Membrane fractionation of *Cynara cardunculus* swine blood hydrolysate: ingredients of high nutritional and nutraceutical value

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Abstract

This work proposes an innovative approach to valorise swine blood based on enzymatic hydrolysis and membrane fractionations. Hydrolysis with *Cynara cardunculus* enzymes, followed by microfiltration and double nanofiltration generated three high protein fractions, retentate of microfiltration (RMF; >0.5 µm) and retentate of nanofiltration

(RNF; >3 kDa) with approximately 90% of protein on a dry basis and filtrate of nanofiltrate (FNF; <3 kDa) with 65%. FNF, rich in low molecular weight peptides, showed excellent antioxidants (ABTS and ORAC of 911.81 and 532.82 µmol TE g^{-1} DB, respectively) and antihypertensive (IC_{50} of 28.51 µg mL^{-1}) potential. By peptidomics and *in silico* analysis, 43 unique sequences of interest were found, among which LVV-Hemorphin-7 was identified. This hemorphin was demonstrated as the main responsible for the observed bioactivity. Complementary results showed a prebiotic effect mainly for the growth of *Bifidobacterium animalis* Bo, as well as interesting free amino acids (mainly glutamic acid, leucine, alanine, phenylalanine and aspartic acid) and mineral (e.g., Ca, Mg, P, K and Na) profiles. No antibacterial effect was verified for the seven pathogenic bacteria tested. This study allowed obtaining new ingredient of high nutritional and nutraceutical value for human consumption, with a perspective of sustainability and industrial viability.

Keywords (8/8): swine blood; enzymatic hydrolysis; bioactive peptides; ACE inhibitor; antioxidant activity; BIOPEP; functional ingredient; waste valorisation.

1. Introduction

The increasing demand for meat and meat products has led to exponential livestock production (Alao et al., 2017). About half of the EU's annual meat production was ascribed to the pork industry, with 22.8 million tons reported in this last report (Eurostat, 2020). Daily, the meat industry generates high amounts of by-products such as blood, bones and skin (Bah et al., 2013; Toldrà et al., 2018). In the EU, over 20 million tons of animal by-products are reported annually (Jędrejek et al., 2016), while globally, approximately 60 million are estimated (Hou et al., 2017). Developing strategies to

51 valorise these losses will increase the food system's efficiency and sustainability (Araújo-
52 Rodrigues et al., 2021).

53 Blood is one of the main meat industry by-products, possessing an attractive
54 nutritional value and functional properties (Alao et al., 2017; Bah et al., 2013; Borrajo et
55 al., 2019; Hou et al., 2017; Toldrà et al., 2018). Raw blood is a homogenous plasma and
56 blood cells mixture (Toldrà et al., 2018). According to Gorbatov (1988), swine blood
57 composition contains 79.2% of moisture, 18.5% of protein, 0.9% of minerals, 0.15% of
58 fat and 0.07% of carbohydrates. Thus, blood possesses a high protein content, comprising
59 all essential amino acids (AA) and several bioactive peptides. This by-product is also rich
60 in minerals, mainly iron (Alao et al., 2017; Appiah & Peggy, 2012; Toldrà et al., 2018).

61 Beyond the blood's nutritional and functional profile, the high amounts generated
62 and associated environmental pollution result in a high economic potential (Appiah &
63 Peggy, 2012; Bah et al., 2013; Toldrà et al., 2018). Several applications have been
64 described for blood by-products, for instance, animal feed, food ingredients,
65 nutraceuticals, pharmaceuticals and fertilisers (Toldrà et al., 2018). According to recent
66 reports, although blood is a good and sustainable source of functional proteins, only a
67 small fraction (approximately 30%) is directed toward the food industry (Bah et al., 2013;
68 Toldrà et al., 2018). The main products using blood and its derivatives are related to the
69 pet food industry, followed by the fertilisers sector (Toldrà et al., 2018). This fact could
70 be explained by the consumers' reluctance concerning blood-derived products (e.g.,
71 transmission risk of spongiform encephalopathies) (Bah et al., 2013; Jędrejek et al., 2016;
72 Toldrà et al., 2018) and stricter regulations for human food application (European
73 Parliament and Council, 2009; Toldrà et al., 2018). Regarding the fertiliser industry, low
74 processing and research costs are generally involved (Toldrà et al., 2018).

75 The reported attractive value and the need for more sustainable protein sources
76 for human nutrition raise the importance of developing effective strategies to increase
77 blood added value and valorisation for human application (Toldrà et al., 2018). In the last
78 two decades, advancements in blood collection and processing strategies increased blood
79 use (Appiah & Peggy, 2012). Firstly, the microbial and chemical quality should be
80 ensured, guaranteeing its safety and quality (Jędrejek et al., 2016). Some blood processing
81 approaches, such as concentration, separation, hydrolysis, purification and others,
82 significantly increase blood market value. These processing strategies typically result in
83 protein and peptide extracts with higher functional properties (Jin et al., 2020; Nedjar-
84 Arroume et al., 2020; Sun & Luo, 2011; Toldrà et al., 2018). The drying techniques are
85 widely used to produce highly stable and longer shelf-life blood products (Appiah &
86 Peggy, 2012; Jędrejek et al., 2016; Toldrà et al., 2018).

87 Result from protein hydrolysis, the bioactive peptides (generally with 2 to 20 AA
88 residues; <3 kDa) may possess better solubility, techno-functional and biological
89 properties (Borrajo et al., 2019; Coscueta et al., 2019; Toldrà et al., 2018; Verma et al.,
90 2017). Antioxidant, antimicrobial (antibacterial or antifungal) and other promising
91 properties have been reported for blood hydrolysates, being advantages of blood
92 hydrolysates over those derived from other sources (Abou-Diab et al., 2020; Appiah &
93 Peggy, 2012; Borrajo et al., 2019; Sanchez-Reinoso et al., 2021). In addition, peptides
94 from natural sources may be safer than synthetic (Appiah & Peggy, 2012; Bah et al.,
95 2013). Enzymatic hydrolysis is one of the most described techniques for generating
96 bioactive peptides from different protein sources (Borrajo et al., 2019; Cunha & Pintado,
97 2021; Verma et al., 2017). Swine blood hydrolysates have been produced using
98 pancreatin and papain, alcalase, flavourzyme and bromelain, resulting in antioxidant and

99 antimicrobial hydrolysates (Abou-Diab et al., 2020; Sanchez-Reinoso et al., 2021; Sun &
100 Luo, 2011; Verma et al., 2019).

