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1 **Functional, nutritional, antioxidant and sensory properties of faba bean (*Vicia faba*, L.) seed**  
2 **protein hydrolysates and fortified apple juice, and comparative peptidomic profile**

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31 **Abstract**

32 Enzymatic hydrolysis of plant-derived proteins can improve their quality offering opportunities for  
33 food applications. In this study, three proteolytic enzymes (pepsin, trypsin, alcalase) were used, alone  
34 or combined, to produce faba bean protein hydrolysates (PHs). Their functional, nutritional and  
35 antioxidant properties were evaluated, and the peptidomic profile was assessed by LC-ESI-QO-  
36 MS/MS. Hydrolysis improved solubility of faba proteins at acidic and neutral pH, as well as their  
37 antioxidant properties. Peptidomic analysis identified 2031 peptides in the different PHs. Among  
38 them, 9 showed 100% homology with previously known antioxidant peptides and several others  
39 include in their sequences antioxidant motifs. Sensory data analysis showed that after addition of PHs  
40 to apple juice, no significant differences were perceived between control and some of the PHs. This  
41 study demonstrates that enzymatic hydrolysis enhances the functional and antioxidant properties of  
42 faba bean proteins. Specifically, hydrolysates can be used as functional food ingredients to produce  
43 fortified beverages.

44  
45 **Keywords**

46 Faba proteins; enzymatic hydrolysis; mass spectrometry; bioactive peptides; apple juice; functional  
47 food; sensory analysis.

48  
49 **Abbreviations**

50 AA: ascorbic acid; AAeq: ascorbic acid equivalents; AAS: amino acid score; ABTS: 2,2,-azino-bis(3-  
51 ethylbenz-thiazoline-6-sulfonic) acid; BV: biological value; DPPH: 1,1-DiPhenyl-2-PicrylHydrazyl;  
52 EAAI: essential amino acid index; (E/T): essential amino acids to total amino acids FC: foaming  
53 capacity; FS: foaming stability; MW: molecular weight; PAGE: polyacrylamide gel electrophoresis;  
54 PE: Faba bean protein extract; PER: protein efficiency ratio; PHs: Faba bean protein hydrolysates;  
55 SDS: sodium dodecyl sulfate; TSS: total soluble solids; WHO: World Health Organization.

## 56 **1. Introduction**

57 The faba bean (*Vicia faba*, L.) is an annual legume that grows in different climatic zones from Europe  
58 to Africa and Asia. Consumed as a food in many countries, it is noteworthy for its low cost and  
59 valuable nutritional properties – high in proteins, carbohydrates, vitamins, minerals, and dietary fibers  
60 (Multari, Stewart & Russel, 2015). The ever-increasing demand for substitutes for animal-based  
61 proteins caused by population growth is driving the scientific community's interest toward this  
62 legume. The faba beans protein content ranges from 27% to 34% of the dry weight (depending on the  
63 variety and growing conditions), with the globulin storage proteins, vicilin, and legumin, comprising  
64 about 80% (Vioque, Alaiz & Giron-Calle, 2012). (In fact, research is ongoing to increase the quality  
65 and quantity of its proteins even further through genetic selection and agricultural conditions.)  
66 Compared to the FAO profile of required amino acids, faba beans amino acid composition is low for  
67 the sulphur amino acids cysteine and methionine, as well as tryptophan (Kaldy & Kasting, 1974).  
68 One way to increase the use of faba as a valuable protein source is by combining it with cereals,  
69 which are high in those amino acids, since faba is rich in lysine, which cereals lack, thus  
70 guaranteeing a complete amino acid supply for the human diet. Another way to increase faba  
71 consumption, even in countries where it is not part of the food tradition, is to transform the protein  
72 fraction into high-quality functional food components. Typically, this transformation is accomplished  
73 through microbial fermentation or enzymatic hydrolysis (Rizzello, Tagliazucchi, Babini, Rutella, Saa  
74 & Gianotti, 2016; Wouters, Rombouts, Fierens, Brijs & Delcour, 2016). The second method,  
75 enzymatic hydrolysis, is more widespread, because of the mild processing conditions, easily  
76 controlled reaction, and minimal formation of by-products. Both these proteolytic treatments break  
77 down the primary sequence, producing active amino acids and peptides. Protein hydrolysis can create  
78 new food applications by modifying the biological, nutritional, or functional properties of proteins.  
79 The most significant biological benefits that have been reported are antioxidant, anti-hypertensive,  
80 antimicrobial and anti-carcinogenic activities (Rizzello et al., 2016). In particular, a recent work  
81 showed angiotensin-converting enzyme (ACE)-inhibitory, antioxidant and lipoxygenase-inhibitory

82 activities of faba bean proteins after fermentation with *Lactobacillus plantarum* 299v (Jakubczyk,  
83 Karas, Złotek, Szymanowska, Baraniak & Bochnak, 2019). Pepsin treatment of a faba bean protein  
84 extract significantly increased its antioxidant properties (Ali, 2019). It is worth noting that proteolysis  
85 has improved the biological properties of other foods as well. For example, lima bean (*Phaseolus*  
86 *lunatus*, L.) protein hydrolysates produced with sequential pepsin-pancreatin hydrolysis had high  
87 ACE-inhibitory activity (Chel-Guerrero, Dominguez-Magana, Martinez-Ayala, Davila-Ortiz &  
88 Betancur-Ancona, 2012). Moreover, the hydrolysis of other plant proteins such as rice, rice bran, and  
89 hemp seed with proteolytic enzymes (neutrase, pepsin, alcalase, and pancreatin) has produced  
90 peptides with antioxidant activity (Rizzello et al., 2016).

91 Second, the ability to modify nutritional properties offers the significant advantage of eliminating  
92 anti-nutritional compounds. For faba beans, in particular, this means removing the favism-inducing  
93 glycosides, vicine and convicine (Vioque et al., 2012).

94 Lastly, the third advantage of proteolysis is improving the functional properties of plant proteins. For  
95 example, the bioavailability of plant proteins is often limited because of their low solubility in  
96 aqueous media (Wouters et al., 2016). However, it was recently demonstrated that the enzymatic  
97 treatment and ultrafiltration of faba bean protein extract significantly increased its protein solubility,  
98 foaming and oil-holding capacity (Eckert, Han, Swallow, Tian, Jarpa-Parra & Chen, 2019).

99 Additionally, alcalase hydrolysis of a faba bean protein isolate increased the physical and oxidative  
100 stability of oil/water emulsions, and markedly reduced lipid oxidation during storage (Liu, Bhattarai,  
101 Mikkonen & Heinonen, 2019). Thus, enzymatic hydrolysis can create new products with enhanced  
102 bioactivity and superior nutritional and physicochemical properties compared to the original proteins.

103 The aim of the present study was: first, to evaluate the nutritional, functional, antioxidant and sensory  
104 properties of faba bean protein hydrolysates (PH) obtained using different enzymes; and second, to  
105 assess the sensorial properties of apple juice enriched with the PH. The peptidomic profiles of the PH  
106 were determined by high-resolution mass spectrometry to correlate biological activity with the  
107 released bioactive peptides. Apple juice was chosen since the apple (*Malus domestica*) is a leading

108 fruit variety in terms of world production, and its most important industrial application is the creation  
109 of juice. Adding hydrolyzed faba bean proteins addition to natural apple juice is an innovative  
110 approach to the goal of introducing a new viable protein source, increasing the commercial value of  
111 this legume while producing a healthy new drink.

112

## 113 **2. Materials and Methods**

### 114 **2.1 Raw material and chemicals**

115 The dried and completely matured Faba bean seeds (variety of Barkat) were bought from Provincial  
116 Agricultural Organization at Gorgan (Iran). Reagents were analytical grade, from Sigma (Saint Louis,  
117 MO, USA) and Merck (Darmstadt, Germany). Pre-cast gels, the MW marker for sodium dodecyl  
118 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bovine serum albumin (BSA), mass  
119 spectrometry solvents and related reagents were from Bio-Rad (Hercules, CA, USA).

120

### 121 **2.2 Preparation of faba bean protein extract (PE)**

122 Faba seeds were ground using a mill 1000 Asan Tus (Iranian Model) and passed through a 50-mesh  
123 sieve. The powder was defatted 3 times with hexane in the ratio of 1:3. The saturated solvent was  
124 replaced every 2 h. The sample was dried at room temperature and then stored at -18°C. The defatted  
125 flour was dispersed in distilled water (1:10), the pH was adjusted to 11 and the mixture was stirred  
126 for 60 min at room temperature. After centrifugation at 10518 g for 20 min, the supernatant was  
127 collected, and the pH was adjusted to 3. The precipitated proteins were recovered by centrifugation  
128 at 10518g for 20 min and then freeze-dried in an FDB 5503 dryer (Operon, Korea).

129

### 130 **2.3 Preparation of faba bean protein hydrolysates (PHs) by protease treatments**

131 The freeze-dried PE was dissolved at 4% (w/v) concentration in 100 mM phosphate buffer. The  
132 hydrolysis with single enzymes was performed using 3% (w/v) enzyme concentration, 180 min  
133 reaction time, and pH and temperature conditions optimal for each enzyme (respectively: for alcalase

134 8.5 and 50°C; for trypsin 7 and 37°C; for pepsin 2 and 37°C). The digestion reaction was stopped by  
135 inactivating the enzyme at 85°C for 15 min. After centrifugation at 10518g for 10 min, the supernatant  
136 was freeze-dried and stored at -18°C until use. Hydrolysis with two enzymes was performed  
137 sequentially, heat inactivating the first enzyme before addition of the second one. Nine faba bean  
138 protein hydrolysates were obtained, and named with the codes: **P** (PE hydrolyzed with pepsin 3%);  
139 **T** (PE hydrolyzed with trypsin 3%); **A** (PE hydrolyzed with alcalase 3%); **PT** (PE hydrolyzed with  
140 pepsin 1.5% and trypsin 1.5%); **TP** (PE hydrolyzed with trypsin 1.5% and pepsin 1.5%); **TA** (PE  
141 hydrolyzed with trypsin 1.5% and alcalase 1.5%); **AT** (PE hydrolyzed with alcalase 1.5% and trypsin  
142 1.5%); **TAd** (PE hydrolyzed with trypsin 3% and alcalase 3%); **ATd** (PE hydrolyzed with alcalase  
143 3% and trypsin 3%).

144

#### 145 **2.4 Protein pattern analysis by SDS-PAGE**

146 Protein pattern of PE and PHs was analyzed on hand-cast 14% (v/v) SDS-polyacrylamide gels using  
147 Mini-PROTEAN<sup>®</sup> equipment from Bio-Rad (Hercules, CA, USA). The Precision Plus Protein  
148 Standard from the same company was selected as MW marker.

