used were of analytical quality
118 (Ciccarelli, Argentina). Commercial freeze-dried lactic acid bacteria starters (a mixture of
119 *Lactiplantibacillus plantarum* CH6072, and *Lactococcus Lactis* SR3.54) (CHR Hansen,
120 Denmark) were used.

121 **2.2 Methods**

122 Each experimental run followed the processing steps shown in Figure 1.

123 **2.2.1 Soybean expeller processing and characterization**

124 Expeller pellets were ground at room temperature using a laboratory mill (Bühler, Germany),
125 and sieved through ASTM-standard sieves to achieve a particle size that passed through a 25-
126 mesh (710 μm) sieve and was retained by a 100-mesh (150 μm) sieve.

127 The nitrogen content of the expeller was assessed through AOAC method 2001.11 (AOAC,
128 2005), and subsequently reported as % db (i.e. on a dry basis or a moisture-free basis).
129 Solubility of protein in KOH was evaluated according to the methodology described by Araba
130 & Dale, (1990). Moisture content was assessed with AOAC method 925.10 (AOAC, 2005), and
131 expressed as % wb (i.e. on a wet basis).

132 **2.2.2 Alkaline extraction**

133 The alkaline extraction process was carried out using a batch-type extractor equipped with a 6-
134 blade impeller (Precylec, Argentina), and involved three 15-min extraction cycles using water
135 as the solvent. The pH was set to either 8.5 or 10 using 1 N NaOH, and the temperature was
136 kept constant at 60 ºC. Various solid-to-liquid ratios (w/v), denoted as E1, E2, E3, and E4, and
137 outlined in Figure 2, were used in each extraction cycle to explore the potential for minimizing
138 water usage in the protein recovery process. After each cycle, the expeller was separated from
139 the protein solution, and fresh water at 60 ºC was added at the beginning of the second and
140 third cycles to adjust the solid-to-liquid ratio to the required value. The three protein solutions
141 obtained from each extraction cycle were combined into a liquid pool and transferred to a
142 beaker for subsequent precipitation.

143 **2.2.3 Isoelectric precipitation**

144 In the first two alternatives for the isoelectric precipitation, coded as HCL and LA, the liquid
145 pool was acidified at a temperature of 20 °C until the pH reached 4.5, using 0.1 N hydrochloric
146 acid (as it is a strong acid commonly used in practice), coded as HCL and lactic acid (85 %,

147 food grade), coded as LA. In both cases, the resulting mixture was allowed to settle inside a
148 refrigerator until it reached a temperature of 4 °C, which facilitated the decantation of the
149 protein product.

150 The third alternative, coded as LAB, implied an acidification with a mixture of two freeze-
151 dried lactic acid bacteria, *Lactiplantibacillus plantarum* and *Lactococcus Lactis*. These bacteria
152 were added in a proportion of 0.003 g/l in the liquid pool at a temperature of 35-37 °C for 18 h
153 in a thermostatic bath under aerobic conditions (Tecno Dalvo, Argentina). If necessary, lactic
154 acid (85 %, food grade) was used for final adjustments of the pH value to reach the isoelectric
155 point. Subsequently, the mixture was left to settle in a refrigerator until it reached 4 °C, which
156 facilitated the decantation of the protein product.

157 **2.2.4 Decantation, neutralization and spray drying**

158 The liquid supernatant of the mixture obtained at the isoelectric precipitation stage was
159 separated by decantation. Next, 5 N NaOH was added to the remaining mixture until reaching
160 a pH of 7, with stirring for 1 h at room temperature.

161 Drying was done in a co-current spray dryer (TP-S15, XI'An Toption Instrument Co., Ltd,
162 China) using a nozzle of 0.5 mm. The peristaltic pump used to feed the suspension was set at
163 15% of the maximum flow (2 l/h). The inlet air temperature was fixed at 180 °C, and the
164 resulting outlet air temperature was measured at an average value of 54-60 °C. The dried
165 powder was gathered from both the cyclone and the cylindrical components of the dryer
166 chamber and stored in sterilized flasks.

167 **2.2.5 Bacterial cell counts**

168 The concentration of lactic acid bacteria was evaluated in experiments where they were used
169 as the precipitant agent. Representative samples were cultured on MSR agar plates and
170 incubated at 37 °C for 72 h under microaerophilic conditions. Viable cell counts were
171 determined through visual inspection and expressed as colony-forming units per gram of
172 sample (CFU/g).

173 **2.2.6. Performance of the protein recovery process**

174 The evaluation of the protein recovery was conducted by assessing the nitrogen content, yield
175 of recovery, productivity, and specific water usage. Additionally, an analysis of the functional
176 characteristics and antioxidant capabilities of the resulting protein products was carried out to
177 obtain a comprehensive insight of the extraction and precipitation process.

178 The nitrogen content of the protein products was assessed through AOAC method 2001.11
179 (AOAC, 2005), and subsequently reported as % db (i.e. on a dry basis or a moisture-free basis).
180 Moisture content was assessed with AOAC method 925.10 (AOAC, 2005), and expressed as %
181 wb (i.e. on a wet basis).

182 The protein recovery yield Y_T (% db) was computed as the quantity of protein in the product
183 relative to the initial protein content in the flour, as defined in Eq. (1).

184 $$Y_T = \frac{mass\ of\ protein\ in\ the\ final\ product\ (kg\ db)}{mass\ of\ protein\ in\ the\ initial\ flour\ (kg\ db)} \quad (1)$$

185 The productivity of each protein recovery process P_T (kg product db/kg flour db) was computed
186 as the amount of product yielded per kilogram of flour, as defined in Eq. (2).

187 $$P_T = \frac{mass\ of\ final\ product\ (kg\ product\ db)}{mass\ of\ initial\ flour\ (kg\ flour\ db)} \quad (2)$$

188 The specific water consumption G_W (kg water/kg final product db) was computed as the water
189 consumed for obtaining each kilogram of the final protein product, as defined in Eq. (3).

190 $$G_W = \frac{mass\ of\ consumed\ water\ (kg\ water)}{mass\ of\ final\ product\ (kg\ product\ db)} \quad (3)$$

191 **2.2.7 Functional and antioxidant properties**

192 **2.2.7.1 Water holding capacity and oil holding capacity**

193 Water and oil holding capacities of the soybean expeller protein products were determined
194 according to Boye et al., (2010); Garcia-Vaquero et al., (2017); Stone et al., (2015) with
195 modifications. A sample (0.5 g) of the protein product was mixed in 15 ml centrifuge tubes
196 with distilled water (3 ml) or high oleic sunflower oil (3 ml) in a vortex mixer (Precytec,
197 Argentina). The pH was fixed to 2, 4.5, 7, 9.5, or 12, using 1 N HCl or 1 N NaOH, and mixed
198 with a vortex mixer for 30 sec. The pH-adjusted sample was centrifuged at 2200×g for 30 min
199 in a laboratory centrifuge (Rolco, Argentina). The liquid portion was discarded, and the
200 centrifuge tube holding the remaining solid was weighed. The water holding capacity WHC
201 (%) and oil holding capacity OHC (%) were computed as the quantity of water or sunflower
202 oil held per gram of protein product, as defined in Eq. (4-5).