101 Different proteases may produce hydrolysates with varied peptide types and
102 molecular weights (MW), resulting in distinct biological and functional properties
103 (Borrajo et al., 2019; Cunha & Pintado, 2021; Verma et al., 2017; Voss et al., 2019).
104 *Cynara* sp., *Ficus* sp. and other plant extracts are reported as very proteolytic (Araújo-
105 Rodrigues et al., 2020). *Cynara cardunculus* L. is a flower found in southern and western
106 Mediterranean regions, Portugal and Canary Islands, associated with traditional cheese
107 coagulation due to its intense proteolytic action and similar characteristics to other
108 aspartic proteinases (Araújo-Rodrigues et al., 2020; Voss et al., 2019). The violet flowers
109 of *C. cardunculus* L. contain proteolytic enzymes (cyprosins, cardosins or cynarase
110 groups). This plant grows spontaneously (Araújo-Rodrigues et al., 2020), which may be
111 a more sustainable source than animal sources.

112 Specifically, Cardosin A and Cardosin B are proteolytic enzymes that possess
113 different AA sequences, comparable with animal enzymes chymosin and pepsin,
114 respectively. Nevertheless, the Cardosin B enzyme has less specificity than the
115 gastrointestinal pepsin enzyme and hydrolyses phenylalanine, leucine, tyrosine or valine
116 bonds (Voss et al., 2019). *C. cardunculus* isolated enzymes also possess lower specificity
117 than chymosin, however, these exhibit higher proteolytic activity and higher secondary
118 proteolytic specificity regarding the hydrolysis of α_S- and β-caseins (Araújo-Rodrigues et
119 al., 2020). Until now, no studies have reported the release of bioactive peptides from
120 blood by-products based on *C. cardunculus* enzymes. The broadening of its use will boost
121 its cultivation, which has particular importance in the scenario of climate changes.

122 Accordingly, an innovative approach based on enzymatic hydrolysis and
123 membrane technology system was developed in the present study. This work tested a

124 vegetable enzymatic extract obtained from the flower of *C. cardunculus* since it has not
125 been previously reported for blood hydrolysates production. The hydrolysis,
126 microfiltration and nanofiltration membrane systems produced three novel blood
127 products, whose biological and bioactive potential was investigated.

128

129 ## 2. Materials and methods

130 ### 2.1. Materials and chemicals

131 Cooked swine blood was kindly provided by Primor Group (Portugal) as raw
132 material. *C. cardunculus* extract was acquired from Formulab (Portugal). Sulphuric acid,
133 Kjeldahl tablets (Catalyst with 0.3% $CuSO_4 \cdot 5\,H_2O$) and petroleum ether (40-60 ºC) were
134 provided by VWR Scientific (VWR chemicals, Karlsruhe, Germany). Boric acid, nitric
135 acid (65%), hydrogen peroxide (30%) and hydrochloric acid (32%) were purchased from
136 Merck (Damdstadt, Germany), while sodium hydroxide from LabChem (USA). The plate
137 count agar (PCA), rose bengal chloramphenicol agar (RBCA) and Mueller Hinton agar
138 (MHA) were purchased from Biokar (France) and violet red bile glucose agar (VRBGA)
139 from VWR Scientific.

140 Concerning the proteins standards used namely, Thyroglobulin (669 kDa),
141 Ferritin (440 kDa), Aldolase (158 kDa), Conalbumin (75 kDa), Ovalbumin (43 kDa),
142 Carbonic anhydrase (29 kDa), Ribonuclease A (13.7 kDa), Aprotinin (6.5 kDa) were
143 obtained from GE Healthcare (USA) and an antihypertensive peptide with sequence
144 KGYGGVSLPEW (99.7%; 1.2 kDa) from GenScript (China).

145 ABTS diammonium salt (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic
146 acid)), phosphate-buffered saline (≥ 99%; PBS), fluorescein, 2,2′-azo-bis-(2-
147 methylpropionamidine)-dihydrochloride (≥ 97%; AAPH), 6-hydroxy-2,5,7,8-
148 tetramethylbroman-2-carboxylic acid (≥ 97%; Trolox) and angiotensin-I converting

149 enzyme (peptidyl-dipeptidase A, EC 3.4.15.1, 5.1 U mg^{-1}) were supplied by Sigma-
150 Aldrich (St. Louis, MO, USA). The peptide Abz-Gly-Phe(NO_2)-Pro was provided by
151 Bachem Feinchemikalien (Switzerland).

152 Benzene-1,2-dicarboxaldehyde (OPA), o-Phthalaldehyde, o-Phthalic
153 dicarboxaldehyde and AA standards were supplied by Sigma-Aldrich. The standard mix
154 of minerals (UCP-3) was provided by Inorganic ventures (USA). The probiotic strains
155 used were *Lactobacillus acidophilus* Ki (LAS) (isolated from fermented milk, CSK,
156 Netherlands), *Lacticaseibacillus casei (L. casei)* L26 (DSM - Moorebank, NSW,
157 Australia), *Bifidobacterium animalis* spp. lactis Bb12 (Chr. Hansen, Denmark) and
158 *Bifidobacterium animalis* Bo (CSK, Ede, Netherlands).

159

160 **2.2. Blood collection**

161 According to conventional practices used to slaughter pigs, the blood was
162 collected in a licensed slaughterhouse (Matadouro Central de Entre Douro e Minho, S.A;
163 Veterinary Control Number D36). Succinctly, the animals were stunned electrically
164 through scissor-type stunning tongs positioned between both eye and ear sides, for head-
165 only electrical stunning. Posteriorly, the swine were transported to slaughter procedures,
166 suspended by a hind limb and moved down. To guarantee the hygienic, safe and
167 maximum collection of swine blood, the bleeding was carried out using a vampire knife,
168 inserting the knife into the thoracic cavity and severance of the carotid artery and jugular
169 vein. The blood was collected in a special canister, covered with a sterile plastic bag. The
170 swine blood by-product was followed and traceable to collect blood from animals
171 considered safe for human consumption. All the management followed the animal by-
172 products (ABPs) regulation of the European Parliament (EC No 1069/2009) for category
173 3 (European Parliament and Council, 2009).