149

#### 150 **2.5 Protein concentration by Bradford and Kjeldahl assays**

151 Soluble protein concentration and total protein content of the PE and PHs were analyzed using  
152 Bradford and Kjeldahl methods, respectively. The first assay was performed using the Quick Start  
153 Bradford Protein Assay kit from Bio-Rad (Hercules, CA, USA). The standard curve was obtained  
154 with BSA from 0.5 to 10 µg/mL. Total protein amount was determined by the Kjeldahl method  
155 (Schuman, Stanley & Knudsen, 1973) by mineralizing 1.0 g (d.w) of sample with 10 mL of 95:5 (v/v)  
156 sulphuric acid:phosphoric acid (H<sub>2</sub>SO<sub>4</sub>:H<sub>3</sub>PO<sub>4</sub>) mixture at 420°C for 180 min and subsequent  
157 distillation with 32% (v/v) sodium hydroxide (NaOH) and titration with 0.1 N H<sub>2</sub>SO<sub>4</sub>.

158

#### 159 **2.6 Analysis of functional properties**

### 160 **2.6.1 Protein solubility**

161 Solubility was determined by the method of Klompong, Benjakul, Kantachote & Shahidi (2007).  
162 Briefly, 5 mg of protein hydrolysate were dispersed in 500  $\mu$ L of deionized water and well mixed for  
163 10 min. Samples with pH values from 2 to 12 were obtained by addition of HCl or NaOH. After  
164 correction of the pH, each sample was shaken for 30 min and centrifuged at 27440 g for 5 min. Protein  
165 content in the supernatant was determined using the Bradford method, while total protein content in  
166 the sample was determined using Kjeldahl method.

167 Protein solubility (%) was calculated according to the following equation:

$$168 \text{ Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein in sample}} \times 100$$

169

### 170 **2.6.2 Foaming capacity (FC) and foaming stability (FS)**

171 FC and FS of protein extract and protein hydrolysates were determined according to the method of  
172 Jamdar, Rajalakshmi, Pednekar, Juan & Sharma (2010), with some modifications. Aliquots (20 mL)  
173 of 0.5% (w/v) sample solution were adjusted to pH 4, 6, 8 and 10, and then transferred into a 50 mL  
174 cylinder for homogenization with Ultra-Turrax T25 (IKA-Werke, Germany), at 16.000 rpm, for 2  
175 min at room temperature. The total volume was read after 30 sec. The FC (%) was calculated as  
176 follows:

$$177 \text{ FC (\%)} = \frac{(A_0 - B)}{B} \times 100$$

178 where  $A_0$  and B are the volumes (mL), respectively, after and before whipping.

179 The whipped sample was left stand at 25°C for 10 min and the volume of whipped sample was then  
180 recorded. The FS (%) was calculated as follows:

$$181 \text{ FS (\%)} = \frac{(A_t - B)}{B} \times 100$$

182 where  $A_t$  is the volume after standing (mL) and B is the initial volume, before whipping (mL).

183

## 184 2.7 Amino acid analysis and evaluation of nutritional parameters

185 A standard oven acidic hydrolysis of the proteins at high temperature was applied for the analysis of  
186 the amino acid composition (Weiss, Manneberg, Juranville, Lahm & Fountoulakis, 1998).

187 Five mg of PE and PHs were accurately transferred in an ampule and phenol was added at 0.5% (w/v)  
188 concentration. After addition of 0.6 mL HCl 6 M, the mixture was ultrasonicated for 15 min until  
189 complete dissolution. The ampule was sealed and placed in the oven for hydrolysis at 110°C for 22  
190 h. The sample was then neutralized by addition of 0.8 mL 6 M KOH, and transferred into a 5 mL  
191 volumetric flask, brought to volume with 0.1 M potassium borate buffer (pH 8.5), and finally filtered  
192 with a 0.45 µm syringe filter. For derivatization, a 90 µL aliquot of the hydrolyzed sample was  
193 transferred into a 1.5 mL vial in the presence of 10 µL of internal standard (Norvaline, IS) solution.  
194 After addition of 100 µL aliquot of 0.1 M potassium borate buffer (pH 8.5) and 200 µL aliquot of the  
195 derivatization reagent solution 9-fluorenylmethylchloroformate (FMOC-Cl, 20 mM in acetonitrile),  
196 the mixture was stirred and kept at room temperature for 20 min. Finally, formic acid (250 µL, 2%  
197 w/v) was added to terminate the derivatization reaction. The final solution was filtered through a 0.45  
198 µm syringe filter, before injection (20 µL) on a Phenomenex Kinetex Core-shell 5 µm C18 column  
199 (150 x 4.6 mm i.d.). Separations were performed using a Jasco Model LG-980-02S gradient unit, a  
200 Jasco PU-1580 pump and a Jasco UV-1575 UV/Vis detector (Jasco corporation, Tokyo, Japan) under  
201 a ternary gradient elution using aqueous ammonium formate at two different pH values (5.5 and pH  
202 7.5) and in the presence of formic acid (0.1%)/acetonitrile, 10/90 (v/v). The flow rate was 1.2 mL/min  
203 and the detection wavelength was 265 nm (Themelis, Gotti, Orlandini & Gatti, 2019).

204 Nutritional properties of PHs were determined based on their amino acid profiles. Amino acid score  
205 (AAS) was calculated using the FAO/WHO/UNU (1973) reference pattern.

$$206 \text{ AAS} = \frac{\text{mg of amino acid in 1g total protein}}{\text{mg of amino acid in requirement pattern}} \times 100$$

207 Essential Amino Acid Index (EAAI) was measured by using the amino acid composition of the whole  
208 egg protein as standard (Amza, Balla, Tounkara, Man & Zhou, 2013).

$$209 \quad EAAI = \sqrt[9]{\frac{(\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Trp})_a}{(\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Trp})_b}}$$

210 In this equation, “a” represents the content of amino acids specified in the formula in test sample  
211 and “b” the content of the same amino acids in egg standard protein (%), respectively.

212 Biological value (BV) and Protein efficiency ratio (PER) values were calculated according to Amza,  
213 Balla, Tounkara, Man & Zhou (2013).

$$214 \quad BV = 1.09 \times (EAAI) - 11.7$$

$$215 \quad PER = \begin{cases} Eq. 1 = -0.684 + 0.456 (Leu) - 0.047(Pro) \\ Eq. 2 = -0.468 + 0.453(Leu) - 0.04(Tyr) \\ Eq. 3 = -1.816 + 0.435(Met) + 0.780(Leu) + 0.211(His) - 0.944(Tyr) \\ Eq. 4 = 0.08084 (Thr + Val + Met + Ile + Leu + Phe + Lys) - 0.1094 \\ Eq. 5 = 0.06320 (Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Tyr) - 0.1539 \end{cases}$$

216 The proportion of essential amino acids to total amino acids (E/T) of the test protein was calculated  
217 as follow (Wani, Sogi, Singh & Shivhare, 2011):

$$218 \quad E/T\% = \frac{(\text{Ile} + \text{Leu} + \text{Lys} + \text{Met} + \text{Cys} + \text{Phe} + \text{Tyr} + \text{Thr} + \text{Trp} + \text{Val} + \text{His})}{(\text{Ala} + \text{Asp} + \text{Arg} + \text{Gly} + \text{Glu} + \text{His} + \text{Ile} + \text{Leu} + \text{Lys} + \text{Met} + \text{Cys} + \text{Phe} + \text{Tyr} + \text{Pro} + \text{Ser} + \text{Thr} + \text{Trp} + \text{Val})} \times 100$$

219

## 220 **2.8 In vitro antioxidant activity assays**

221 The antioxidant activity assays were performed on a microplate scale and absorbance was measured  
222 using SPARK 10M microplate reader (TECAN, Mannedorf, CH). Results were expressed as mean  
223 values of three replicates.

224 The ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity was  
225 determined according to the procedure of Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans  
226 (1999). Briefly, an ABTS stock solution (7 mM in 2.45 mM K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) was diluted with sodium acetate  
227 20 mM pH 4.5, to an absorbance at 734 nm of 0.70 ± 0.02. The solution was mixed with the sample  
228 and incubated for 30 min in the dark. The absorbance was measured at 734 nm, and value was  
229 corrected with a water blank. Activity was expressed as mg ascorbic acid (AA) eq/L by means of a  
230 calibration curve, with AA from 0 to 5 mg/L.

231 The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according  
232 to the method of Sharma & Bhat (2009). An aliquot of sample was mixed with 50 µM DPPH solution  
233 in methanol and the mixture was incubated for 30 min in the dark. The absorbance was read at 517  
234 nm and corrected with a water blank. Activity was expressed as mg AA eq/L by means of a dose-  
235 response calibration curve (from 0 to 5 mg/L) of AA.

236 Ferrous ion-chelating activity was measured according to the method reported in Tang, Kerry,  
237 Sheehan & Buckley (2002). Sample aliquot was mixed with 50 µM ferrous sulfate (FeSO<sub>4</sub>) and 300  
238 µM ferrozine. After incubation for 10 min, the absorbance was measured at 562 nm. The percentage  
239 of inhibition of ferrozine-Fe<sup>2+</sup> complex formation was calculated as follows:

$$240 \text{ Ferrous ion – chelating activity (\%)} = (A_0 - A_s)/A_0 \times 100$$

241 where A<sub>0</sub> is absorbance of the control and A<sub>s</sub> is absorbance in the presence of sample.

242

## 243 **2.9 Sensory evaluation of protein hydrolysates and fortified juices**

244 Samples were evaluated by a panel of trained judges for selecting, among PHs, the most promising  
245 to be tested diluted in apple juices. Subsequently, the selected samples were used to fortify apple  
246 juices at 1% (w/v) to be tested by consumers, by comparing them with the sole apple juice (control  
247 sample). Study protocol followed the ethical guidelines of the sensory laboratory, approved by the  
248 University of Bologna, and a written informed consent was obtained from each participant before  
249 they entered the first test.

250 Samples were firstly evaluated asking to a panel to compare each of the PHs, diluted in water with  
251 one (T) randomly selected as control. The panel consisted of 11 trained members from different  
252 sections of the Food Science Department, Cesena, Italy and the test was conducted in a sensory  
253 laboratory. Trained judges (n=11, 7 females, 4 males, age from 21 to 60; students and employees of  
254 the University of Bologna) served as panelists. Each sample was prepared by adding 0.5% (w/v) of  
255 the PHs to distilled water, and 15 mL of this solution were distributed to the assessor, in a white  
256 plastic cup. Water and unsalted breadsticks were provided for cleaning mouth during tests. After this

257 preliminary test served as screening, 30 consumers (19 female, 11 males; age from 22 to 58) were  
258 recruited to participate a test in which they were firstly encouraged to describe if they normally  
259 consumed apple juices and/or products enriched in antioxidant compounds. Then they were asked to  
260 assess the juices with an addition of PHs of 1% (w/v) and to express and to mark on a 9-point hedonic  
261 scale the previously selected attributes of: sourness, sweetness, salty taste, apple aroma and overall  
262 liking. Apple juice without any addition of the PHs was used as control. Samples were randomly  
263 distributed in 10 mL white plastic cups. The pH of the fortified apple juices was determined by a pH  
264 Meter (AMEL 33-B, Italy) and a refractometer (Kruss DR-301, Germany) was used to determine the  
265 total soluble solids (TSS) at room temperature.