203 $$WHC = \frac{mass\ of\ water\ retained\ (g)}{mass\ of\ protein\ product\ (g)} \quad (4)$$

204 $$OHC = \frac{mass\ of\ sunflower\ oil\ retained\ (g)}{mass\ of\ protein\ product\ (g)} \quad (5)$$

205 **2.2.7.2 Emulsifying capacity and emulsion stability**

206 Emulsifying capacity and emulsion stability of the soybean expeller protein products were
207 determined according to Garcia-Vaquero et al. (2017) with modifications. A protein product
208 sample (containing 0.5 g of protein db according to Kjeldahl analysis) was mixed in 50 ml
209 laboratory tubes with distilled water (10 ml) in a vortex mixer (Precytec, Argentina). The pH
210 was fixed to 2, 4.5, 7, 9.5, or 12, using 1 N HCl or 1 N NaOH, while mixed in the vortex mixer
211 for 30 sec. To create an emulsion, high oleic sunflower oil (7.5 ml) was incorporated and
212 homogenized for 30 sec at 14,000 rpm in a laboratory homogenizer (Labortechnik, Germany).
213 An equal volume of high oleic sunflower oil (7.5 ml) was again added and homogenized for
214 90 sec at 14,000 rpm in the laboratory homogenizer. Then, the homogenized sample was
215 centrifuged at 1100×g for 5 min in a laboratory centrifuge (Rolco, Argentina). The volume of
216 the emulsion layer was determined. Finally, the emulsifying capacity EC (%) was calculated
217 as the volume of the emulsion layer relative to the total volume, as defined in Eq. (6).

218 $$EC = \frac{volume\ of\ the\ emulsion\ layer\ (ml)}{total\ volumen\ (ml)} \quad (6)$$

219 Afterwards, the previously prepared emulsion was heated at 85 °C in a thermostatic bath
220 (Tecno Dalvo, Argentina) for 15 min, allowed to cool at room temperature for 10 min, and then
221 subjected to centrifugation at 1100×g for 5 min in the laboratory centrifuge. The emulsion
222 stability ES (%) was computed as the volume of the emulsion layer after heating relative to the
223 original volume of the emulsion layer, as defined in Eq. (7).

224 $$ES = \frac{volume\ of\ the\ emulsion\ layer\ after\ heating\ (ml)}{volume\ of\ the\ emulsion\ layer\ (ml)} \quad (7)$$

225 **2.2.7.3 Foaming capacity and foam stability**

226 Foaming capacity and foam stability of the soybean expeller protein products were determined
227 according to Garcia-Vaquero et al. (2017); Stone et al. (2015) with slight modifications. A
228 protein product sample (containing 0.75 g of protein db according to Kjeldahl analysis) was
229 mixed in 50 ml laboratory tubes with distilled water (10 ml) in a vortex mixer (Precytec,
230 Argentina). The pH was fixed to 2, 4.5, 7, 9.5, or 12, using 1 N HCl or 1 N NaOH, while mixed
231 in the vortex mixer for 30 sec. The mixture was homogenized for 60 s at 9,000 rpm in a
232 laboratory homogenizer (Labortechnik, Germany). The foam layer volume was determined.
233 Then, the foaming capacity FC (%) was computed as the volume of the foam layer relative to
234 the total volume, as defined in Eq. (8).

235 $FC = \frac{volume\ of\ the\ foam\ layer\ (ml)}{total\ volumen\ (ml)}$ (8)

236 Afterwards, the previously prepared mixture was left undisturbed at ambient temperature. The
237 remaining volume of the foam layer was determined at specified intervals (30 and 60 min). The
238 foam stability FS (%) was computed as the emulsion layer volume after a given time relative
239 to the original foam layer volume, as defined in Eq. (9).

240 $FS = \frac{volume\ of\ the\ foam\ layer\ after\ heating\ (ml)}{volume\ of\ the\ foam\ layer\ (ml)}$ (9)

241 **2.2.7.4 Solubility**

242 Solubility of the soybean expeller protein products was determined according to Garcia-
243 Vaquero et al. (2017); Stone et al. (2015) with modifications. A product sample (containing
244 0.1 g of protein db according to Kjeldahl analysis) was mixed in 50 ml centrifuge tube with
245 distilled water (10 ml) in a vortex mixer (Precytec, Argentina). The pH was fixed to 7, using 1
246 N HCl or 1 N NaOH, while mixed in the vortex mixer for 30 sec. The suspension underwent
247 centrifugation at 4000×g for 30 min in a laboratory centrifuge (Rolco, Argentina). A 1 g sample
248 of the supernatant was separated, and its nitrogen content was assessed through AOAC method
249 2001.11 (AOAC, 2005), and subsequently reported as % db (i.e. on a dry basis or a moisture-
250 free basis). The solubility S (%) was computed as the protein content post-centrifugation of
251 the solution compared to the protein content of the dispersion, as defined in Eq. (10).

252 $S = \frac{protein\ content\ after\ centrifugation\ of\ the\ supernatant\ of\ the\ pH\ adjusted\ solution\ (\%\ db)}{protein\ content\ of\ the\ full\ dispersion\ (\%\ db)}$ (10)

253 **2.2.7.5 Protein profiles by SDS-PAGE**

254 The protein powders obtained with HCL, LA, and LAB were analyzed using sodium dodecyl
255 sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A suspension (containing 1 mg of
256 protein db according to Kjeldahl analysis) was solubilized in 0.125 M Tris–HCl buffer with a
257 pH of 7.5 and dyed with Coommasie blue R-250. The sample was left to incubate at 85 °C for
258 15 min, and then centrifuged at 8000g for 5 min at ambient temperature. A 20 µg sample was
259 loaded into 12 % polyacrylamide gel slabs. The peptide electrophoretic pattern determination
260 was conducted employing a stable current of 20 mA per gel. All Blue Marker (Biorad, US) and
261 Protein Marker II (Serva, Germany) were used as molecular weight protein markers.