174 After collection, the swine blood by-product was subjected to a cooking process
175 at 100 °C for approximately 45 min. This process was performed at the slaughterhouse
176 and envisaged stabilising the product before being transported to another company that
177 treats by-products from the meat industry. After the cooling process in refrigeration, the
178 cooked blood was adequately stored and identified as "*Category M3*- Products not taken
179 care of for human consumption- Intended for research and diagnosis". This product was
180 refrigerated until the processing step, within a maximum period of 24 h. The cooked
181 swine blood was analysed in three different batches.
182

183 **2.3. Physicochemical and microbiological characterisation of cooked blood**

184 The dry matter and ashes of cooked swine blood were determined according to
185 NP1614-1:2009 (Instituto Português da Qualidade, 2009) and NP1615:2002 (Instituto
186 Português da Qualidade, 2002) Portuguese standards, respectively. The total fat content
187 was determined by the Soxhlet method based on NP1613:1979 (Instituto Português da
188 Qualidade, 1979). The Kjeldahl method was used concerning the total nitrogen content
189 of cooked swine blood based on NP1612 (Instituto Português da Qualidade, 2006). All
190 analyses were performed in duplicate.
191 For microbiological analysis, total aerobic bacteria (TAB) were evaluated
192 according to the international standard (ISO) 4833-1, where TAB was enumerated in PCA
193 media after incubation for 72 h at 30 °C. *Enterobacteriaceae* were grown at 37 °C for 24
194 h in VRBGA, while yeasts and moulds were enumerated on RBCA at 30 °C for 3 to 5
195 days. All analyses were performed in duplicate, and the microbial counts average was
196 expressed in log (CFU g^{-1}).
197

198 **2.4. Enzymatic hydrolysis and membrane technology**

199 Swine cooked blood was enzymatically hydrolysed with 4% of *C. cardunculus*
200 enzymes for 4 h at 55 ºC and pH 5.2. 200 kg of cooked swine blood were homogenised
201 and thoroughly mixed with 300 kg of water. After hydrolysis, the resultant product was
202 filtered with a sieve (0.5 mm) and the solid (blood cells) and liquid fractions (350 L) were
203 separated. The resultant liquid fraction was separated by microfiltration (MF) with a
204 spiral-wound MF module (0.5 µm; 5.5 m^2). The FMF was subsequently submitted to a
205 nanofiltration (NF), recurring to a cut-off membrane threshold of 3 kDa, resulting in the
206 retentate of nanofiltration (RNF). The other fraction, NF filtrate, was resubmitted to a
207 sequential step of NF with a cut-off membrane threshold of 120 g mol^{-1}. The retentate of
208 this stage was filtrate of NF 3 kDa (FNF). To reduce water, the fractions obtained - RMF,
209 RNF (>3 kDa) and concentrated FNF (<3 kDa) - were concentrated by reverse osmosis
210 and dehydrated by freeze-drying. This experimental procedure is schematised and
211 summarised in Figure 1.
212
213 **2.5. Characterisation of hydrolysate fractions**
214 **2.5.1. Total protein content and peptide profile**
215 As described previously, the total protein content of resultant hydrolysates was
216 determined by the Kjeldahl method. All experiments were carried out in triplicate and
217 expressed as % on a dry basis (DB). The MW of RMF, RNF and FNF hydrolysates was
218 determined by size exclusion chromatography (SEC) using the AKTA Pure 25 system
219 (GE Healthcare Life Sciences, Freiburg, Germany), as described by Voss et al. (2019).
220
221 **2.5.2. Total antioxidant capacity**
222 To evaluate the antioxidant capacity and antihypertensive potential of blood
223 hydrolysates, these were dissolved in 10 mL of phosphate buffer pH 7.5 and
224 homogenised, recurring to a vortex for 2 min. The mixture was incubated at room

225 temperature for 40 min under stirring. Then, the mixture was centrifuged for 10 min at
226 4000 *g* and 4 ºC, being the supernatant harvested. The antioxidant capacity of
227 hydrolysates was evaluated by ABTS and ORAC assays.

228 For the ABTS assay, the method established by Coscueta et al. (2020) was
229 followed. All hydrolysates were analysed in triplicate and the results were expressed in
230 µmol Trolox equivalent (TE) per g of DB, after the calculation of Trolox concentration
231 through regression curves equations. Regarding the ORAC method, this assay was
232 performed according to Coscueta, Brassesco, & Pintado (2021). All hydrolysates were
233 analysed in triplicate. After calculating Trolox concentration through regression
234 equations, the results were expressed in µmol TE by g of DB (µmol TE g^{-1} DB).

235

236 **2.5.3. Angiotensin-converting-enzyme inhibitory effect**

237 The ACE-inhibitory activity (iACE) was assayed by the fluorimetric assay
238 described by Coscueta, Brassesco & Pintado (2021). Each sample was evaluated in
239 triplicate and iACE was expressed as the concentration capable of inhibiting 50% of the
240 ACE activity (IC_{50}). Non-linear modelling was applied to determine the IC_{50}, and the
241 results were expressed as µg mL^{-1} to inhibit 50% of the enzymatic activity.

242

243 **2.6. Biological potential of FNF**

244 The FNF, rich in low MW compounds, was characterised by peptidomics for its
245 free AA (FAA) and mineral profile, as well as its antibacterial and prebiotic potential.