266

## 267 **2.10 Peptide identification and sequencing by LC-ESI-QO-MS/MS analysis**

268 The PHs were submitted to high-resolution LC-ESI-QO-MS analysis for peptide identification. The  
269 LC-ESI-QO-MS analysis was performed with a UHPLC Ultimate 3000 coupled to a Q Exactive  
270 Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA), using the  
271 C18 column (Zorbax SB-C18 Reversed phase,  $2.1 \times 50$  mm,  $1.8 \mu\text{m}$  particle size, Agilent  
272 Technologies, Santa Clara, CA, USA). The mobile phase consisted of (A) H<sub>2</sub>O/formic acid (99.9:0.1,  
273 v/v) and (B) acetonitrile. The sample (10  $\mu\text{L}$ , 20-fold diluted) was loaded into the column at a flow  
274 rate of 0.3 mL/min. The gradient started at 2% B, and grew to 3% B in 2 min. The mobile phase  
275 composition was raised to 27% B in 19 min and then to 90% in 4 min. The mass spectrometer was  
276 tuned and calibrated according to the manufacturer's instructions. The, MS/MS spectra were then  
277 converted to .mgf files and the peptides were identified by using the Swiss-Prot database through  
278 MASCOT (Matrix Science, Boston, MA, USA) protein identification software. The following  
279 parameters were considered: enzyme, none; peptide mass tolerance,  $\pm 5$  ppm; fragment mass  
280 tolerance,  $\pm 0.12$  Da; variable modification, oxidation (M) and phosphorylation (ST); maximal  
281 number of post-translational modifications permitted in a single peptide, 4. Only peptides with a best  
282 expected value lower than 0.05 that corresponded to  $P < 0.01$  were considered. For the analysis of

283 short peptides (< 5 amino acids length), the .mgf files were analyzed by a de novo peptide sequencing  
284 approach performed by Pepnovo software (<http://proteomics.ucsd.edu/ProteoSAFe/>) using the same  
285 parameters as reported above. The assignment process was complemented and validated by the  
286 manual inspection of MS/MS spectra. The peptides identified in PHs samples were investigated in  
287 relation to their bioactivity by comparison with previously identified bioactive peptides using the  
288 BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba & Darewicz, 2008).

289

## 290 **2.11 Statistical analysis**

291 All analyses were performed in triplicate. Statistical tests were performed using Microsoft Excel  
292 statistical software XLSTAT version 2018.5 and SPSS software (SPSS16, Inc, USA). Statistical  
293 significance of differences among several means was determined using one-way analysis of variance  
294 ANOVA with Tukey test, with a significant level of  $P < 0.05$ . Sensory data were analyzed with LSD  
295 Fisher test,  $P < 0.05$ .

296

## 297 **3. Results and discussion**

### 298 **3.1 Preparation of PHs**

299 Protein hydrolysates were produced by three proteases, alcalase, pepsin and trypsin, alone or in  
300 combination. Alcalase is an endoprotease that has been widely used for the generation of protein  
301 hydrolysates, for its high activity and low cost. Alcalase-derived hydrolysates are also more resistant  
302 to digestive enzymes (Sarmadi & Ismail, 2010). Pepsin and trypsin, which are endoproteases as well,  
303 are generally used to simulate human digestion.

304 The efficacy of the hydrolytic process was checked comparing the protein/peptide profile of  
305 hydrolysates to that of the substrate on SDS-PAGE (Fig. 1). The results indicate that all of the  
306 enzymatic treatments were able to degrade high MW proteins to smaller peptides. Pepsin, alone or in  
307 combination with trypsin (samples P, PT and TP), was particularly active, producing peptides with  
308 MW lower than 15 kDa. In the alcalase hydrolysate, peptides with low MW were strongly

309 predominant. In all the other samples, some non-hydrolyzed or partially hydrolyzed proteins were  
310 still present (bands with MW between 20 and 37 kDa), indicating a lower hydrolytic efficacy.

311

### 312 **3.2 Solubility, foaming capacity and stability**

313 Solubility is one of the most important functional property of proteins. The high solubility of a  
314 protein-based product is necessary for its application in manufactured foods, especially for  
315 rheological properties such as foaming capacity. The solubility of PE and PHs at pH values in the  
316 range of 2-12, is reported in Table 1. The results indicated that solubility of both PE and PHs was  
317 pH-dependent. In the pH range of 3-7, faba bean proteins had very low solubility (from 0.07 to  
318 1.45%), but after enzymatic hydrolysis solubility significantly increased, especially in pepsin and  
319 trypsin hydrolysates (sample P, values from 41.52 to 57.54%; sample T from 8.43% to 44.03%,  
320  $P<0.05$ ). Similarly, solubility of faba bean proteins notably increased after enzymatic hydrolysis with  
321 pepsin and neutrase at pH 5 and 7 (Eckert et al., 2019). Hydrolysis of faba bean proteins by alcalase  
322 at pH 8 increased solubility of about 6-10% (Liu et al., 2019). A similar trend was also observed for  
323 peanut proteins, where hydrolysis improved the solubility up to 80%, in the pH range of 4–6 (Jamdar  
324 et al., 2010). In alkaline conditions, solubility of PE increased, as well as in pepsin and trypsin  
325 hydrolysates with maximum values at pH 12 for samples PE and T (75.75% and 56.07%,  
326 respectively) and at pH 11 for sample P (66.85%). For alcalase hydrolysate, the maximum solubility  
327 (45%) was obtained at pH 8 and 12. For all the other samples, resulted from sequential hydrolysis  
328 with two peptidases, the solubility remained significantly lower in all the pH-range evaluated  
329 ( $P>0.05$ ). These results indicated that hydrolysis with selected enzymes was a useful method to  
330 increase solubility of faba bean protein extract in the pH range of 3-7. Generally, proteins solubility  
331 depends on several factors such as pH, polarity, molecular size and hydrophilic sites. The peptides  
332 produced through hydrolysis have smaller molecular masses and less tertiary structure than parental  
333 proteins. Besides, hydrolysis liberates ionizable groups that can interact with water molecules. All  
334 these factors can improve proteins solubility (Wouters et al., 2016). The different solubility observed

335 for different PHs can be due to the specific peptide profile (molecular size and exposure of hydrophilic  
336 or hydrophobic groups) generated by each enzyme or combination of enzymes. Samples obtained by  
337 sequential hydrolysis with alcalase and trypsin (samples AT, ATd and TAd), which had low  
338 solubility, showed indeed a protein pattern with lower degree of hydrolysis on SDS-PAGE (Fig. 1).  
339 In association to solubility, another important characteristic of proteins is the ability to create stable  
340 foams. Foaming capacity provides unique texture in various foods including bread, cakes and ice  
341 cream. Foaming capacity and stability of PE and PHs are shown in Table 1. At pH 4 and 6 the PE  
342 showed poor foaming capacity, with values of 25 and 50%, respectively, which are similar to those  
343 reported for faba bean at pH 5 and 7 (31 and 67%) by Eckert et al. (2019). In acidic conditions, all  
344 PHs had higher foaming capacity than PE. Most plant proteins have limited foaming properties due  
345 to their compact structure or low solubility. Hydrolytic treatments disrupt the compact tertiary  
346 structure of native proteins and decrease their MW, facilitating their diffusion and adsorption to air-  
347 water interface, resulting in higher foaming capacity (Wouters et al., 2016; Eckert et al., 2019). The  
348 highest effect was observed for TP treatment (250% at pH 4 and 200% at pH 6), but values about  
349 three times higher than those of PE were observed for sample PT (170% at pH 6), TA (155% at pH  
350 4), AT (155% at pH 4) and ATd (160% at pH 4). At pH values of 8 and 10, the foaming capacity of  
351 PE increased to 125 and 150% respectively. Similar values were observed for PHs, with the exception  
352 of samples PT (180% at pH 8 and 230% at pH 10) and TA (200% at pH 8). Hydrolysis of faba bean  
353 proteins with different enzymes is therefore recommended for increasing the foaming capacity at low  
354 pH values. The stability of foam containing PE after 10 min was low at pH 4 and 6 (15 and 20%  
355 respectively), but increased up to 100 and 125%, at pH 8 and 10. Hydrolysis generally improved foam  
356 stability at pH 4 and 6, particularly for samples PT (60 and 140%), TP (170 and 125%) and AT (135  
357 and 100%). In basic solution, the stability of PHs was lower or in the range of PE determinations.  
358 Therefore, pH had significant effect on foaming stability, with a significant increase at acidic pH  
359 values. Reported data already confirmed that hydrolysis increased foaming stability in other plant

360 protein sources, like amaranth, bean, pumpkin, rice bran, lupin protein and corn glutelin (Wouters et  
361 al., 2016, and references therein).

362

### 363 **3.3 Amino acid composition and nutritional properties**

364 The amino acid composition of a food protein source is an essential feature in determining its  
365 nutritional value. Seeds are known to be a rich source of proteins, but they are in general deficient in  
366 some essential amino acids, which compromise their nutritional quality. For example, legumes are  
367 generally deficient in sulfur-containing amino acids (cysteine and methionine), and cereals in lysine  
368 and tryptophan. Amino acid contents of PE and PHs (in %, w/w), are reported in Table 2. The amino  
369 acid content of PE was referred to all the possible sources of amino acids in the extract (i.e. soluble  
370 and insoluble proteins, peptides and free amino acids). Whereas, the amino acid content of PHs was  
371 referred to the amino acid sources that are soluble after hydrolysis, and thus available in fortified food  
372 prepared by the addition of these hydrolysates. The amino acid profile of PE was similar to that  
373 reported from various authors on different faba cultivars (Kaldy et al., 1974 and references therein;  
374 Palander, Laurinen, Perttila, Valaja & Partanen, 2006; Vioque et al., 2012; Hendawey & Younes,  
375 2013; Eckert et al., 2019; Tab S1, supplementary material). The PE was rich in acidic aspartate  
376 (18.28%) and glutamate (22.23%) residues, which are the most abundant amino acids of globulins,  
377 the main proteins of the seed. All the essential amino acids, with the exception of tryptophan, were  
378 present in the extract. Of these, methionine was the only limiting amino acids, while all the others  
379 were present at concentrations higher than the WHO values, with leucine being the most concentrated

380 (9.27%). Among conditionally essential amino acids, arginine was the most abundant (12.55%) while  
381 cysteine was not detected, as well as the non-essential amino acids glutamine and asparagine.  
382 Hydrolysis had not an appreciable impact on the percentage content of most amino acids. An  
383 exception, common to all PHs, was the strong decrease in alanine concentration, from 14.87 to values  
384 of 3.86-4.77%. Besides, the concentration of aspartic acid was almost halved in all the hydrolysates.  
385 This amino acid, together with glutamic acid, arginine, and leucine was anyway the most abundant

386 in PHs, similarly to what observed for PE. Methionine, which was present at low concentration before  
387 hydrolysis (0.68%) and which is considered a limiting amino acid of faba bean (Kaldy et al., 1974;  
388 Hendawey et al., 2013), was not detected in the hydrolyzed samples, probably because of oxidation  
389 processes. Other significant variations were the strong increases in histidine content in pepsin  
390 hydrolysate (sample P, from 3.54 to 9.92%) and of tyrosine in hydrolysates obtained with double  
391 concentrations of combined alcalase and trypsin (samples ATd and TAd, from 3.54 to 5.34 and  
392 6.67%, respectively). The different amino acid profile of PHs can be attribute to the specificity of  
393 catalytic site and reaction mechanism of enzymes, generating peptides with different solubility.