262 **2.3 Statistical analysis**

263 Results were assessed utilizing one-way and/or two-way ANOVA assuming normal
264 distribution with confidence level of 95%. Each experimental measurement was done at least

265 in duplicate. Results were presented as the mean value and standard deviation. Statistically
266 significant differences ($p < 0.05$) were detected among the experimental data when different
267 letters are shown next to them, according to post-hoc Tukey tests. Statistical analyses were
268 done in R-3.6.0 software.

269

270 **3 Results & discussion**

271 **3.1 Performance evaluation of the protein recovery process from soybean expeller**

272 The soybean expeller used as raw material was characterized as having a protein content of
273 43.76 ± 0.23 % db, a protein solubility in KOH of 67.11 ± 0.35 %, and a moisture content of
274 5.80 ± 0.16 % wb.

275 Table 1 shows the protein recovery performance from soybean expeller for experimental runs
276 using pH values of 8.5 or 10 in the alkaline extraction stage, and using hydrochloric acid, lactic
277 acid (food grade), or a combination of lactic acid bacteria along with lactic acid as precipitating
278 agent in the isoelectric precipitation stage. A pH value of 8.5 allowed to obtain a product with
279 higher protein content ($p < 0.05$, one-way ANOVA of protein content with respect to pH) and
280 expectedly better market value. Therefore, a pH value of 8.5 was selected to be used in the rest
281 of the experimental runs, since the protein recovery yield did not significantly differ from the
282 experiences where a pH value of 10 was used ($p > 0.05$, one-way ANOVA of recovery yield
283 with respect to pH), although the productivity was lower ($p < 0.05$, one-way ANOVA of
284 productivity with respect to pH) and the specific water consumption was higher ($p < 0.05$, one-
285 way ANOVA of specific water consumption with respect to pH). Mardiah et al. (2014) also
286 found no effect of alkaline extraction pH values from 8 to 10 on the final protein content of
287 protein products obtained from soybean. Accoroni et al. (2020) reported protein concentrations
288 of 60-65 % for the alkali extraction of proteins from soybean expeller flour at a pH value of
289 8.5, with recovery yields of 46-48 %. For two extruded expelled soy meals, Wang et al. (2004)
290 obtained isolates with a protein content of 79.61-80.82 % through an alkaline extraction at a
291 pH value of 8.5, with values of the protein yield of 40.46-60.89 %.

292 Table 2 shows the protein content, protein recovery yield, productivity and specific water
293 consumption for the different alternatives in the protein recovery process from soybean
294 expeller, including four different combinations of solid-to-liquid ratios in each of the 3 cycles
295 of the alkaline extraction stage, and three precipitant agents in the isoelectric precipitation
296 stage. No significant differences ($p > 0.05$, two-way ANOVA for each response with respect

to solid-to-liquid ratio and precipitant agent) were found for the protein content, yield, productivity, and specific water consumption of the protein recovery process when different flour-to-water ratios or different precipitants were used. The implemented extraction strategy with the addition of fresh water in each cycle intends to simulate a continuous counter-current extraction effect, which implies improvements of the protein recovery yield with respect to the standard process with 1 or 2 extraction cycles, Accoroni et al. (2020) as the addition of fresh solvent enhances the driving force to further extract soluble proteins that are still tightly bound (Sunley, 1995). However, significant differences ($p < 0.05$, one-way ANOVA for specific water consumption respect to solid-to-liquid ratio) were found for the specific water consumption of the protein recovery process. As a general trend, the process alternatives with three 1:10 (w/v) extraction cycles used the lowest amount of water per kilogram of obtained protein product.

The efficiency of recovery process from different matrices was previously discussed in the literature. For one extraction cycle, Preece et al., (2017) proposed a model in which the results indicate that larger volumes of water used at higher solid-to-liquid ratios lead to a significant loss of proteins in the waste stream, along with its water content, thereby reducing the extraction yield. However, Sari et al., (2015) reported higher protein yields for higher solid-to-liquid ratios, reaching a recovery maximum value for ratios higher than 1:40 (with no significant differences). Results here obtained for the recovery of proteins from soybean expeller show that larger volumes of water are not required for achieving a better protein recovery performance. Contrary to the high solid-to-liquid ratio extractions usually reported in the literature which may be suitable for protein recovery from valuable matrices at laboratory scale, the methodology here proposed could be more easily implemented at medium size scale processing plant for obtaining a food grade protein product.

For the protein recovery experiment labeled E1, LAB cell counts were assessed and recorded as follows: the original commercial freeze-dried LAB starter had 6×10^{13} CFU/g, the isoelectric precipitation stage yielded a wet product with 3×10^{12} CFU/g, and the spray drying stage produced a protein product with 1.5×10^{12} CFU/g. Notably, a marked increase in viable LAB mass was observed during the isoelectric precipitation phase. Consistent with this observation, Rezvani et al. (2017) reported that various Lactobacilli species exhibited exponential growth after 10 h of fermentation, a duration comparable to our study, despite a decline in lactic acid production. The final pH, influenced by the bacterial strain and fermentation specifics, as noted by Engels et al. (2022), hovered around 4.5 optimal for

330 isoelectric precipitation in the pH-shifting method. In contrast, the spray drying process led to
331 a decrease in viable LAB mass in the protein product, albeit to a lesser extent due to the
332 laboratory spray dryer's limited efficiency, as Moreira et al. (2021) indicated. Mora-Villalobos
333 et al. (2020) have suggested that the residual bacterial cells could affect the functional and
334 probiotic qualities of the final protein products.

335 **3.2 Evaluation of functional properties of soybean expeller protein products**

336 The advantages of incorporating soybean expeller proteins to food products like emulsions,
337 foams, or gels could potentially be enhanced by gaining a deeper understanding of how pH
338 affects their properties (Benelhadj et al., 2016). Given that target food products will vary in
339 their pH, it becomes essential to assess how pH influences the functional properties of the
340 resulting protein products.

341 Table 3 shows the experimental values for the functional attributes of the protein products
342 derived from soybean expeller. For evaluating these properties, three 15-min extraction cycles
343 using water as solvent, 1:10 (w/v) solid-to-liquid ratio, and a pH value of 8.5, were adopted in
344 the alkaline extraction stage (previously coded as E1). The isoelectric precipitation stage was
345 performed using hydrochloric acid (HCL), lactic acid (LA), or a combination of lactic acid
346 bacteria starters and lactic acid (LAB). The other processing parameters were kept at the values
347 previously stated at section 2.2. Here, water and oil holding capacities, emulsifying capacity
348 and its stability, and foaming capacity and its stability were determined at five pH values: 2,
349 4.5, 7, 9.5, and 12, while the solubility was evaluated at a pH value of 7.