246

247 **2.6.1. Peptidomics analysis**

248 Protein identification and quantitation were performed by nanoLC-MS/MS as
249 described by Osório et al. (2021). Proteome Discoverer 2.5.0.400 software (Thermo

250 Scientific, Bremen, Germany) was used to process raw data. For protein identification,
251 the data available in the UniProt protein sequence database was used for the Sus scrofa
252 Proteome together with sample-specific entries (cardosin) and a common contaminant
253 database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry,
254 Munich, Germany). The Sequest HT tandem mass spectrometry peptide database search
255 program performed the analysis, considering a no-enzyme (unspecific) search with an ion
256 mass tolerance of 10 ppm for precursor ions and 0.02 Da for fragment ions. The maximum
257 allowed missing cleavage sites was set as 2. Methionine oxidation was defined as variable
258 modification. Peptide confidence was set to high. The processing node Target-Decoy
259 PSM validator was enabled with the following settings: target/decoy selection
260 concatenated; FDR target (strict) 0.01, (relaxed) 0.05. Protein-label-free quantitation was
261 performed with the Minora feature detector node at the processing step. Precursor ion
262 quantification was performed at the processing step with the following parameters:
263 Peptides: unique plus razor; precursor abundance was based on intensity.

264 To predict the bioactivity *in silico,* all the identified peptides were analysed with
265 the AHTpin web server (http://crdd.osdd.net/raghava/ahtpin) to predict potential
266 antihypertensive activity (Kumar et al., 2015). Peptides with high support vector machine
267 (SVM) scores (> 1.0) were considered as predicted with antihypertensive properties. At
268 the same time, the most promising and the most abundant peptides were analysed using
269 the database available online, BIOPEP
270 (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), estimating potential
271 bioactivities (Minkiewicz et al., 2008).

272

273 **2.6.2. FAA profile**

274 The FAA profile of FNF hydrolysate was evaluated by high-performance liquid
275 chromatography (Beckman coulter, California, USA) coupled with a fluorescence
276 detector (Waters, Milford, MA, USA), following the orthophthalaldehyde (OPA)
277 derivatisation methodology described by Voss et al. (Voss et al., 2019). For FAA
278 identification and quantification, calibration curves of pure AA standards were prepared,
279 and homoserine and norvaline were used as internal standards. The analysis was
280 performed in duplicate, and the results were presented as mean ± standard deviation (SD)
281 and expressed in mg g^{-1} of DB.
282

283 **2.6.3. Mineral profile**

284 The digestion of 300 mg of hydrolysate with 9 mL of HNO_3 (65%) and 1 mL of
285 H_2O_2 (30%) was performed in a Teflon reaction vessel, recurring to a microwave digestor
286 (Berghof, Germany). The digestion program was conducted with the first stage of 30 min
287 to reach 130 ºC, followed by 40 min to reach 170 ºC and 50 min to reach 200 ºC. Then,
288 the digestion program continued with 30 min to reach 100 ºC and was finalised with 20
289 min at 100 ºC. The samples were then cooled on ice and collected to polyethylene bottles,
290 where the digested volume was adjusted to 20 mL with water. Quantitative mineral profile
291 was monitored through inductively coupled plasma-optical emission spectrometry (ICP-
292 OES, Perkin Elmer, Optical Emission Spectrometer, Optima 7000 DV). Calibration
293 curves were established from a standard mix prepared in 5% of HNO_3 namely, sodium
294 (Na; 0.25 - 25 mg L^{-1}; 589.592 nm), iron (Fe; 0.15 - 15 mg L^{-1}; 259.939 nm), calcium
295 (Ca; 5 - 500 mg L^{-1}; 317.933 nm), phosphorus (P; 2.5 - 250 mg L^{-1}; 214.914 nm), zinc
296 (Zn; 0.06 - 6 mg L^{-1}; 213.857 nm), magnesium (Mg; 1.5 - 150 mg L^{-1}; 279.077 nm),
297 potassium (K; 6 - 600 mg L^{-1}; 769.896 nm), cadmium (Cd; 0.0001 - 0.01 mg L^{-1}; 214.440
298 nm), molybdenum (Mo; 0.01 - 1 mg L^{-1}; 203.845 nm), lead (Pb; 0.0005 - 0.05 mg L^{-1};

299 220.353 nm), cobalt (Co; 0.0001 - 0.01 mg L^{-1}; 230.786 nm), nickel (Ni; 0.0003 - 0.03
300 mg L^{-1}; 231.604 nm), aluminum (Al; 0.025 - 2.5 mg L^{-1}; 394.401 nm), boron (B; 0.005 -
301 0.5 mg L^{-1}; 249.772 nm), copper (Cu; 0.015 - 1.5 mg L^{-1}; 324.752 nm), manganese (Mn;
302 0.025 - 2.5 mg L^{-1}; 257.610 nm), to quantify milligrams of mineral per grams of dried
303 sample (mg g^{-1} DB). The analysis was performed in duplicate and the final results were
304 presented in mean ± SD.
305
306 **2.6.4. Antibacterial activity**
307 The antibacterial potential of the FNF fraction was screened against three Gram-
308 negative (*Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa)* and three
309 Gram-positive bacteria (*Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus*
310 *aureus)*, adapting the Minimum Inhibitory Concentration (MIC) determination method
311 described by (Wiegand et al., 2008) and p-iodonitrotetrazolium chloride (INT)
312 colourimetric assay described by Alves et al. (2012).
313
314 **2.6.5. Prebiotic activity**
315 The prebiotic potential of FNF was analysed according to Bordiga et al. (2019).
316 The prebiotic strains used were *L. acidophilus* Ki (LAS), *L. casei* L26, *B. animalis* spp.
317 lactis Bb12 and *B. animalis* Bo. All the assays were performed using MRS, but
318 *Bifidobacterium* was supplemented with 0.5 g L^{-1} of L-cysteine-HCl.
319 FNF was tested, in triplicate, in a medium with and without a sugar source. So,
320 for each bacteria, the following tests were performed (in a final volume of 200 µL): (a)
321 1% FNF, 2% glucose; (b) 1% FNF, 0% glucose; (c) 2% FNF, 2% glucose, ; (d) 2% FNF,
322 0% glucose. Bacterial inoculum was added at 2%. Fructooligosaccharide (FOS; 2%) was
323 used as a positive control. Wells with *Bifidobacterium* were sealed with paraffin to assure

anaerobiosis conditions. The bacterial growth was monitored at 660 nm for 48 h at 37 ºC in a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA), and optical density (OD) was measured hourly. Growth curves were created, and growth rates (GR) were determined by exponential regression in the logarithmic phase.