394 Nutritional parameters of PE and PHs expressed as E/T (%), AAS (%), EAAI (%), BV (%) and PER  
395 are reported in Table 2. All PHs had E/T values higher compared to PE, overcoming the recommended  
396 value by FAO/WHO/UNU (36%), especially samples P, ATd and TAd (40.9, 41.6 and 41.7%,  
397 respectively), with the only exception of sample T (35.8%). The amino acid score (AAS) in PE and  
398 TAd was approximately equal (119%), and close to that recently reported by other authors (127%,  
399 Eckert et al., 2019). All the other PHs had AAS higher than 100%. EAAIs of PHs were in the range  
400 of 70.0–94.9%, while the BVs were in the range of 91.8 to 64.5%, with the highest values for pepsin  
401 hydrolysate (sample P, 94.9 and 91.8%). Usually, a protein source with BV between 70-100% and  
402 EAAI above 90% is assumed to be of good nutritional quality (Amza et al., 2013). All PHs, with  
403 exception of AT and ATd samples, have proven to be useful and high-quality food.

404 Protein efficiency ratio (PER) is a quality index ranging from 0 (low protein quality) up to 2 and  
405 above (high protein quality) (Amza et al., 2013). The PER values of PE and most of PHs were higher  
406 than 2. Most of PHs (except for TA, ATd and TAd) turned out to be of good (samples PE, P, A, PT,  
407 TP) or intermediate (T and AT) quality.

408 All the above results indicate that faba bean PHs represent an interesting protein supply, rich in some  
409 essential amino acids, even if not adequately balanced for human diet. They could become a strategic  
410 alternative to other more consumed plant protein sources, especially in the formulation of new

411 functional products with improved nutritional value. In particular, the high content of lysine suggests  
412 a possible use as supplement to cereal based diets and products, which lack in this amino acid.

413

### 414 **3.4 Antioxidant properties**

415 The antioxidant properties of PE and its hydrolysates were analyzed for their ABTS- and DPPH-  
416 radical scavenging activities and ferrous ion-chelating ability. The first two methods exploit the  
417 scavenging capacity of hydrogen donating-antioxidants towards the free radicals ABTS<sup>•+</sup> and DPPH<sup>•</sup>.  
418 The third one measures the ability of antioxidants to chelate transition metal ions like Fe<sup>2+</sup>. Results  
419 are reported in Fig. 2 and are expressed as mg AA eq/g of protein for ABTS and DPPH assays, and  
420 as IC<sub>50</sub> (mg/L) values for ferrous ion-chelating ability assay.

421 Radical scavenging activity of all hydrolysates was generally higher than that of the original substrate  
422 (Fig. 2, A and B), as recently observed for a faba bean pepsin hydrolysate (Ali, 2019). Among single  
423 enzyme hydrolysates, the most active was the one obtained by alcalase ( $P < 0.05$ ). ABTS value of this  
424 sample was 55.9 mg AA eq/g of protein, while the DPPH value was 26.2 mg AA eq/g of protein, ten  
425 and five times higher than PE, respectively. The higher sensitivity of ABTS method is due to the  
426 preferred interactive reaction between ABTS radical and hydroxylated aromatic compounds present  
427 in the peptides sequences (Li, Shen, Deng, Li & Ding, 2014). The differences may be related to the  
428 changes in protein composition and surface hydrophobicity values for the respective hydrolysates.  
429 Thus, peptides in hydrolysates might differently scavenge the two ABTS<sup>•+</sup> and DPPH<sup>•</sup> radicals. The  
430 combination of alcalase with trypsin, even in double concentration, and independently on the  
431 sequential order the enzymes were added to the substrate (samples TA, AT, ATd, TAd), produced  
432 hydrolysates with strongly lower antioxidant properties, with respect to the alcalase hydrolysate  
433 ( $P < 0.05$ ). These results could be correlated to the lower degree of hydrolysis observed for these  
434 samples on SDS-PAGE. Peptide bioactivity is in fact dependent on the MW of peptides, besides on  
435 the amino acid composition and sequence, being higher for smaller peptides (Rizzello et al., 2016).  
436 The highest radical scavenging activity in double enzyme hydrolysates, were obtained with the

437 combination of pepsin and trypsin (sample TP, ABTS value 59,7 mg AA eq/g of protein, and sample  
438 PT, DPPH value 28.2 mg AA eq/g of protein) enhancing the effect produced by the single enzyme  
439 hydrolysis ( $P<0.05$ ). Similar results were reported in many other studies. For instance, date protein  
440 hydrolysates produced with a mixture of enzymes (alcalase and thermolysin) had higher DPPH  
441 scavenging activity than hydrolysates produced with each enzyme separately (Ambigaipalan, Al-  
442 Khalifa & Shahidi, 2015).

443 Transition metal ions are known to stimulate lipid oxidation producing reactive peroxy and alkoxy  
444 radicals. Chelation of transition metal ions like  $Fe^{2+}$  by antioxidative peptides would prevent  
445 oxidation (Klompong et al., 2007). The  $IC_{50}$  values reported in Fig. 2C, indicate that enzymatic  
446 hydrolysis increased the  $Fe^{2+}$  chelating property of the undigested substrate up to fifty times ( $P<0.05$ ).  
447 The lowest  $IC_{50}$  values (meaning higher chelating activity) were observed for alcalase hydrolysates  
448 (sample A, 4.19 mg/L) and for combined pepsin and trypsin hydrolysates (samples PT and TP, 3.50  
449 and 3.88 mg/L, respectively). Hydrolysates prepared with trypsin alone or combined with alcalase  
450 had the lowest  $Fe^{2+}$  chelating activity (samples T and AT, 8.62 and 7.86 mg/L, respectively) ( $P<0.05$ ).  
451 Similar results were obtained by other authors on different substrates. Flaxseed protein hydrolysates  
452 produced with papain, trypsin, pancreatin, alcalase and flavourzyme were more effective as  $Fe^{2+}$   
453 chelators than the protein isolate (Karamac, Kosinska-Cagnazzo & Kulczyk, 2016). Date seed flour  
454 protein hydrolysates prepared using a mixture of alcalase and flavourzyme had the highest chelating  
455 activity compared to single enzymes (Ambigaipalan et al., 2015). During hydrolysis, peptide bond  
456 cleavage increase metal ions binding due to an increased concentration of carboxylic and amino  
457 groups from acidic and basic amino acids, respectively. This metal ions binding ability may lead to a  
458 reduction of prooxidative metal ions from the system (Liu, Kong, Xiong & Xia, 2010).

459 The above results indicate that enzymatic hydrolysis significantly improved the antioxidant potential  
460 of faba bean proteins in terms of both radical scavenging activity and transition metal ion chelation.  
461 These effects were greatly affected by the type of protease, the proteases combination as well as the

462 sequential order they were added to the substrate. Alcalase and the combination of pepsin and trypsin  
463 produced the most bioactive hydrolysates.

464

### 465 **3.5 Peptidomic profile of PHs**

466 The peptidomic profile of the different hydrolysates was analyzed by means of high-resolution mass  
467 spectrometry. The complete list of identified peptides together with the MS data is reported in  
468 supplementary material (Table S2). A total of 2031 unique peptides were identified in the different  
469 hydrolysates. The highest amount of peptides was found in the hydrolysates obtained with pepsin and  
470 trypsin in combination (PT and TP) with 656 and 659 identified peptides, respectively. According to  
471 the SDS-PAGE results, hydrolysis by combination of trypsin and alcalase (samples TA, AT, TAd and  
472 ATd) resulted in a lower amount of identified peptides respect to pepsin/trypsin combinations.  
473 Among the hydrolysates obtained after incubation with the 3 individual enzymes, alcalase was found  
474 to have the highest hydrolytic efficiency, as already proved by electrophoresis analysis.

475 As expected, Venn diagram (Figure S1, supplementary material) revealed great differences in the  
476 peptidomic profile of the sample hydrolyzed with the 3 different enzymes. Only 2 peptides  
477 (corresponding to the 0.2% of peptides identified in the three samples) were found commonly released  
478 by the three enzymes. No more peptides were found in common between peptic and tryptic  
479 hydrolysates whereas 18 peptides were commonly found in peptic and alcalase hydrolysates and 16  
480 peptides in tryptic and alcalase hydrolysates. A comparison between the peptidomic profiles of PT  
481 and TP samples indicated that the order of addition of the enzymes was of paramount importance in  
482 the release of specific peptide fragments with just 272 peptides (corresponding to the 26.1% of total  
483 peptides) commonly found in the two samples (Figure S1, supplementary material). The same  
484 consideration can be made for the hydrolysates obtained by combination of trypsin and alcalase with  
485 only 220 peptides (25.2% of total peptides) in common between TP and PT samples (Figure S1,  
486 supplementary material).

487 Sequence matching suggested that most of the released peptides were included in the sequence of  
488 faba bean vicilin and legumin B-types (Figure S2, supplementary material). The highest number of  
489 peptides originated from vicilin and legumin B-types was found in the samples hydrolyzed with both  
490 the combination of pepsin and trypsin (PT and TP samples) suggesting that the combined use of these  
491 enzymes cleaved more efficiently both the proteins.