350 *Water holding capacity (WHC) and oil holding capacity (OHC)*

351 The water and oil holding capacities of the protein products showed significant differences (p
352 < 0.05, two-way ANOVA for each response with respect to precipitant agent and pH) with
353 respect to some combinations of the precipitant agent for all five tested pH values, as shown in
354 Table 3. Both water and oil holding capacities of plant proteins play a crucial role determining
355 the textural qualities, such as juiciness and tenderness, of health-focused food products, making
356 them a viable alternative to meat proteins (Ashaolu, 2020; Ma et al., 2022).

357 As general trend, the protein products showed significantly larger water holding capacity ($p <$
358 0.05, two-way ANOVA for water holding capacity with respect to precipitant agent and pH)
359 when LAB was used as the precipitating agent, and specifically when the pH of the solution
360 was alkaline (fixed to 9.5 and 12). Yang et al. (2021) discussed that lactic acid bacteria
361 fermentation led to the denaturation of soybean protein isolates, which induced structural

362 alterations and prompted the formation of aggregates. These aggregates retain water, forming
363 the fundamental basis for enhancing the water holding capacity compared to non-fermented
364 samples.

365 The oil holding capacity had a comparable magnitude to the values previously reported by Ma
366 et al., (2022) for faba bean, pea, lentil and soybean protein isolates. The experimental values
367 of this functional property are determined by the arrangement of amino acids, whether charged
368 and polar or nonpolar (i.e., surface hydrophilicity vs. hydrophobicity), as well as surface
369 chemistry and porosity of the protein powders, among other factors.

370 *Emulsifying capacity (EC) and emulsion stability (ES)*

371 The emulsifying capacity and the corresponding emulsion stability of the products presented
372 significant differences ($p < 0.05$, two-way ANOVA for each response with respect to
373 precipitant agent and pH) with respect to some combinations of the precipitant agent for all
374 five tested pH values, as shown in Table 3. The emulsifying capacity indicates a sample's ability
375 to swiftly adhere to the interfaces between oil and water during the emulsification process,
376 preventing flocculation and coalescence. This property is relevant to the elaboration of many
377 food products by improving texture, preventing oil/water phases separation, and retaining
378 flavors. Stable emulsions aid in distributing flavors, thus making food products more palatable
379 (Naurzbayeva et al., 2023).

380 It is noted that no emulsion was formed at a pH value of 4.5 when hydrochloric acid or lactic
381 acid was used as the precipitant agent during the protein recovery process. Under these
382 experimental conditions, the solubilized protein product rapidly coalesced and precipitated
383 when the pH was adjusted to this value, which is the protein's isoelectric point, thus no emulsion
384 could be formed. Similarly, Wang et al., (2010) observed that an emulsion stabilized with
385 soybean protein concentrate at pH 4.5 using HCl is potentially less stable, due to the proximity
386 to the isoelectric point, where the Zeta potential tends to approach zero, indicating reduced
387 electrostatic repulsion among the colloidal particles in the emulsion, thus increasing the
388 influence of hydrophobic interactions and Van der Waals forces (McClements, 2004). A link
389 between emulsifying capacity and zeta potential was evidenced by Wang et al., (2010),
390 suggesting that emulsions exhibited enhanced stability against droplet aggregation or
391 coalescence at elevated pH levels, because of heightened electrostatic repulsion.

392 On the other hand, the LAB-precipitated products exhibited some emulsifying capacity even at
393 the isoelectric pH value. According to Aluko et al., (2009), pea protein products from lactic

394 acid bacteria precipitation presented higher sugars contents, which may potentially enhance the
395 protein solubility, thus improving the emulsifying capacity. In addition, the LAB-precipitated
396 products presented similar values of the emulsion stability than the HCL-precipitated ones, and
397 larger than the LA-precipitated ones ($p < 0.05$, one-way ANOVA for emulsion stability with
398 respect to precipitant agent). From the previous literature, contradictory accounts were found
399 regarding the influence of the production method of legumes protein products on their emulsion
400 capacity and stability, where some authors found an improvement of these properties when
401 using salt extraction, ultrafiltration, micellar precipitation, or lactic acid fermentation, while
402 other works didn't report such differences (Adebowale et al., 2011; Boye et al., 2010; Karaca
403 et al., 2011; Stone et al., 2015).

404 *Foaming capacity (FC) and foam stability (FS)*

405 Table 3 shows that the foaming capacity and the corresponding foam stability presented
406 significant differences ($p < 0.05$, two-way ANOVA for each response with respect to
407 precipitant agent and pH) with respect to some combinations of the precipitant agent for all
408 five tested pH values, as shown in Table 3. The ability of flours to form foams could be deemed
409 essential for their application in the production of non-dairy foods, as foam formation is
410 essential in the manufacturing of various food products, including ice cream, cakes, fruit
411 snacks, and foams, which underscores the significance of this functional property in the food
412 industry (Jarpa-Parra & Chen, 2021).

413 As a general trend, it is observed that the foam is more stable ($p < 0.05$, one-way ANOVA for
414 foam stability with respect to precipitant agent) when formed with protein products obtained
415 using lactic acid bacteria rather than hydrochloric acid or lactic acid as precipitant agent during
416 the protein recovery process. Foaming properties rely on protein features (migration to the
417 interface, surface tension, and alignment of hydrophobic and hydrophilic components),
418 endogenous factors (temperature, pH, protein content, and interactions with other components),
419 and parameters influencing foam creation (Emkani et al., 2022). Fermentation induces
420 alterations in the electrostatic properties of macromolecules like proteins, allowing them to
421 create dense films around each air bubble, consequently lowering the surface tension and
422 enhancing the foam capacity and stability. Consequently, the synergy between heightened
423 electrostatic charges and water-holding capacity contributes to an augmented foam stability
424 (Awuchi et al., 2019).

425 The foaming capacity values here obtained are in agreement to the ones reported by Foh et al., 426 (2012) for soybean protein products from a pH-shifting process, which ranged from about 4 % 427 at a pH value of 4 to about 102 % at a pH value of 10. However, Foh et al., (2012) presented 428 better foam stability values of 45 % at 30 min and 25 % at 60 min. The increase in foam capacity 429 at higher pH is probably a consequence of the heightened net charges on the protein, leading 430 to improved protein flexibility and a reduction in hydrophobic interactions. This facilitates 431 quicker protein diffusion to the interface between air and water, resulting in improved 432 encapsulation of air particles and enhanced foam formation, as described by Wierenga & 433 Gruppen, (2010).