2.7. Statistical analysis

In all cases, the comparison of means was conveniently performed by one-way analysis of variance (ANOVA), followed by Tukey's Post Hoc test, using a level of significance of 5% ($p < 0.05$). Statistical analysis was performed recurring to the SPSS statistical software (27.0).

3. Results and discussion

Microbiological and physicochemical characterisation of cooked blood

The chemical composition and microbiological characterisation of the three batches of cooked swine blood are presented in Table 1. Generally, the nutritional composition of the present study agrees with swine blood literature data (Alao et al., 2017; Appiah & Peggy, 2012; Toldrà et al., 2018). Previous studies have focused on microbiological parameters of raw swine blood and blood cell concentrates. TAB values below 5.00 x 10^6 CFU g^{-1} are recommended in raw blood (Nowak et al., 2007), being the present results below this limit (Table 1).

Enzymatic hydrolysis and membrane technology

To improve the functional profile of cooked swine blood, this product was submitted to enzymatic hydrolysis combined with membrane systems for fractionation of hydrolysates (Figure 1). The enzymatic hydrolysis was already processed at a pilot scale using the optimal hydrolysis conditions of the *C. cardunculus* commercial extract, with

time previously determined in laboratory conditions to generate the best hydrolysis (DH) in a shorter time. The mixture with water before hydrolysis allowed the extraction of many blood components as possible. Microfiltration, ultrafiltration, nanofiltration and reverse osmosis have been acquiring significant relevance in the food industry due to the large-scale processing and economic advantages compared with preparative chromatographic approaches (Tavares et al., 2011; Tolkach & Kulozik, 2005).

The filtration with a sieve was also an important stage that partially clarified the blood hydrolysate, facilitating the microfiltration step. The microfiltration membrane system, to reduce the microbial load, resulted in 70 L (with 15.52% of total solids) of RMF and 280 L of FMF. Membrane filtration systems separate particles according to their size and shape. Regarding RNF and NF filtrate resulting from nanofiltration with a 3 kDa cut-off membrane, the processes originated 15 L and 265 L, respectively. This nanofiltration allowed for separating two fractions, the RNF and the FNF. As previously mentioned, several studies have associated biological potential with smaller peptides (<3 kDa) (Coscueta et al., 2019; Toldrà et al., 2018). To increase the concentration of low MW peptides, the NF filtrate was further concentrated, without altering the mineral concentration of this fraction and concentrated FNF corresponded to 50 L, with 0.78% of total solids.

The powders of RMF, RNF and FNF possess a free-flowing aspect and pleasant odour with dark red, dark orange and golden yellowish appearance (Figure 1), respectively. In the FNF fraction, the enzymatic and membrane technology allowed to remove the blood's dark red colour, making the application of this hydrolysate more transversal, without the need to apply an additional step to colour remotion.

Protein content and peptide profile of blood hydrolysates

375 Regarding total protein measured in the freeze-dried hydrolysates, all fractions
376 possessed a high protein content. Significant variations were registered in the total protein
377 content of RMF, RNF and FNF fractions. The results suggested that the RMF fraction
378 possesses a high protein content (94.34%), followed by the RNF fraction (91.21%), while
379 the FNF fraction showed a lower value of 64.01% (Figure 2a). As expected, the
380 processing strategies used significantly concentrate the blood protein (approximately
381 29% on a wet basis in cooked blood - Table 1) (Toldrà et al., 2018). During membrane
382 fractionation, mainly structural proteins (e.g., erythrocytes) were removed. Accordingly,
383 there is a concentration of other compounds such as minerals, fatty acids and
384 carbohydrates, increasing their relative percentage and contributing to a lower relative
385 rate of protein, more significant in the FNF fraction.

386 The protein and peptide chromatograms of solubilised RMF, RNF and FNF
387 suggested a similar profile, as is present in Figure 2b. However, the concentrations of
388 proteins and peptides of each fraction were very different. All fractions had the highest
389 peaks in the region between 1200 and 14000 Da, but the FNF fraction had a high content
390 of peptides smaller than 1200 Da. This resulted from the retention of higher MW (> 3
391 kDa) protein components in the first NF step and the permeation of low MW peptides (<
392 3 kDa). These low MW protein components were further concentrated by the second NF
393 step, while low MW components (< 120 g mol^{-1}) were lost in the permeate and not
394 concentrated in this step as occurred with low MW peptides.

395

396 ***Bioactive potential***

397 The FNF fraction showed the more significant antioxidant activity analysed by
398 ABTS and ORAC (Table 2) assays. These results aligned with the higher concentration
399 of lower MW peptides present in this fraction. Small peptides may be associated with

400 higher antioxidant capacities due to their stronger ability to interact with free radicals
401 (Bah et al., 2015). The RNF fraction also had an attractive antioxidant potential, although
402 lower than FNF. The RMF fraction had the lowest antioxidant capacity of all fractions
403 tested. Literature results reported significant antioxidant activity in animal by-products,
404 including swine and bovine blood hydrolysates (Abou-Diab et al., 2020; Borrajo et al.,
405 2019; J. T. Wei & Chiang, 2009), aligning with the present results. Based on literature
406 data, the current results suggest that FNF and RNF hydrolysates possess a very relevant
407 antioxidant capacity. These fractions showed higher ORAC values than those obtained
408 from other animal-derived hydrolysates, such as fish protein hydrolysates (225 µmol TE
409 g^{-1}) (Samaranayaka et al., 2010) and salmon hydrolysates (19.45 µmol of TE g^{-1}) (Neves
410 et al., 2017).