492

### 493 **3.5.1 Identification of antioxidant peptides in PHs**

494 Several peptides with previously demonstrated antioxidant activity or sharing homology of sequence  
495 with known antioxidant peptides were identified in the different hydrolysates (Table 3). Nine  
496 peptides, sharing 100% of homology with peptides previously characterized for their antioxidant  
497 properties, were identified in the different hydrolysates. The peptides TETWNPNHPEL and FVPH  
498 have been previously reported as responsible for the antioxidant properties of chickpea protein  
499 hydrolysates (Torres-Fuentes, Contreras, Recio, Alaiz & Vioque, 2015). The remaining identified  
500 antioxidant peptides were di-peptides with a Y or W residue in their sequences. The presence of Y  
501 and/or W residues in the sequence of peptides is considered of paramount importance in determining  
502 the antioxidant effect of a peptide, because of their strong ability to donate a proton (Rival, Boeriu &  
503 Wichers, 2001; Tagliazucchi, Helal, Verzelloni & Conte, 2016). Their occurrence in the sequences  
504 of antioxidant di- and tri- peptides is actually recognized as responsible for their antioxidant activity.  
505 Several other peptides depicting structural homology with previously described antioxidant peptides  
506 were detected in the different faba beans hydrolysates. For example, as reported in Table 3, 17 and  
507 26 peptides shared the antioxidant sequences TETWNPNHPEL and ALEPDHR, respectively. All of  
508 these peptides were from the hydrolysis of faba bean legumin B-types. Both these sequences were  
509 identified as antioxidant peptides in chickpea protein hydrolysates (Torres-Fuentes et al., 2015). The  
510 antioxidant property of ALEPDHR may be due to the presence of the basic amino acids H and R.  
511 The imidazole and guanidine group of H and R, respectively, may act as donors or acceptors of

512 protons, especially when they were at the C-terminus position (Suetsuna, Ukeda & Ochi, 2000;  
513 Wallner, Hermetter, Mayer & Wascher, 2001).

514 Additional 17 peptides, released after the hydrolysis of faba bean vicilin shared in their structure the  
515 antioxidant sequence VIPAGYP. This peptide was identified as responsible for the antioxidant  
516 properties of a hydrolysate from soybean  $\beta$ -conglycinin prepared with protease S (Chen, Muramoto  
517 & Yamauchi, 1995).

518 Peptides displaying the sequence TETWNPNHPEL can be the most relevant to the total antioxidant  
519 activity of faba bean proteins hydrolyzed with pepsin and trypsin (PT). In fact, sample PT was  
520 characterized for the presence of 15 out of 17 peptides sharing this sequence. Sample PT, which was  
521 characterized for the highest DPPH radical scavenging activity, also contained the antioxidant  
522 sequences TETWNPNHPEL, FVPH, LY, IY, VY and YV. The sample hydrolyzed with trypsin and  
523 pepsin (TP), instead, showed the highest ABTS radical scavenging activity. This sample contained  
524 both the antioxidant peptides TETWNPNHPEL and FVPH as well as the antioxidant di-peptides YV.  
525 Indeed, it contained 8 and 7 peptides sharing the antioxidant sequences TETWNPNHPEL and  
526 VIPAGYP, respectively. Moreover, it was also the richest in peptides containing the antioxidant  
527 sequences PHW and IY (Saito et al., 2003; Beermann, Euler, Herzberg & Stahl, 2009). Faba beans  
528 proteins hydrolyzed with alcalase (sample A) also released peptides with high ABTS and DPPH  
529 radical scavenging activities. This sample did not contain peptides with 100% of homology with  
530 previously known antioxidant peptides. However, this sample was rich in VIPAGYP- and PHY-  
531 containing peptides (Saito et al., 2003). Most of these peptides also contained the antioxidant  
532 sequence YVE (Tian, Fang, Jiang, Guo, Cui & Ren, 2015).

533

### 534 **3.5.2 Identification of other bioactive peptides in PHs**

535 Additional 31 peptides with previously demonstrated bioactivities have been identified in the  
536 different hydrolysates prepared from faba bean protein extract (Table S3, supplementary material).  
537 The majority of the peptides (13 peptides) were both dipeptidyl-peptidase-IV (DPP-IV)- and

538 angiotensin-converting enzyme (ACE)-inhibitors whereas 12 and 6 peptides were exclusively DPP-  
539 IV- or ACE-inhibitor, respectively. The hydrolyzed samples with the highest number of identified  
540 bioactive peptides were PT and ATd, followed by sample TA. All of the three samples contained the  
541 potent ACE-inhibitory peptide VY ( $IC_{50} = 7 \mu\text{mol/L}$ ), previously isolated from brewed sake, sardine  
542 muscle proteins hydrolysates and *in vitro* digested milk. Interestingly, this dipeptide was effective  
543 also *in vivo* by decreasing blood pressure in spontaneously hypertensive rats and mild hypertensive  
544 human subjects (Saito, Wanezaki, Kawato & Imayasu, 1994; Kawasaki et al., 2000; Tagliacruzchi,  
545 Martini, Shamsia, Helal & Conte, 2018). Sample PT and TA also included the dipeptide VK, which  
546 showed very low  $IC_{50}$  value ( $13 \mu\text{mol/L}$ ). Indeed, the peptide AW ( $IC_{50} = 10 \mu\text{mol/L}$ ) was found  
547 exclusively in ATd sample. Moreover, these three samples were also characterized for the highest  
548 number of DPP-IV-inhibitory peptides.

549

### 550 **3.6 Sensory properties of PHs and fortified orange juice**

551 The literature reports that bioactive hydrolysates can produce off-flavors and bitter taste (Moller,  
552 Scholz-Ahrens, Roos & Schrezenmeir, 2008). So, to set the maximum amount of PHs to be possibly  
553 added to the apple juices, a preliminary sensory evaluation by a trained panel (11 assessors) was  
554 performed and showed that there was no significant difference between the diverse PHs solutions in  
555 water at 0.5% ( $P < 0.05$ , data not reported). Since no off flavors were detected by the panelist at this  
556 dilution, all the PHs were tested in apple juice to check the sensory consequences of their addition in  
557 a real case. As PE had no satisfactory solubility, this sample was not tested. Assuming a masking  
558 effect of the apple juice and considering the need of adding a higher amount of PHs to reach a  
559 fortifying effect, the test with the consumers was realized preparing juices added with 1% of PHs.  
560 Sensory evaluation demonstrated that the use of samples with different PHs for enrichment of apple  
561 juices, caused low turbidity with some insoluble particles (Table 4). The only exception is represented  
562 by apple juice fortified with P, which showed more sour taste without any significant difference in  
563 terms of turbidity, when compared to control ( $P > 0.05$ ). Apple juices added with TA and AT were

564 rated as the highest in sweetness (6.1 and 6, respectively) and significantly different to the samples  
565 obtained by pepsin, that showed the lowest intensity of sweet attribute ( $P<0.05$ ). The instrumental  
566 evaluation of the acidity (pH) did not show significant differences with control sample ( $P<0.05$ ). The  
567 salty taste increased, by adding PHs, but only the juice added with AT showed a significant difference  
568 if compared to the others ( $P<0.05$ ). In all apple juices, except those fortified with TP, TA and AT, no  
569 significant differences were reported ( $P>0.05$ ). The main problem, highlighted, only for a number of  
570 the supplemented apple juices, during the sensory evaluation, was the perception of bitterness, often  
571 reported by the consumers in the comments (Table 4). In particular, this was evident for juices added  
572 with P, A (reported as bitterness) and TP, AT and ATd (reported as low bitterness). The overall liking  
573 of juices fortified with ATd was even higher than control (even if not significantly), while P, TAd  
574 and A showed no significant difference with control apple juice ( $P>0.05$ ). The TSS of fortified apple  
575 juices were increased by adding PHs but they were not significantly different if compared to control  
576 apple juice ( $P>0.05$ ). Khairallah, Hettiarachchy & Rayaprolu (2016) reported that no significant  
577 differences were perceived between the freshly prepared control juice and the samples prepared by  
578 adding peptide fraction into orange juice (0.3%). The pH of the orange juice alone and with peptide  
579 fractions did not show any significant changes demonstrating that orange juice can be a potential  
580 vehicle for application of bioactive peptide fractions (Khairallah et al., 2016). Results described in  
581 the present work indicate that apple juice, similarly to the orange one, can be effectively used to  
582 incorporate hydrolysates of faba proteins as a source of amino acids and peptides with antioxidant  
583 properties.

584

#### 585 **4. Conclusions**

586 This study showed that faba beans are a suitable source of proteins of high nutritional quality,  
587 containing all essential amino acids (except tryptophan) at concentrations higher than the WHO  
588 values. It is particularly rich in leucine and arginine. Enzymatic hydrolysis improved some nutritional  
589 parameters, overcoming the problem of faba protein's poor solubility. Moreover, it produced low

590 MW peptides with increased solubility at acidic and neutral pH, improving the rheological properties  
591 such as foaming capacity and stability. All PHs showed antioxidant properties in terms of radical  
592 scavenging activity and ferrous ion chelation capacity. A complete peptidomic analysis identified  
593 several peptides with previously demonstrated antioxidant activity or sharing sequence homology  
594 with known antioxidant peptides. In order to test consumer acceptability, we conducted a sensory  
595 analysis of apple juice supplemented with faba hydrolysates, which revealed no significant  
596 differences compared to the majority of the other hydrolysates tested. Hydrolysates obtained with  
597 pepsin (alone or combined with trypsin) or alcalase might be preferable for application as food  
598 ingredients, due to their combination of nutritional, functional and bioactive properties. Importantly,  
599 the pepsin hydrolysate has the additional advantage of not altering the sensory acceptability of apple  
600 juice. These faba protein hydrolysates could be therefore an innovative ingredient in the preparation  
601 of functional foods due to their amino acid content and antioxidant properties.

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610

611 **Conflict of interest**

612 The authors declare that there are no conflicts of interest.

613

614 **Supplementary data**

615 Supplementary data associated with this article can be found in the online version.

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801 **Figure captions**

802

803 **Fig. 1.** SDS-PAGE of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v):  
804 Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5%  
805 (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd);  
806 Trypsin 3% and Alcalase 3% (ATd). pST: peptide MW marker; St: MW marker.

807

808 **Fig. 2.** ABTS radical scavenging activity (mg AAeq/g protein, **A**) DPPH radical scavenging (mg AAeq/g protein, **B**) and  
809 Ferrous ion-chelating activity (IC<sub>50</sub>, mg/L, **C**), of faba bean protein extract (PE) and its hydrolysates with enzymes  
810 (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT);  
811 Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT);  
812 Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (ATd). Means followed by the same letter did not  
813 differ significantly (Tukey test,  $P>0.05$ ).

814

**Table 1.** Functional properties (solubility, foaming capacity and foaming stability %) at different pH of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (TAd).