434 *Protein solubility (S)*

435 The protein solubility was significantly higher ($p < 0.05$, one-way ANOVA for protein 436 solubility with respect to precipitant agent) for protein products obtained using hydrochloric 437 acid and lactic acid bacteria as a precipitant agent during the protein recovery process, with 438 respect to the solubility of protein products obtained using lactic acid, as seen in Table 3.

439 The state of the art regarding legume fermentation with lactic acid was reviewed by Emkani et 440 al., (2022), as a strategy to enhance sensory and functional properties. The authors found that 441 protein solubility depends upon factors such as production of acid by certain types of 442 microorganisms, changes in the proteins surface, occurrence of proteolysis, diminution in the 443 protein size, and others. Peng et al., (2020) reported a solubility index of around 90 % at a pH 444 value of 7.5 for protein products obtained from soybean cultivars with high initial protein 445 content of 80 %, using hydrochloric acid as precipitant agent. Even so, they concluded that the 446 solubility for different pH values is case-dependent as a function of the specific protein 447 conformation, protein surface charge, and ionic strengths during protein extraction. For the 448 nitrogen solubility of soybean protein meal samples fermented with *Lactobacillus plantarum*, 449 Amadou et al., (2010) reported values of 23-25 % at pH 7, and an increase to 99 % at pH 12. 450 Soybean protein powders commonly exhibit limited solubility in water, especially when 451 approaching neutral pH levels, posing a challenge for their integration into complex nutritional 452 formulations. O′Flynn et al., (2021) obtained solubility values of unheated soybean protein 453 isolate solutions at pH 9.0 of 28.8 %, which were notably greater compared to dispersions at 454 pH 6.9 with a solubility of 17.8 %. In contrast, Meinlschmidt et al., (2016) reported solubility 455 values of about 44, 16 and 18 % for a soybean protein isolate without fermentation and 456 fermented with *Lactobacillus helveticus* for 24 and 48 h, respectively.

457 As many authors reported for soybean products Das et al. (2022); Peng et al. (2020); Song et
458 al. (2023), the solubility profile of the alkaline-soluble proteins (i.e., the ones recovered through
459 the pH-shifting process) exhibit a bell-shaped curve, with a minimum solubility index around
460 the isoelectric point (4.5-5) and maximum values under alkaline (pH 8) and very acidic (pH 2)
461 conditions. The solubility of proteins is primarily determined by the equilibrium between
462 interactions among proteins and interactions with the solvent. As reported by Chang et al.,
463 (2015), a Pearson correlation analysis revealed a strong association between solubility and
464 protein charge, indicating that proteins with higher positive or negative charges tend to be more
465 soluble. Conversely, hydrophobicity was not found to be significantly correlated with
466 solubility. However, it is important to note that hydrophobic interactions are likely to aid in the
467 stabilization of aggregates among proteins, particularly under neutral conditions.

468 *Protein profiles by SDS-PAGE*

469 To determine the impact of the precipitant agent on polypeptide composition of the recovered
470 protein products, SDS-PAGE electrophoresis was used for the protein powders obtained with
471 HCL, LA and LAB, as shown in Figure 3. It is observed that the electrophoretic profile of the
472 protein product obtained using LAB (Lane 3) exhibits a higher intensity band of the β-
473 conglycinin subunit β (~50 kDa) and similar intensity bands of the β-conglycinin subunits α'
474 (~71 kDa) and α (~67 kDa) compared to the product obtained using HCL (Lane 1) and LA
475 (Lane 2). Moreover, the intensity of the band for glycinin, primarily composed of the acidic
476 subunit A (29–33 kDa), is higher for LAB products.

477 Meanwhile, the basic subunit B (18–22 kDa) and the 2S fraction for conglycin (lower than 18
478 kDa) do not visually show differences among the three protein products. In general terms, the
479 choice of precipitant agent does not appear to affect the subunits of peptides. These
480 observations are analogous to those reported by Meinlschmidt et al., (2016), where glycinin
481 remained unaffected by LAB fermentation, possibly attributed to the proteins structure and the
482 presence of disulfide bonds in glycinin, which impede enzymatic protein hydrolysis. Aguirre
483 et al., (2008) examined the proteolytic potential of 12 LAB strains, through a 6-h incubation at
484 37 °C of a soybean protein extract, where no discernible enzymatic activity from *Lactobacillus*
485 *fermentum*, *Lactobacillus plantarum*, or *Pediococcus pentosaceus* was observed on the
486 resultant peptide composition of the protein products.

487

488 **4. Conclusions**

489 The alternative pH-shifting processes for the recovery of soybean expeller proteins resulted in
490 products with comparable protein contents and similar protein recovery yields, when different
491 flour-water ratios were employed in the alkaline extraction or when different precipitants were
492 used for the isoelectric precipitation. No significant differences ($p > 0.05$) were found in
493 process indicators including protein content, productivity and specific water consumption of
494 the protein recovery process, which implies that a lower water volume and alternative
495 precipitant agents can be effectively employed in the protein recovery process without
496 negatively impacting its performance.

497 Furthermore, protein products obtained with lactic acid bacteria exhibited improved functional
498 properties compared to those obtained with hydrochloric acid as the precipitant agent,
499 particularly in water holding capacity and foaming capacity. Similar values were observed for
500 emulsion capacity, stability, and protein solubility across the analyzed pH range. These
501 enhanced functional attributes position lactic acid bacteria as a sustainable alternative to
502 inorganic acids as precipitant agents, which would enable the utilization of the obtained protein
503 products in diverse food technology applications. Moreover, the resultant pH-shifting process
504 could be used by small and medium-sized enterprises for onsite value adding to soybean
505 expeller through a sustainable and technically feasible protein recovery alternative.

506

507 **Declaration of Competing Interests**

508 The authors declare that they have no known competing financial interests or personal
509 relationships that could have influenced the work and results reported in this paper.

510

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701
702

703 **Tables**

704 **Table 1.** Performance of the protein recovery process from soybean expeller for different pH
705 values in the alkaline extraction stage.