411 The main peptidases reported in animal by-products valorisation are papain,
412 bromelain, thermolysin, alcalase, flavourzyme, pepsin and trypsin (Borrajo et al., 2019).
413 Bah et al. (2015) studied the bioactive potential of swine plasma hydrolysates resultant
414 from a plant (papain and bromelain) and fungal hydrolysis. The results suggested that
415 fungal proteases resulted in hydrolysates with higher antioxidant activities due to the
416 production of smaller peptides by fungal proteases. As previously mentioned, the
417 proteases from *C. cardunculus* are reported as very proteolytic (Araújo-Rodrigues et al.,
418 2020; Voss et al., 2019) due to the lower specificity of Cardosin B, which hydrolyses
419 phenylalanine, leucine, tyrosine or valine bonds (Voss et al., 2019) but was never applied
420 to blood by-product hydrolysis.

421 The ACE is the main target of antihypertensive peptides, being also the target of
422 drugs used in the clinical treatment of antihypertensive related diseases (Wei & Chiang,
423 2009). The results of this assay also suggested that shorter peptides of FNF possessed
424 very stronger iACE (Table 2). The iACE is generally associated with short-chain peptides

425 between 2 and 12 AA (Wei & Chiang, 2009). The present results suggested that to inhibit
426 50% of the angiotensin-converting enzyme (IC_{50}) are only necessary 28.51 µg mL^{-1} of
427 FNF fraction. Based on literature, this fraction revealed an excellent antihypertensive
428 potential, since it has a much lower IC_{50} than the reference value of 500 µg protein mL^{-1}
429 (Coscueta et al., 2016). This blood fraction has revealed higher potential than obtained
430 for other animal-derived peptide hydrolysates such as that obtained from the mussel
431 *Mytillus galloprovincialis* (IC_{50} of 1000 µg protein mL^{-1}) (Cunha et al., 2021), fish protein
432 hydrolysate (FPH) (IC_{50} of 161 µg peptide mL^{-1}), FPH < 1 kDa (IC_{50} of 115 µg protein
433 mL^{-1}) (Samaranayaka et al., 2010).

434 A previous study reported the continuous production of swine blood hydrolysates
435 by enzymatic hydrolysis in a membrane reactor (Wei & Chiang, 2009). The mixture of
436 trypsin, chymotrypsin and thermolysin proteases resulted in swine blood hydrolysates
437 with lower half-maximal inhibitory concentration (IC_{50}) of 580.0 µg mL^{-1}. Comparing
438 this result with the IC_{50} of FNF in the present study, in the study of Wei & Chiang (2009),
439 approximately 20 times more hydrolysate was required to inhibit 50% of the ACE
440 activity. Other authors studied the iACE of swine globin hydrolysates resulting from
441 pepsin, trypsin and papain hydrolysis. The IC_{50} was 1190.0, 8790.0, and 2210.0 µg mL^{-}
442 ^{1}, respectively, even much lower, corresponding to no antihypertensive activity. Also,
443 Ren et al. used pepsin protease and studied this bioactive property in swine globin
444 hydrolysates. The results indicated an IC_{50} of 4370.0 µg mL^{-1}. In all cases, the results
445 suggested that hydrolysates possessed much lower antihypertensive potential than the
446 FNF. So, this is the first study where swine blood hydrolysates presented an excellent
447 ACE-inhibitory potential, which is associated with the specific activity of *C. cardunculus*
448 enzymes, also tested for the first time in this substrate.

449 Thus, the present antioxidant and ACE-inhibitory results raised the high potential
450 of FNF for functional ingredient and nutraceuticals formulation. The bioactive peptides
451 responsible for these bioactivities are equally present in RMF and RNF fractions.
452 However, in the FNF, their relative abundance is higher, contributing to better antioxidant
453 and antihypertensive activities. According to their protein content and bioactive potential,
454 the hypothesis of RMF and RNF being used in aquaculture feed was also raised. The
455 remaining work was focused on characterising the FNF assuming human nutrition
456 applications.

457

458 ***Identification of peptides in FNF hydrolysate***

459 FNF presented an ACE IC_{50} comparable to pure peptides considered potent
460 antihypertensives. Kohmura et al. (1989) reported the antihypertensive oligopeptides
461 VMP (IC_{50} of 10.0 µg mL^{-1}), TVYTKGRVMP (IC_{50} 43.7 µg mL^{-1}), PAVVLP (IC_{50} of
462 26.8 µg mL^{-1}), LPQNILP (IC_{50} of 36.5 µg mL^{-1}), PHQIYP (IC_{50} of 22.6 µg mL^{-1}),
463 NPPHQIYP (IC_{50} of 35.7 µg mL^{-1}), VLPYP (IC_{50} of 21.1 µg mL^{-1}), and VLPIP (IC_{50} of
464 16.7 µg mL^{-1}), sequences encoded in the human β-casein. In another paper, Kohmura,
465 Nio, and Ariyoshi (1990) reported the oligopeptides SLVYP (IC_{50} of 23.7 µg mL^{-1}),
466 FAQTQSLVYP (IC_{50} of 28.8 µg mL^{-1}), and HPFAQTQQSLVYP (IC_{50} of 36.1 µg mL^{-1}
467), as excellent antihypertensives encoded in various β-caseins. Wako, Ishikawa, and
468 Muramoto (1996) identified in autolysates of squid liver and mantle muscle the
469 antihypertensive peptide GYALPHA, with an IC_{50} 19.9 µg mL^{-1}. Comparison with these
470 examples highlights the strong antihypertensive potential of FNF and points to the need
471 to analyse in detail the sequences of the peptides present in the fraction.

472 Peptides arising from FNF were sequenced by MS/MS to identify their nature and
473 then analysed to find the presence of potentially bioactive sequences in their structures.

474 536 unique peptide sequences were identified. Figure 3 shows the size distribution profile
475 of the peptides, with the composition ranging from 3 to 39 AA residues (i.e. between 403
476 and 3556 Da).