Solubility (%)										
pH	PE	P	T	A	PT	TP	TA	AT	ATd	TAd
2	33.58±0.29 <sup>b</sup>	41.95±0.16 <sup>a</sup>	11.02±0.16 <sup>d</sup>	16.73±0.12 <sup>c</sup>	8.01±0.17 <sup>d</sup>	7.99±0.12 <sup>d</sup>	10.25±0.06 <sup>d</sup>	2.96±0.16 <sup>e</sup>	3.81±0.12 <sup>e</sup>	4.66±0.04 <sup>e</sup>
3	1.445±0.05 <sup>d</sup>	41.52±0.12 <sup>a</sup>	8.43±0.64 <sup>c</sup>	12.39±0.02 <sup>b</sup>	3.20±0.18 <sup>d</sup>	7.87±0.06 <sup>c</sup>	9.26±0.04 <sup>bc</sup>	1.05±0.12 <sup>d</sup>	2.36±0.08 <sup>d</sup>	3.21±0.13 <sup>d</sup>
4	0.23±0.39 <sup>e</sup>	43.74±0.23 <sup>a</sup>	13.50±2.19 <sup>b</sup>	12.94±0.36 <sup>b</sup>	6.85±0.08 <sup>cd</sup>	11.56±0.07 <sup>bc</sup>	7.49±0.15 <sup>cd</sup>	1.06±0.10 <sup>e</sup>	2.71±0.14 <sup>de</sup>	3.28±0.11 <sup>de</sup>
5	0.07±0.62 <sup>g</sup>	55.27±0.06 <sup>a</sup>	13.78±0.02 <sup>cd</sup>	24.66±0.06 <sup>b</sup>	12.57±0.25 <sup>cd</sup>	16.73±0.24 <sup>c</sup>	15.80±0.27 <sup>c</sup>	3.97±0.15 <sup>fg</sup>	9.50±0.80 <sup>de</sup>	6.41±0.28 <sup>ef</sup>
6	0.93±0.01 <sup>f</sup>	44.31±0.23 <sup>a</sup>	25.94±0.09 <sup>b</sup>	18.31±0.33 <sup>c</sup>	16.92±0.05 <sup>cd</sup>	16.13±0.22 <sup>cd</sup>	27.63±0.36 <sup>b</sup>	10.15±0.08 <sup>e</sup>	11.55±0.04 <sup>e</sup>	13.76±0.11 <sup>de</sup>
7	1.05±0.28 <sup>g</sup>	57.54±1.15 <sup>a</sup>	44.03±0.10 <sup>b</sup>	23.90±0.27 <sup>d</sup>	19.54±0.17 <sup>de</sup>	19.95±0.00 <sup>de</sup>	32.19±0.28 <sup>c</sup>	14.16±0.02 <sup>f</sup>	17.53±0.01 <sup>ef</sup>	15.36±0.19 <sup>ef</sup>
8	18.32±0.15 <sup>de</sup>	47.88±0.24 <sup>a</sup>	48.44±1.26 <sup>a</sup>	45.42±0.22 <sup>a</sup>	21.43±0.06 <sup>c</sup>	20.72±0.15 <sup>cd</sup>	35.44±0.16 <sup>b</sup>	13.59±0.08 <sup>f</sup>	18.03±0.02 <sup>de</sup>	15.82±0.16 <sup>ef</sup>
9	48.85±0.04 <sup>a</sup>	46.67±0.38 <sup>a</sup>	41.58±0.69 <sup>b</sup>	25.42±0.83 <sup>d</sup>	23.05±0.45 <sup>d</sup>	19.66±0.19 <sup>e</sup>	35.79±0.14 <sup>c</sup>	13.88±0.19 <sup>f</sup>	18.23±0.19 <sup>e</sup>	18.34±0.00 <sup>e</sup>
10	24.02±0.49 <sup>d</sup>	49.53±0.02 <sup>a</sup>	43.80±0.15 <sup>b</sup>	26.44±0.52 <sup>d</sup>	23.51±0.03 <sup>d</sup>	20.00±0.00 <sup>e</sup>	36.10±0.04 <sup>c</sup>	13.95±0.13 <sup>f</sup>	18.98±0.14 <sup>e</sup>	17.30±0.01 <sup>ef</sup>
11	66.14±0.18 <sup>a</sup>	66.85±0.07 <sup>a</sup>	45.71±0.27 <sup>b</sup>	29.38±1.44 <sup>d</sup>	22.20±0.72 <sup>e</sup>	19.53±0.13 <sup>e</sup>	35.11±0.01 <sup>c</sup>	14.26±0.00 <sup>f</sup>	20.47±0.19 <sup>e</sup>	18.59±0.18 <sup>e</sup>
12	75.75±0.30 <sup>a</sup>	55.43±0.18 <sup>b</sup>	56.07±0.07 <sup>b</sup>	43.54±1.07 <sup>c</sup>	23.85±0.16 <sup>d</sup>	20.45±0.21 <sup>de</sup>	43.45±1.73 <sup>c</sup>	14.26±0.27 <sup>f</sup>	19.35±0.05 <sup>def</sup>	15.50±0.31 <sup>ef</sup>
Foaming capacity (%)										
4	25±2.50 <sup>i</sup>	70±14.00 <sup>g</sup>	140±20.00 <sup>d</sup>	65±7.00 <sup>h</sup>	80±5.00 <sup>f</sup>	250±15.00 <sup>a</sup>	155±15.00 <sup>c</sup>	155±5.00 <sup>c</sup>	160±12.00 <sup>b</sup>	105±5.00 <sup>e</sup>
6	50±5.00 <sup>i</sup>	125±10.00 <sup>e</sup>	140±14.00 <sup>c</sup>	125±5.00 <sup>e</sup>	170±15.00 <sup>b</sup>	200±12.00 <sup>a</sup>	90±5.00 <sup>g</sup>	130±12.00 <sup>d</sup>	120±6.00 <sup>f</sup>	85±10.00 <sup>h</sup>
8	125±12.00 <sup>g</sup>	105±5.00 <sup>h</sup>	125±8.00 <sup>g</sup>	140±10.00 <sup>e</sup>	180±5.00 <sup>b</sup>	125±5.00 <sup>g</sup>	200±20.00 <sup>a</sup>	135±9.00 <sup>f</sup>	150±10.00 <sup>d</sup>	160±7.00 <sup>c</sup>
10	150±10.00 <sup>d</sup>	150±20.00 <sup>d</sup>	145±12.00 <sup>e</sup>	125±5.00 <sup>f</sup>	230±20.00 <sup>a</sup>	125±10.00 <sup>f</sup>	145±10.00 <sup>e</sup>	170±5.00 <sup>b</sup>	155±15.00 <sup>c</sup>	125±5.00 <sup>f</sup>
Foaming stability after 10 min (%)										
4	15±3.00 <sup>j</sup>	25±4.00 <sup>i</sup>	80±11.00 <sup>e</sup>	35±5.00 <sup>h</sup>	60±5.00 <sup>g</sup>	170±20.00 <sup>a</sup>	90±7.00 <sup>d</sup>	135±13.00 <sup>b</sup>	130±10.00 <sup>c</sup>	65±5.00 <sup>f</sup>
6	20±2.50 <sup>h</sup>	35±5.00 <sup>f</sup>	45±7.00 <sup>e</sup>	35±4.00 <sup>f</sup>	140±15.00 <sup>a</sup>	125±8.00 <sup>b</sup>	25±4.00 <sup>g</sup>	100±15.00 <sup>c</sup>	85±10.00 <sup>d</sup>	45±11.00 <sup>e</sup>
8	100±7.00 <sup>b</sup>	20±2.50 <sup>f</sup>	40±5.00 <sup>e</sup>	60±6.00 <sup>d</sup>	100±10.00 <sup>b</sup>	40±5.00 <sup>e</sup>	100±15.00 <sup>b</sup>	80±9.00 <sup>c</sup>	80±5.00 <sup>c</sup>	105±7.00 <sup>a</sup>
10	125±10.00 <sup>b</sup>	55±6.00 <sup>f</sup>	60±7.0 <sup>e</sup>	40±5.00 <sup>h</sup>	135±9.00 <sup>a</sup>	45±2.50 <sup>g</sup>	55±7.00 <sup>f</sup>	120±10.00 <sup>c</sup>	105±11.00 <sup>d</sup>	30±5.00 <sup>i</sup>

Means followed by the same letter did not differ significantly (Tukey test,  $P>0.05$ ).

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**Table 2.** Total amino acid composition (% w/w) and nutritional parameters (PER: protein efficiency ratio; E/T: essential to total amino acids; AAS: amino acid score; EAAI: essential amino acid index; BV: biological value), of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (TAd).