	Protein content (% db)			**Protein recovery yield, Y_T (%db)**			**Productivity, P_T (kg product db/kg flour db)**			**Specific water consumption, G_W (kg water/kg final product db)**		
	HCL	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**
E1 - pH 8.5	66.78 ± 0.19 a	68.38 ± 4.50 a	59.05 ± 5.25 a	31.77 ± 6.99 a	34.14 ± 7.84 a	24.13 ± 4.58 a	0.25 ± 0.02 a	0.26 ± 0.02 a	0.21 ± 0.02 a	143.80 ± 15.70 a	140.08 ± 12.39 a	174.89 ± 11.34 a
E1- pH 10	57.94 ± 6.80 a	57.06 ± 5.20 a	54.45 ± 2.64 a	33.05 ± 7.69 a	33.74 ± 0.46 a	35.47 ± 2.21 a	0.25 ± 0.08 a	0.25 ± 0.02 a	0.28 ± 0.00 a	132.90 ± 45.90 a	123.01 ± 9.56 a	111.76 ± 1.57 a

706 Two-way ANOVA for each variable, where different letters represent significant differences
707 between experimental results. The flour-to-water ratio used in the alkaline extraction stage is
708 indicated as: E1, E2, E3, E4. The precipitant agent used in the isolectric precipitation stage is
709 indicated as: HCl, hydrochloric acid; LA, lactic acid; LAB, lactic acid bacteria.

710

Table 2. Performance of the protein recovery process from soybean expeller.

	Protein content (% db)			**Protein recovery yield, Y_T (%db)**			**Productivity, P_T (kg product db/kg flour db)**			**Specific water consumption, G_W (kg water/kg final product db)**		
	HCL	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**
E1	66.78 ± 0.19 a	68.38± 4.50 a	59.05 ± 5.25 a	31.77 ± 6.99 a	34.11 ± 7.84 a	24.13 ± 4.58 a	0.20 ± 0.04 a	0.21 ± 0.03 a	0.17 ± 0.01 a	156.80 ± 34.00 a	148.70 ± 24.58 a	179.80 ± 18.30 a
E2	68.30 ± 0.50 a	53.01 ± 0.48 a	62.38 ± 2.04 a	40.21 ± 4.00 a	30.80 ± 5.64 a	30.49 ± 2.60 a	0.25 ± 0.02 a	0.25 ± 0.04 a	0.21 ± 0.02 a	165.60 ± 15.20 a	169.806 ± 29.60 a	199.50± 23.40 a
E3	66.47 ± 9.93 a	58.97 ± 2.38 a	56.65 ± 6.79 a	34.89 ± 6.04 a	41.09 ± 2.81 a	32.17 ± 5.56 a	0.23 ± 0.07 a	0.30 ± 0.00 a	0.24 ± 0.01 a	237.60 ± 75.70 a	174.29 ± 4.91 a	214.65 ± 11.50 a
E4	61.55 ± 4.52 a	57.23 ± 2.24 a	61.56 ± 9.17 a	31.89 ± 4.49 a	39.69 ± 4.95 a	35.59 ± 6.65 a	0.22 ± 0.04 a	0.30 ± 0.05 a	0.25 ± 0.00 a	285.20 ± 60.70 a	211.70 ± 35.20 a	252.62 ± 9.72 a

Two-way ANOVA for each variable, where different letters represent significant differences between experimental results. The flour-to-water ratio used in the alkaline extraction stage is indicated as: E1, E2, E3, E4. The precipitant agent used in the isolectric precipitation stage is indicated as: HCl, hydrochloric acid; LA, lactic acid; LAB, lactic acid bacteria.

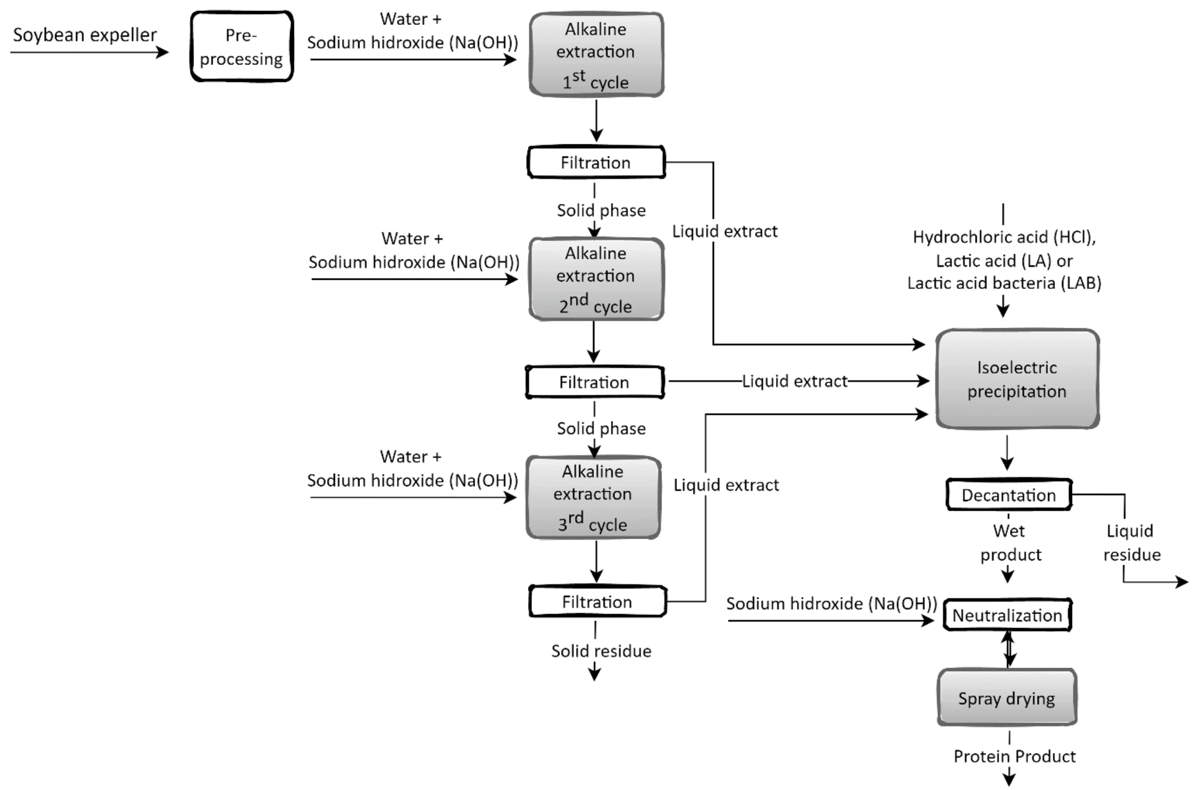
717 **Table 3.** Functional properties of protein products obtained from soybean expeller.