477 All peptide sequences were analysed *in silico* to predict antihypertensive
478 properties. AHTpin is a web-based software that uses a supervised machine learning
479 technique to predict the antihypertensive property of a peptide based on the type of AA
480 and its position in the primary structure relative to previously reported antihypertensive
481 peptides (Kumar et al., 2015). Peptides predicted as antihypertensive, Support Vector
482 Machine (SVM) > 1.0, by AHTpin were selected to be analysed individually in the
483 BIOPEP database. The complete simulation results in AHTpin are in an open repository
484 (https://doi.org/10.5281/zenodo.5821650). From this first selection, 23 unique sequences
485 in the range of 530 to 2745 Da resulted, as shown in Table 3 in decreasing order according
486 to their relative abundance. For each of these 23 peptides, the potential biological activity
487 parameter (B) was calculated in BIOPEP (Coscueta et al., 2019; Minkiewicz et al., 2008),
488 ranking in descending order at this value. The results returned by the database for each
489 peptide can be seen in an open repository (https://doi.org/10.5281/zenodo.5821909).
490 Based on predictions, both rankings matched at position 3 (Table 3), occupied by the
491 sequence FPSVLQPSG with an SVM value of 1.91 and a B value of 0.0590 μM^{-1}. About
492 the others, in general, the positions did not demarcate a notable difference, only for the
493 first two positions of each ranking (Table 3). However, when calculating the joint
494 abundance of these 23 peptides, about 0.2% of the total was covered, which apparently
495 would not be enough to explain the high bioactivity observed *in vitro*.

496 So, we extended the analysis to the 20 most abundant peptides, in addition to the
497 23 peptides predicted as antihypertensive. Considering these 43 sequences, about 74.0%
498 of the total abundance was covered. Table 4 shows the ranking of these 43 peptides in

499 decreasing order concerning the relative abundance of each sequence. The 20 most
500 abundant peptides, according to the AHTpin simulation, have a very low antihypertensive
501 capacity, remaining between positions 144 and 515, except for a single sequence
502 positioned as 33rd. Then, the analysis in BIOPEP for the new peptides was extended and
503 ranked among the 43 sequences concerning the B value. The results thrown by the
504 database for each one of the other 20 peptides are in an open repository (Coscueta,
505 Araújo-Rodrigues & Pintado, 2022). In this new ranking, the peptide ranked at position
506 33rd of AHTpin was in the first position for BIOPEP. This peptide, with the sequence
507 LVVYPWTQRF, represents about 2.2% of the total peptide abundance of FNF. It has a
508 potential ACE-inhibitory activity value of 0.2991 μM^{-1}, and, in addition, interesting
509 values of potential antidiabetic activity (alpha-glucosidase inhibitor of 5.9524 10^{-5} μM^{-1};
510 dipeptidyl peptidase IV inhibitor of 0.0037 μM^{-1}) and opioid activity (0.0697 μM^{-1})
511 (Coscueta, Araújo-Rodrigues & Pintado, 2022).

512 LVVYPWTQRF is a sequence already reported in the literature, also known as
513 LVV-Hemorphin-7, an opioid-like peptide derived from the degradation of hemoglobin
514 β-globin chain, particularly from positions 33-42 in the species under study (Accession
515 F1RII7). LVV-Hemorphin-7 is the most stable form of hemorphin, and was isolated, in
516 1992, from the ventricular cerebrospinal fluid of patients with cerebrovascular bleedings
517 (Glämsta et al., 1992). This peptide is found in high concentrations in human plasma and
518 exhibits different affinities to many receptors, including opioid receptors, insulin-
519 regulated aminopeptidase (IRAP; angiotensin IV receptors), among others (Wei et al.,
520 2020). In addition to its antihypertensive, antidiabetic, and opioid properties, LVV-
521 Hemorphin-7 could induce anxiolytic-like effects, having a potential application for
522 mood regulation (Wei et al., 2020) as well as in disorders such as alcoholism (Hung et
523 al., 2021). Hemorphins have been frequently studied in the last two decades, given that

524 they have many properties of interest for health. However, they will continue to be
525 investigated since the mechanisms by which they act and their potential properties and
526 applications remain to be revealed. For this reason, the fact that FNF has a promising
527 relative abundance of LVV-Hemorphin-7 results in a substantial and novel contribution.
528

529 ***FAA profile of FNF hydrolysate***

530 Protein hydrolysis results in peptides and FAA. The AA, similarly to bioactive
531 peptides, may also have antioxidant activity. Other health-related functions such as blood
532 flow regulation, cell signalling, immune responses, and immunity against viral infections
533 have been associated with dietary supplementation with AA (Wu, 2013). FAA present in
534 FNF fraction were identified and quantified (Table 5), being among the most predominant
535 glutamic acid, leucine, alanine, phenylalanine and aspartic acid, with concentrations
536 between 1.10 and 1.91 mg g^{-1} on a DB. Between the nine essential AA, isoleucine,
537 leucine, methionine, phenylalanine, threonine, tryptophan and valine were identified and
538 quantified. For example, glutamic acid, leucine and other AA have been reported in gene
539 expression modulation and growth of skeletal muscle and small intestine. Also, D-AA
540 such as D-alanine and D-aspartate have been associated with regulating neurological
541 development and function (Wu, 2013). Thus, the FAA profile suggests that FNF may be
542 an interesting AA source, with functional properties, and most essential AA.
543

544 ***Mineral profile of FNF hydrolysate***

545 Minerals are essential compounds in the human diet (Guiné et al., 2011). Mineral
546 content was also monitored (Table 5), with Ca, Mg, P, K and Na as the most incident
547 minerals, being their concentrations of approximately 1.5, 2, 10, 28 and 45 mg g^{-1} DB,
548 respectively. These minerals are required in large amounts and play essential roles in the

549 human body, making the target fraction (FNF) an interesting source of minerals. Ca is
550 associated with preventing and treating some diseases (e.g., osteoporosis). Mg is involved
551 in diabetes, heart diseases and other clinical conditions prevention and treatment. In
552 contrast, K is involved in preventing osteoporosis and treating high blood pressure (Guiné
553 et al., 2011). It should be mentioned that the FNF fraction at the end was concentrated by
554 nanofiltration using a membrane of 120 Da, unaffecting its mineral composition.
555

556 ***Antibacterial effect of FNF hydrolysate***

557 The emergence of antibiotic resistance requires developing new and effective
558 antibacterial solutions (Borrajo et al., 2019). In this context, the antimicrobial potential
559 of FNF was also evaluated. Literature data reported the highest antibacterial activity in
560 peptide hydrolysates with molecular weight between 400 and 1400 Da (Borrajo et al.,
561 2019). Although the protein and peptide profile suggested a higher relative presence of
562 peptides lower than 1200 Da in the FNF fraction, no antibacterial effect was verified for
563 the seven pathogenic bacteria tested at a maximum concentration of 200 mg mL^{-1}.