g AA/ 100 g protein	Symbol	PE	P	T	A	PT	TP	TA	AT	ATd	TAd	WHO*
Arginine	Arg	12.55±3.02 <sup>a</sup>	11.5±3.91 <sup>a</sup>	13.12±3.51 <sup>a</sup>	11.48±4.82 <sup>a</sup>	11.75±0.52 <sup>a</sup>	11.69±3.55 <sup>a</sup>	11.39±0.11 <sup>a</sup>	11.27±0.02 <sup>a</sup>	10.31±0.20 <sup>a</sup>	11.45±0.42 <sup>a</sup>	
Serine	Ser	5.86±2.67 <sup>a</sup>	4.77±5.63 <sup>ab</sup>	4.77±2.28 <sup>ab</sup>	3.95±1.29 <sup>ab</sup>	4.03±5.20 <sup>ab</sup>	3.96±1.89 <sup>ab</sup>	3.14±4.68 <sup>ab</sup>	2.86±0.70 <sup>b</sup>	2.39±3.15 <sup>b</sup>	2.48±3.16 <sup>b</sup>	
Aspartic acid	Asp	18.28±1.49 <sup>a</sup>	9.37±3.09 <sup>bc</sup>	10.33±2.61 <sup>b</sup>	9.22±2.54 <sup>bc</sup>	8.77±4.27 <sup>bcd</sup>	8.86±3.77 <sup>bcd</sup>	7.46±1.64 <sup>bcd</sup>	6.90±0.24 <sup>cd</sup>	6.07±0.13 <sup>d</sup>	6.48±0.87 <sup>cd</sup>	
Glutamic acid	Glu	22.23±2.23 <sup>d</sup>	23.89±1.73 <sup>bcd</sup>	28.82±4.93 <sup>a</sup>	25.42±4.65 <sup>bc</sup>	25.78±0.16 <sup>b</sup>	25.28±1.25 <sup>bc</sup>	25.14±0.09 <sup>bc</sup>	24.41±2.22 <sup>bcd</sup>	22.65±0.17 <sup>cd</sup>	25.19±0.53 <sup>bc</sup>	
Threonine	Thr	5.45±2.65 <sup>a</sup>	3.86±3.37 <sup>a</sup>	4.17±1.64 <sup>a</sup>	3.76±4.11 <sup>a</sup>	3.50±0.63 <sup>a</sup>	3.58±1.61 <sup>a</sup>	3.92±0.18 <sup>a</sup>	3.70±0.44 <sup>a</sup>	3.49±0.61 <sup>a</sup>	4.00±0.27 <sup>a</sup>	2.3
Glycine	Gly	4.77±1.16 <sup>a</sup>	4.41±3.66 <sup>a</sup>	5.16±1.23 <sup>a</sup>	4.70±2.94 <sup>a</sup>	4.21±2.41 <sup>a</sup>	4.15±2.61 <sup>a</sup>	4.51±0.16 <sup>a</sup>	4.37±0.94 <sup>a</sup>	4.05±0.66 <sup>a</sup>	4.58±1.61 <sup>a</sup>	
Alanine	Ala	14.87±1.83 <sup>a</sup>	4.59±3.48 <sup>b</sup>	4.77±3.42 <sup>b</sup>	4.51±0.29 <sup>b</sup>	4.21±3.24 <sup>b</sup>	4.15±1.30 <sup>b</sup>	4.32±0.44 <sup>b</sup>	4.20±0.72 <sup>b</sup>	3.86±0.09 <sup>b</sup>	4.58±1.14 <sup>b</sup>	
Tyrosine	Tyr	3.00±4.55 <sup>bc</sup>	2.38±5.92 <sup>c</sup>	3.77±4.23 <sup>bc</sup>	3.20±3.85 <sup>bc</sup>	2.45±2.88 <sup>bc</sup>	2.26±5.74 <sup>c</sup>	4.71±1.77 <sup>abc</sup>	3.87±2.52 <sup>abc</sup>	5.34±0.35 <sup>ab</sup>	6.67±0.71 <sup>a</sup>	
Proline	Pro	5.45±1.51 <sup>a</sup>	6.06±4.36 <sup>a</sup>	5.76±1.24 <sup>a</sup>	4.70±4.99 <sup>a</sup>	4.38±5.48 <sup>a</sup>	4.52±5.01 <sup>a</sup>	5.10±5.14 <sup>a</sup>	4.88±3.97 <sup>a</sup>	4.41±4.46 <sup>a</sup>	5.15±4.82 <sup>a</sup>	
Methionine	Met	0.68±4.08 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	1.6
Valine	Val	5.32±2.33 <sup>a</sup>	5.14±1.77 <sup>a</sup>	5.56±4.46 <sup>a</sup>	5.27±4.57 <sup>a</sup>	4.73±0.86 <sup>a</sup>	4.71±1.89 <sup>a</sup>	5.10±0.88 <sup>a</sup>	5.21±0.28 <sup>a</sup>	4.78±0.65 <sup>a</sup>	5.34±0.55 <sup>a</sup>	3.9
Phenylalanine	Phe	4.77±3.78 <sup>a</sup>	5.33±4.74 <sup>a</sup>	5.16±2.59 <sup>a</sup>	5.46±3.46 <sup>a</sup>	5.08±4.28 <sup>a</sup>	5.09±3.44 <sup>a</sup>	4.91±0.40 <sup>a</sup>	5.05±1.15 <sup>a</sup>	4.78±1.00 <sup>a</sup>	5.15±1.45 <sup>a</sup>	
Isoleucine	Ile	5.04±2.49 <sup>a</sup>	3.86±5.15 <sup>a</sup>	4.77±3.19 <sup>a</sup>	4.51±4.10 <sup>a</sup>	4.21±0.62 <sup>a</sup>	3.96±3.27 <sup>a</sup>	4.51±0.11 <sup>a</sup>	4.54±0.74 <sup>a</sup>	4.23±1.64 <sup>a</sup>	4.58±0.98 <sup>a</sup>	3.0
Leucine	Leu	9.27±1.78 <sup>a</sup>	9.19±2.40 <sup>a</sup>	7.75±2.81 <sup>a</sup>	7.90±1.72 <sup>a</sup>	7.36±1.78 <sup>a</sup>	7.73±0.98 <sup>a</sup>	7.85±0.89 <sup>a</sup>	8.24±1.00 <sup>a</sup>	7.55±1.35 <sup>a</sup>	8.20±1.68 <sup>a</sup>	5.9
Histidine	His	3.54±5.6 <sup>b</sup>	9.92±5.63 <sup>a</sup>	4.77±6.05 <sup>b</sup>	4.70±6.22 <sup>b</sup>	4.38±6.47 <sup>b</sup>	4.15±5.95 <sup>b</sup>	4.12±5.09 <sup>b</sup>	3.53±3.55 <sup>b</sup>	3.86±5.41 <sup>b</sup>	4.00±5.07 <sup>b</sup>	1.5
Lysin	Lys	5.32±2.88 <sup>a</sup>	5.14±2.64 <sup>a</sup>	4.57±2.01 <sup>a</sup>	5.46±3.09 <sup>a</sup>	6.31±2.12 <sup>a</sup>	5.28±2.16 <sup>a</sup>	5.30±2.66 <sup>a</sup>	5.21±2.55 <sup>a</sup>	4.23±2.86 <sup>a</sup>	4.96±2.36 <sup>a</sup>	4.5
Tryptophane	Trp	0	0	0	0	0	0	0	0	0	0	0.6
Nutritional parameters		PE	P	T	A	PT	TP	TA	AT	ATd	TAd	
PER-EQ.1		3.27 <sup>a</sup>	3.50 <sup>a</sup>	2.85 <sup>a</sup>	2.92 <sup>a</sup>	2.67 <sup>a</sup>	2.84 <sup>a</sup>	2.89 <sup>a</sup>	3.07 <sup>a</sup>	2.75 <sup>a</sup>	3.05 <sup>a</sup>	
PER-EQ.2		3.51 <sup>a</sup>	3.60 <sup>a</sup>	2.89 <sup>a</sup>	2.98 <sup>a</sup>	2.77 <sup>a</sup>	2.94 <sup>a</sup>	2.90 <sup>a</sup>	3.11 <sup>a</sup>	2.73 <sup>a</sup>	2.98 <sup>a</sup>	
PER-EQ.3		3.62 <sup>ab</sup>	5.19 <sup>a</sup>	1.67 <sup>bcd</sup>	2.32 <sup>bc</sup>	2.53 <sup>bc</sup>	2.95 <sup>abc</sup>	0.73 <sup>cde</sup>	1.70 <sup>bcd</sup>	-0.15 <sup>de</sup>	-0.87 <sup>e</sup>	
PER-EQ.4		2.78 <sup>a</sup>	2.52 <sup>a</sup>	2.47 <sup>a</sup>	2.50 <sup>a</sup>	2.41 <sup>a</sup>	2.34 <sup>a</sup>	2.44 <sup>a</sup>	2.47 <sup>a</sup>	2.24 <sup>a</sup>	2.49 <sup>a</sup>	
PER-EQ.5		3.31 <sup>a</sup>	3.41 <sup>a</sup>	3.08 <sup>a</sup>	3.11 <sup>a</sup>	2.99 <sup>a</sup>	2.91 <sup>a</sup>	3.12 <sup>a</sup>	3.04 <sup>a</sup>	2.91 <sup>a</sup>	3.28 <sup>a</sup>	
E/T%		33.53 <sup>f</sup>	40.93 <sup>ab</sup>	35.78 <sup>ef</sup>	38.62 <sup>bcd</sup>	37.60 <sup>cde</sup>	37.00 <sup>de</sup>	39.84 <sup>abcd</sup>	40.06 <sup>abc</sup>	41.60 <sup>a</sup>	41.74 <sup>a</sup>	
AAS%		119.31 <sup>a</sup>	107.18 <sup>cd</sup>	109.79 <sup>bc</sup>	109.21 <sup>bc</sup>	103.31 <sup>e</sup>	100.15 <sup>f</sup>	111.48 <sup>b</sup>	110.01 <sup>bc</sup>	105.65 <sup>de</sup>	119.47 <sup>a</sup>	
EAAI%		91.48 <sup>b</sup>	94.92 <sup>a</sup>	88.31 <sup>cd</sup>	88.74 <sup>bc</sup>	86.94 <sup>cd</sup>	85.62 <sup>d</sup>	87.11 <sup>cd</sup>	75.39 <sup>e</sup>	69.95 <sup>f</sup>	88.34 <sup>cd</sup>	
BV%		88.01 <sup>b</sup>	91.76 <sup>a</sup>	84.54 <sup>c</sup>	85.02 <sup>c</sup>	83.06 <sup>cd</sup>	81.62 <sup>d</sup>	83.25 <sup>cd</sup>	70.47 <sup>e</sup>	64.54 <sup>f</sup>	84.59 <sup>c</sup>	

\*: Essential amino acid recommendation by FAO/WHO/UNU (1981) for weaned (10–12 years old) children. Means followed by the same letter did not differ significantly (Tukey test,  $P>0.05$ ).

24 **Table 3.** Peptides identified in the different faba bean protein hydrolysates that share structure homology with previously  
 25 described antioxidant peptides  
 26

<i>Peptide</i>	<i>Sample<sup>a</sup></i>
<i>Antioxidant peptides (100% homology)</i>	
<b>TETWNPNHPEL</b>	PT, TP, AT, ATd, TAd
<b>FVPH</b>	PT, TP, AT
<b>LY</b>	PT, ATd, TAd
<b>IY</b>	PT, ATd, TAd
<b>VY</b>	PT, TA, AT, ATd
<b>YV</b>	PT, TP, TA, AT, TAd
<b>LW</b>	AT, ATd
<b>IW</b>	AT, ATd
<b>AW</b>	ATd
<i>Peptides sharing the antioxidant TETWNPNHPEL sequence</i>	
RLDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	ATd
LDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u> R	T, PT, AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
DNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
NIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT
<u>NALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
<u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT
LEPDHRVESEAGL <u>TETWNPNHPEL</u>	PT
EPDHRVESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
SEAGL <u>TETWNPNHPEL</u>	PT, TP, ATd
AGL <u>TETWNPNHPEL</u>	PT, TP, AT, ATd
GL <u>TETWNPNHPEL</u> R	PT
GL <u>TETWNPNHPEL</u>	PT, TP, AT, ATd
L <u>TETWNPNHPEL</u> R	TP
L <u>TETWNPNHPEL</u>	PT, TP
<u>TETWNPNHPEL</u> R	TP
<i>Peptides sharing the antioxidant ALEPDHR sequence</i>	
RLDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	ATd
RLDNIN <u>ALEPDHR</u> VE	A, TA, AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u> R	T, PT, AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGLTET	PT
LDNIN <u>ALEPDHR</u> VESEAGLTE	AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGLT	AT, ATd
LDNIN <u>ALEPDHR</u> VESEAGL	PT, TP, AT, ATd
LDNIN <u>ALEPDHR</u> VESEAG	PT, TP
LDNIN <u>ALEPDHR</u> VESEA	PT, AT, ATd
LDNIN <u>ALEPDHR</u> VESE	AT, ATd
LDNIN <u>ALEPDHR</u> VE	TA, ATd
LDNIN <u>ALEPDHR</u>	AT, ATd
DNIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
DNIN <u>ALEPDHR</u> VESEAG	PT
DNIN <u>ALEPDHR</u> VE	A, TA, AT, ATd
NIN <u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT
<u>NALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT, AT, ATd
<u>NALEPDHR</u> VESEAGL	PT, TP, AT, ATd
<u>NALEPDHR</u> VE	AT, ATd, TAd
<u>NALEPDHR</u>	AT
<u>ALEPDHR</u> VESEAGL <u>TETWNPNHPEL</u>	PT
<u>ALEPDHR</u> VESEAGL	P, PT, TP
<u>ALEPDHR</u> VESEAG	TP