pH	Water holding capacity, WHC (g/g)			Oil holding capacity, OHC (g/g)			Emulsifying capacity, EC (%)			Emulsion stability, ES (%)		
	HCL	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**
2	1.20 ± 0.12 fg	1.59 ± 0.08 cdef	2.08 ± 0.17 bcd	1.92 ± 0.10 bc	2.09 ± 0.12 ab	1.91 ± 0.14 bc	47.70± 1.97 ab	44.69 ± 3.65 abcd	46.49 ± 1.24 abc	86.74 ± 7.44 a	21.95 ± 1.59 c	96.22 ± 0.10 a
4.5	2.07 ± 0.23 bcd	1.03 ± 0.12 fg	2.02 ± 0.17 bcde	1.32 ± 0.05 def	1.70 ± 0.07 bcd	1.43 ± 0.09 de	ND	ND	39.47 ± 1.23 bcd	ND	ND	17.69 ± 5.73 c
7	1.24 ± 0.10 fg	1.03 ± 0.07 fg	2.23 ± 0.10 bc	1.92 ± 0.17 bc	1.36 ± 0.08 def	1.43 ± 0.00 de	46.36 ± 1.28 abc	46.43 ± 0.00 abc	49.04 ± 2.64 a	100.00 ± 0.00 a	46.16 ± 5.44 b	98.92 ± 2.52 a
9.5	0.22 ± 0.01 h	1.38 ± 0.17 ef	2.61 ± 0.23 ab	2.43 ± 0.12 a	1.16 ± 0.02 ef	0.99 ± 0.09 f	48.18 ± 1.28 a	45.04 ± 0.57 abc	48.67 ± 3.17 a	94.37 ± 7.96 a	100.00 ± 0.00 a	98.22 ± 2.52 a
12	1.52 ± 0.12 def	0.73 ± 0.09 gh	2.92 ± 0.39 a	1.48 ± 0.06 de	1.66 ± 0.02 cd	1.43 ± 0.19 de	36.45 ± 1.48 d	38.45 ± 0.74 cd	41.02 ± 4.02 abcd	24.29 ± 6.06 c	42.19± 1.82 b	100.00 ± 0.00 a
pH	**Foaming capacity, FC (%)**			**Foam stability at 30 min, FS (%)**			**Foam stability at 60 min, FS (%)**			**Solubility, S (%)**		
	HCL	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**	**HCL**	**LA**	**LAB**
2	50.00± 4.16 c	27.94 ± 2.08 d	70.59 ± 0.00 ab	3.80 ± 0.42 ef	2.65 ± 0.35 fg	5.30 ± 0.00 bc	2.75 ± 0.21 d	1.80 ± 0.00 e	3.50 ± 0.00 c	-	-	-

4.5	23.53 ± 0.00 d	25.00 ± 2.08 d	67.65 ± 4.16 ab	1.80 ± 0.00 g	ND	4.40 ± 0.42 cde	0.60 ± 0.00 f	ND	3.50 ± 0.00 c	-	-	-
7	29.41 ± 0.00 d	50.00 ± 4.16 c	73.53 ± 4.16 a	2.65 ± 0.35 fg	4.40 ± 0.42 cde	4.10 ± 0.00 de	1.80 ± 0.00 e	3.50 ± 0.00 c	3.50 ± 0.00 c	45.86 ± 7.21 a	21.92 ± 0.46 b	42.08 ± 0.00 a
9.5	73.53 ± 4.16 a	79.41 ± 4.16 a	76.47 ± 0.00 a	5.90 ± 0.00 b	5.30 ± 0.00 bc	5.60 ± 0.42 b	4.10 ± 0.00 b	3.50 ± 0.00 c	4.70 ± 0.00 a	-	-	-
12	55.88 ± 4.16 bc	82.35 ± 8.32 a	79.41 ± 4.16 a	5.00 ± 0.42 bcd	7.10 ± 0.00 a	5.60 ± 0.42 b	4.40 ± 0.42 ab	4.70 ± 0.00 a	3.95 ± 0.21 bc	-	-	-

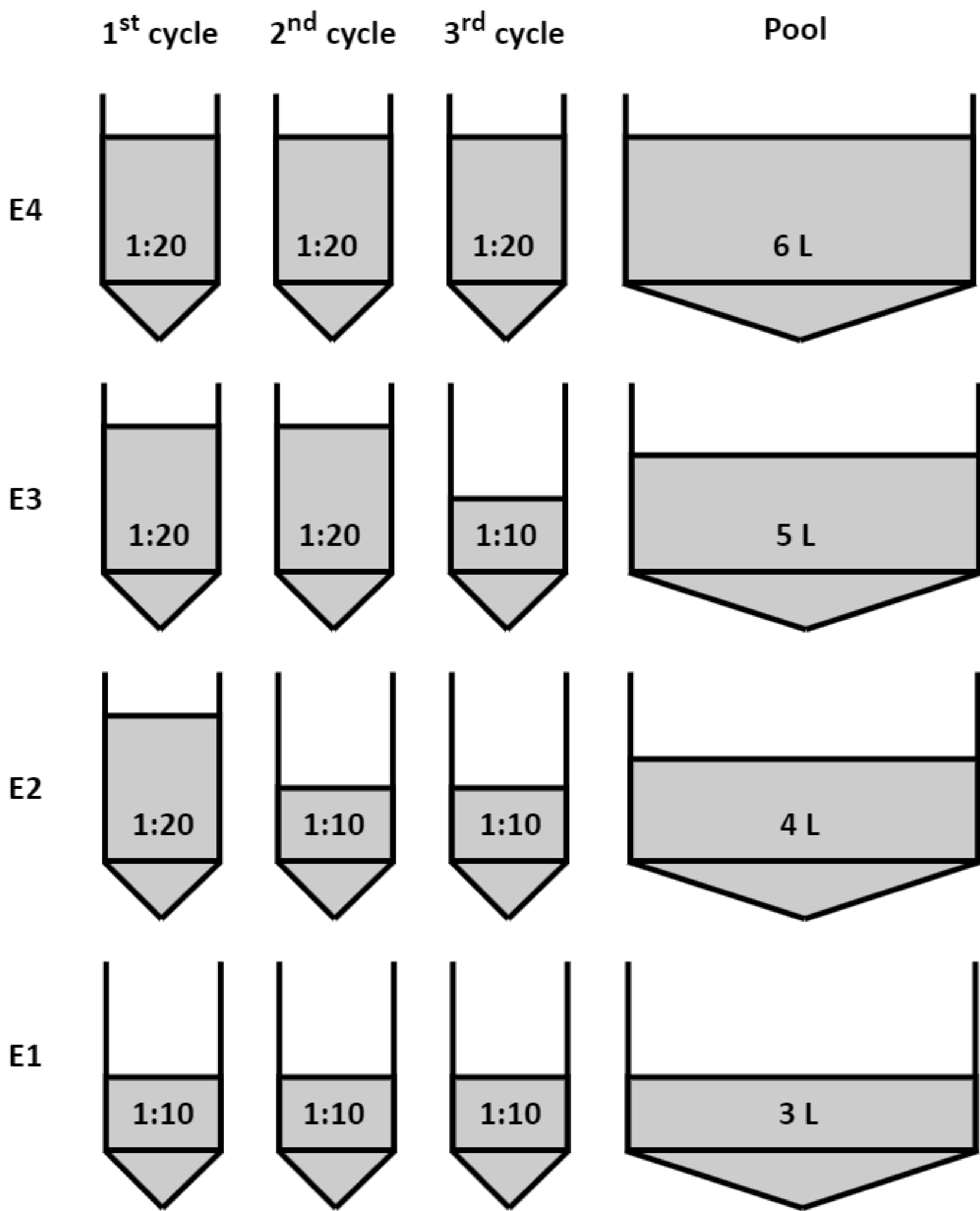
Two-way ANOVA for each variable, where different letters represent significant differences between experimental results. The precipitant agent used in the isolectric precipitation stage is indicated as: HCl, hydrochloric acid; LA, lactic acid; LAB, lactic acid bacteria. ND, i.e. no detected activity, is used to indicate that the functional property could not be determined.