564 Some antibacterial effect was registered, for instance, in some blood hydrolysates
565 resulting from hydrolysis with animal enzymes (e.g., trypsin) (Borrajo et al., 2019).
566 However, no antibacterial activity against typical pathogenic strains found in meat was
567 verified in blood hydrolysates with plant and fungal proteases (Borrajo et al., 2019). The
568 degree and type of hydrolysis influence the hydrophobic and cationic characteristics of
569 resultant peptides and their consequent antimicrobial potential. These properties could
570 favour the interaction of peptides with the microbial membrane, promoting the
571 modification of membrane permeability (Borrajo et al., 2019; Verma et al., 2017). So, the
572 use of a plant-derived protease may explain the lack of antibacterial activity in the present
573 study, according to the findings described by Borrajo et al. (2019).

574 Furthermore, FNF is a mixture of peptides and AA, thus, their joint activity may
575 differ from that observed in isolated peptides. The most abundant peptides on FNF do not
576 appear associated with antimicrobial activity on the DBAASP database
577 (https://dbaasp.org/home), which can also explain the lack of antibacterial activity on this
578 extract. However, isolating peptides may be a further step for confirming if these peptides
579 do not show antimicrobial potential.
580

581 ***Prebiotic potential of FNF hydrolysate***

582 FNF is essentially rich in proteins/peptides and is not a sugar source, being tested
583 in MRS medium with and without glucose to understand better how they affect bacterial
584 growth. When growing these bacterial strains in MRS with glucose, it is expected that
585 they have a high growth rate. Thus, even if FNF could potentiate bacterial growth, it could
586 be difficult to recognise the increase in maximum growth rate. In the medium without a
587 sugar source, it is not expected to have high bacterial growth GR, thus FNF effect on
588 bacterial growth may be easily noticed. The obtained GR's for each bacterium in all tested
589 conditions are described in Table 6. Figures S1 and S2 show the growth curves obtained
590 for all bacteria, obtained using the mean values of the three replicates.

591 For Bb12, in the tests with MRS without glucose, 2% FNF showed GR
592 statistically higher than the control (no glucose). Furthermore, no difference was observed
593 between GR induced by FOS or FNF in 1 or 2%, indicating that FNF showed similar
594 potential to the positive control. Regarding Bo, this bacterium seems to be the one that
595 benefits the most from FNF addition. Compared to the growth controls, a significantly
596 higher GR was observed in FNF tests with and without glucose. Compared to the FOS
597 control, the GR obtained using MRS without glucose was similar (1% FNF) or
598 statistically higher (2% FNF) than that obtained with the FOS. Thus, these results show

599 that FNF may have a prebiotic potential for the growth of Bo. *L. casei* GR is significantly
600 higher in the presence of 1 and 2%, compared to no-glucose control, with GR being higher
601 in the presence of 2% FNF than FOS. LAS GR significantly increased with FNF in the
602 tests with or without glucose compared to the respective controls and similar to FOS GR.
603 In conclusion, the FNF fraction showed potential as prebiotic since it led to GR
604 similar or higher to those obtained with the positive control. Peptide rich extracts were
605 previously studied regarding their prebiotic potential. Yu et al. (2016) showed that a whey
606 peptide extract (WPE) with MW lower than 1 KDa could enhance *Lactobacillus*
607 *acidophilus* Ki and Bb12 growth, especially in the presence of 1% WPE. However, as far
608 as we know, no studies evaluated the prebiotic effect of swine blood hydrolysates, which
609 adds value to this new hydrolysis approach. Furthermore, we did not find other animal
610 blood hydrolysates described for their prebiotic potential, which seems that this
611 bioactivity has been somewhat forgotten in this field. So, the present study results showed
612 that it would be interesting to evaluate the prebiotic potential of animal blood
613 hydrolysates.
614
615 **4. Conclusions**
616 The present work reports a viable alternative for swine blood processing,
617 producing high added-value peptides through microfiltration and nanofiltration
618 membrane fractionations, coupled with *C. cardunculus* enzymatic hydrolysis. This study
619 showed that the lower MW fractions produced (FNF) had high interesting due to the high
620 content and diversity of peptides, but also due to their relevant bioactivity exhibiting high
621 potential antioxidant activity and ACE-inhibitory activity, demonstrating their potential
622 as a functional food ingredient or nutraceutical.

FNF presented an *in vitro* ACE-inhibitory activity comparable to pure/isolated peptides, reported as potent antihypertensives. The sequencing results of the FNF fraction suggested a high diversity of peptide sequences, being identified 536 unique peptide sequences. The analysis was focused on 43 unique sequences, where LVV-Hemorphin-7 was identified as, potentially, the main responsible for the observed bioactivity, given its antihypertensive potential and relative abundance. This peptide is reported as antihypertensive, antidiabetic, opioid and anxiolytic, increasing the high value of the FNF fraction.

The interesting peptide content and profile coupled with the prebiotic effect observed on probiotic strains, the interesting AA concentrations (mainly glutamic acid, leucine, alanine, phenylalanine and aspartic acid) and mineral profiles (Ca, Mg, P, K and Na), represent the first step towards developing a new functional food ingredient or nutraceutical. Due to its high protein content, FNF also represents a more sustainable high value protein source for human consumption. This approach may contribute to a more sustainable world by creating new natural and sustainable bioactive peptide hydrolysates.

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Helena Araújo-Rodrigues: investigation, methodology, formal analysis, writing—original draft preparation. Ezequiel R. Coscueta: methodology, formal analysis, writing—review and editing. Miguel F. Pereira: methodology. Sara A. Cunha: methodology, writing—review and editing. André Almeida: resources; methodology, writing—review and editing. Ana Rosa: methodology. Rui Martins: methodology. Carlos D. Pereira: supervision, methodology, writing—review and editing. Manuela Pintado: conceptualisation, project administration, supervision, writing—review and editing.

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