ALEPDHRVESE  
ALEPDHRVE

A, TP, AT, ATd  
A, TA, AT, ATd, TAd

*Peptides sharing the antioxidant VIPAGYP sequence*

QVQNYKAKLSPGDVLVIPAGYPVAIK  
KAKLSPGDVLVIPAGYPVAIKA  
KAKLSPGDVLVIPAGYPVAIK  
AKLSPGDVLVIPAGYPVAIKASSNLNLVGFGINAENNQR  
AKLSPGDVLVIPAGYPVAIK  
AKLSPGDVLVIPAGYPVVA  
LSPGDVLVIPAGYPVAIKASSNLNLVGFGINAENNQR  
LSPGDVLVIPAGYPVAIKA  
LSPGDVLVIPAGYPVAIK  
LSPGDVLVIPAGYPVVAI  
LVIPAGYPVAIKASSNLNL  
LVIPAGYPVAIK  
LVIPAGYPVVA  
VIPAGYPVAIKASSNLNL  
VIPAGYPVAIKASSNL  
VIPAGYPVAIKA  
VIPAGYPVAIK

T  
A  
A  
T, A  
T, TP, AT  
TP  
T, A, TP  
A  
T, A, TP, AT  
AT, ATd  
P  
TP  
P  
TP  
TP  
A  
TP, A

*Peptides sharing the antioxidant PHW sequence*

LYRNGIYAPHWNINANSLL  
LYRNGIYAPHWNINANSL  
LYRNGIYAPHWNINA  
YRNGIYAPHWNINANSLL  
YRNGIYAPHWNINANSL  
YRNGIYAPHWNINAN  
YRNGIYAPHWNINA  
YRNGIYAPHWNIN  
YRNGIYAPHW  
RNGIYAPHWNIN  
APHWNINAN

TP  
TP  
TP  
TP, P  
TP  
PT  
TP, P  
A  
P, TP  
AT, ATd  
PT

*Peptides sharing the antioxidant PHY sequence*

VNYVEINEGSLLLPHYNSR  
VNYVEINEGSLLLPHYNS  
VNYVEINEGSLLLPHYN  
YVEINEGSLLLPHYNSR  
YVEINEGSLLLPHYNS  
YVEINEGSLLLPHYN  
VEINEGSLLLPHYNSRAIV  
VEINEGSLLLPHYNSR  
VEINEGSLLLPHY  
INEGSLLLPHYNSR  
EGSLLLPHYNSR  
LPHYN

TA  
AT  
A  
TA, TAd, A  
AT  
TA, A  
P  
A  
TP, P  
A  
A  
A

*Peptides sharing the antioxidant YVE sequence*

VNYVEINEGSLLLPHYNSR  
VNYVEINEGSLLLPHYNS  
VNYVEINEGSLLLPHYN  
VNYVEINEGSLLLPH  
VNYVEIN  
VNYVE  
YVEINEGSLLLPHYNSR  
YVEINEGSLLLPHYN  
YVEINEGSLLLPH  
YVEINE  
YVEIN

TA  
AT  
A  
TAd, A  
A  
TA, ATd  
TA, TAd, A  
TA, A  
TAd, A  
A  
TA, ATd, TAd

		27
<i>Peptides sharing other antioxidant sequence</i>		
<u>ETWNP</u> NHPEL	TAd	28
<u>ETWNP</u> NHPE	A, TAd	
<u>TWNP</u> NHPEL	A, AT, TAd	29
<u>TWNP</u> NHPE	A, TAd	
<u>TWNP</u> N	TAd	
PGCPQTY <u>QEPR</u>	PT	30
TY <u>QEPR</u> SS	A	
TY <u>QEPR</u>	AT, ATd	31
<u>YQEPR</u>	TP	
<u>LYVIR</u>	A	32
<u>GTTY</u>	A	
<u>SAY</u>	TAd	33
<u>AYGE</u>	AT	
<u>AYE</u>	TP	34
<u>VTY</u>	TAd	
<u>STY</u>	TAd	35
<i>Peptides fragment of antioxidant sequence</i>		
YVR	A, TA	36
AVPY	T, PT, TA, AT	
YF	T	37
HR	PT, TA, TAd	
YN	PT, AT	38
FY	TA	
NY	TA	39
WT	AT	
SY	AT	40

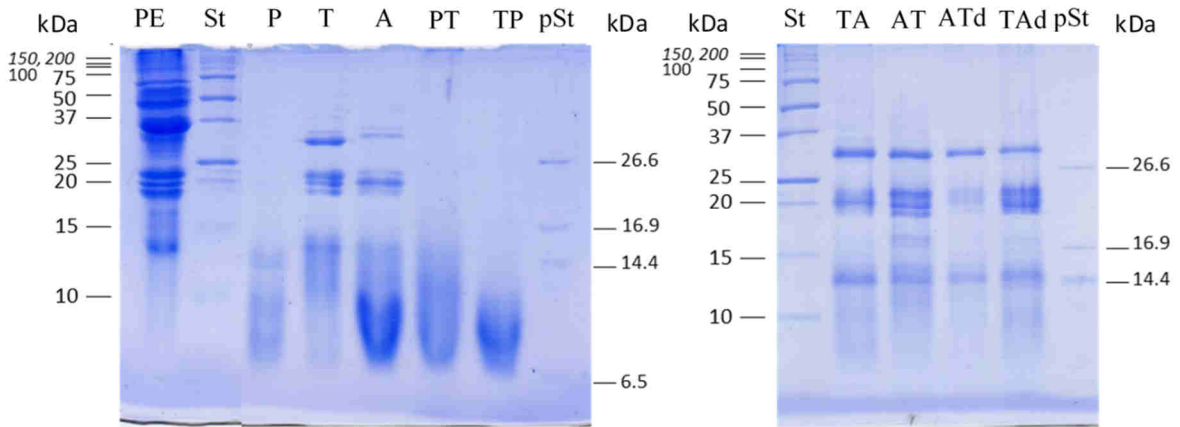
41 <sup>a</sup>Sample in which the peptide was identified (P: Pepsin 3%; T: Trypsin 3%; A: Alcalase 3%; PT: Pepsin 1.5% and  
42 Trypsin 1.5%; TP: Trypsin 1.5% and Pepsin 1.5%; TA: Trypsin 1.5% and Alcalase 1.5%; AT: Alcalase 1.5% and  
43 Trypsin 1.5%; TAd: Trypsin 3% and Alcalase 3%; ATd: Alcalase 3% and Trypsin 3%).

44 **Table 4.** Sensory properties, pH and Total soluble solids (TSS, °Brix) of apple juice fortified with faba bean protein hydrolysates (PHs). P, T, A, PT, TP, TA, AT, ATd and TAd  
 45 represent PE hydrolyzed with enzymes (% , w/v): Pepsin 3%; Trypsin 3%; Alcalase 3%; Pepsin 1.5% and Trypsin 1.5%; Trypsin 1.5% and Pepsin 1.5%; Trypsin 1.5% and Alcalase  
 46 1.5%; Alcalase 1.5% and Trypsin 1.5%; Alcalase 3% and Trypsin 3%; Trypsin 3% and Alcalase 3%. C = Control (apple juice).  
 47

Attribute	C	P	T	A	PT	TP	TA	AT	ATd	TAd
Sour	4.9±2.3 <sup>ab</sup>	5.8±1.8 <sup>a</sup>	3.8±2.2 <sup>cd</sup>	4.1±1.9 <sup>bc</sup>	3.6±1.8 <sup>cd</sup>	5.5±1.9 <sup>a</sup>	2.9±1.7 <sup>d</sup>	3.1±1.7 <sup>cd</sup>	3.1±1.8 <sup>cd</sup>	3.0±1.8 <sup>d</sup>
Sweet	5.4±1.7 <sup>a</sup>	4.1±1.9 <sup>b</sup>	5.4±1.8 <sup>a</sup>	5.5±1.9 <sup>a</sup>	5.9±1.8 <sup>a</sup>	4.4±1.9 <sup>b</sup>	6.0±1.9 <sup>a</sup>	6.1±1.8 <sup>a</sup>	5.8±1.8 <sup>a</sup>	5.9±1.7 <sup>a</sup>
Salty	2.1±1.2 <sup>b</sup>	3.0±1.9 <sup>b</sup>	2.6±1.4 <sup>b</sup>	2.3±1.6 <sup>b</sup>	2.6±1.8 <sup>b</sup>	4.1±2.5 <sup>a</sup>	2.7±1.9 <sup>b</sup>	2.7±1.9 <sup>b</sup>	2.5±1.8 <sup>b</sup>	2.8±1.9 <sup>b</sup>
Apple aroma	6.5±2.1 <sup>a</sup>	4.6±1.9 <sup>c</sup>	6.0±1.9 <sup>ab</sup>	5.6±1.9 <sup>abc</sup>	5.8±2.0 <sup>abc</sup>	4.7±2.0 <sup>c</sup>	5.7±1.9 <sup>abc</sup>	5.8±1.6 <sup>abc</sup>	6.9±5.2 <sup>a</sup>	5.0±2.1 <sup>bc</sup>
Overall acceptance	5.0±1.7 <sup>ab</sup>	5.3±1.5 <sup>ab</sup>	4.8±2.0 <sup>bc</sup>	4.8±2.1 <sup>bc</sup>	4.3±2.2 <sup>bcd</sup>	3.9±2.1 <sup>cd</sup>	3.7±2.2 <sup>d</sup>	3.4±2.1 <sup>d</sup>	6.0±2.1 <sup>a</sup>	5.0±2.0 <sup>ab</sup>
Comments	The most appreciated smell Clear	Bitterness Sour	The most turbid sample; Presence of precipitate	Presence of precipitate; Good smell, the most similar to apple; Low bitterness; Little black points inside	Low turbidity, with fruits pieces,	Low bitterness, Presence of insoluble particles	Low turbidity Unpleasant smell, Salty and sour	Low turbidity Low bitterness, Smell of yeast	Low astringency, low bitterness Turbidity, Flour-like smell	Low turbidity Artificial taste Presence of other fruit smell more than apple, Precipitate
pH	3.41 <sup>a</sup>	3.27 <sup>a</sup>	3.71 <sup>a</sup>	3.78 <sup>a</sup>	3.93 <sup>a</sup>	3.23 <sup>a</sup>	4.13 <sup>a</sup>	4.13 <sup>a</sup>	4.03 <sup>a</sup>	4.13 <sup>a</sup>
TSS	11.2 <sup>b</sup>	12.4 <sup>ab</sup>	11.9 <sup>ab</sup>	13 <sup>a</sup>	11.8 <sup>ab</sup>	12.1 <sup>ab</sup>	11.9 <sup>ab</sup>	11.9 <sup>ab</sup>	12.1 <sup>ab</sup>	11.8 <sup>ab</sup>

48 Means followed by the same letter did not differ significantly in the same raw (for sensory data, LSD Fisher test was applied,  $P>0.05$ ; for pH and TSS values, Tukey test was applied,  $P>0.05$ ).  
 49

1 **Figure 1.**  
2



3 **Figure 2.**  
 4  
 5