723 **Figure 1**



724

725 Figure 2



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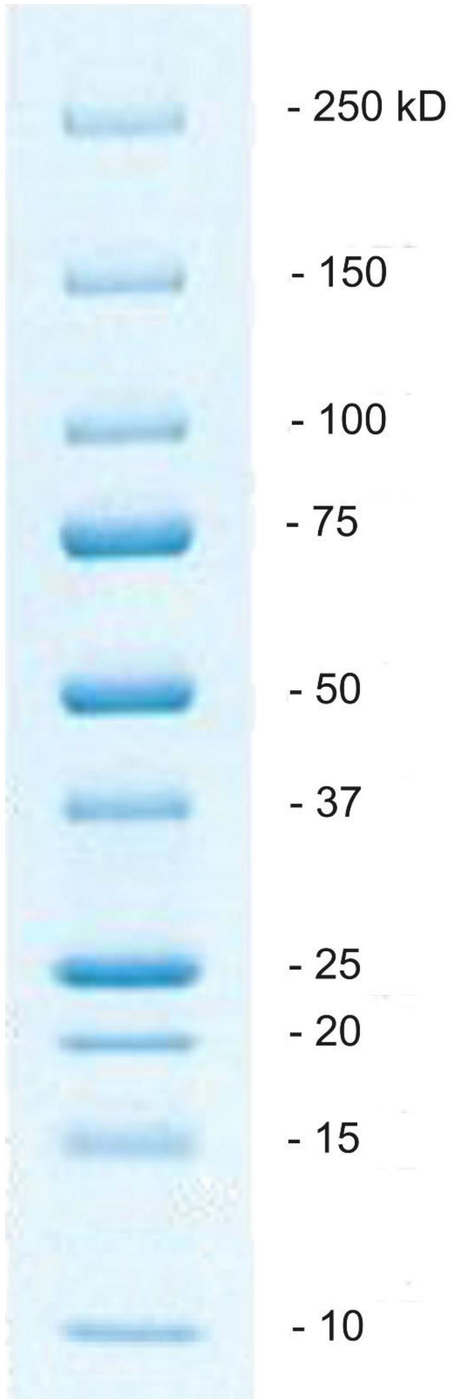
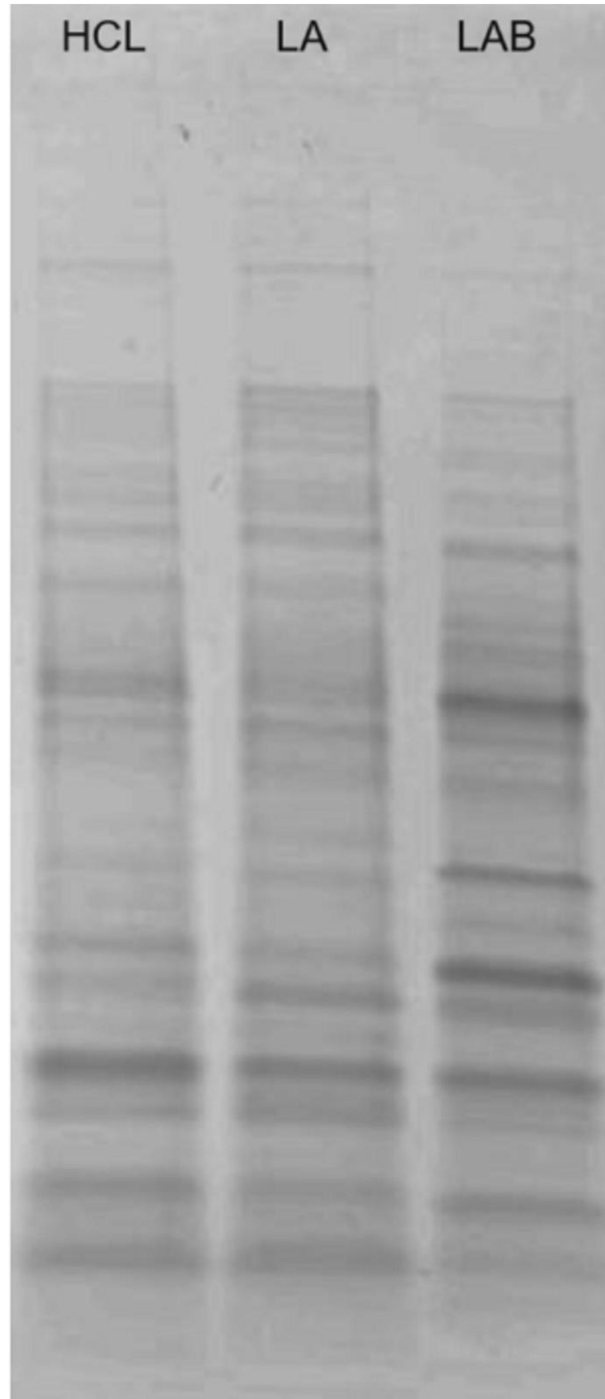
727 **Figure 3**





728

729 **Figure captions**

730 **Figure 1.** Experimental methodology for the recovery of proteins from soybean expeller, using
731 different extraction conditions and precipitant agents.

732 **Figure 2.** Representation of the flour-to-water ratio (w/v) used in each extraction cycle at the
733 alkaline extraction stage, coded as E1, E2, E3, and E4.

734 **Figure 3.** Protein profiles by SDS-PAGE for protein products precipitated with hydrochloric
735 acid (HCl), lactic acid (LA), and lactic acid bacteria (LAB).