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Structural and chemical changes induced by temperature and pH hinder the digestibility of whey proteins

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ABSTRACT

1 In the food and feed industry, protein extraction is commonly performed under acid or basic conditions,
2 combined with heat, in order to increase the extraction yield. Under severe processing conditions, proteins may
3 undergo molecular modifications. Here, the effects of heating (30, 60, 90°C) at different pH values (2, 7, 9,
4 11, 13) were evaluated on commercial whey proteins, used as a simplified protein model. The main structure
5 and chemical modifications concerning protein aggregation, hydrolysis, insolubilization, amino acid
6 degradation and racemization were investigated in detail. Using *in vitro* static models, the degree of protein
7 hydrolysis and the released peptides were determined after the digestive process. Accumulation of molecular
8 modifications was mostly observed after basic pH and high temperatures treatments, together with a marked
9 decrease and modification of the digestibility profile. Instead, protein digestibility increased in neutral and
10 acidic conditions in a temperature-dependent manner, even if some modification in the structure occurs.

11 **KEYWORDS:** protein modification; protein digestion; heat treatment; basic pH; whey proteins; peptide
12 release

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24 **1 INTRODUCTION**

25 Proteins are one of the three main macronutrients and are essential components to be introduced into the human
26 diet. Dietary proteins are found as animal-based (milk, meat, eggs) and plant-based (cereals, legumes). The
27 integrity of the proteins is an important parameter closely related to the quality of their constituents, the amino
28 acids, as well as to their biological use and their digestibility (Friedman, 1996).

29 The Food and Agricultural Organization of the United Nations (FAO) indicated that, with an expected
30 population rise to 9.1 billion, it will be necessary to increase food production by 70% by 2050 (FAO, 2009).

31 Given the demographic increase associated with socio-economic changes (e.g., recognition of the role of
32 proteins in a healthy diet), also the demand for protein is expected to strongly increase (Henchion et al., 2017).

33 It has been estimated that only 35% of the protein produced by the agricultural sector is used for food use,
34 while the majority is used for feed, non-food products or wasted (Leip et al., 2014). To meet the increasing
35 demand for proteins, food and feed industries are persistently aiming to extract additional proteins from
36 traditional protein sources (milk protein, cereals, oil seeds) as well as new or unconventional (insects,
37 microalgae, leaves). Additionally, many new research underscores the importance of using food waste and by-
38 products as protein source in food and also in non-food applications (Torres-León et al., 2018). An example
39 of the valorisation of food waste is represented by whey, the main by-product of the dairy industry, which is
40 widely reused and appreciated for its nutritional, biological and functional properties (Mollea et al., 2013).

41 For this reason, many methods have been developed for the extraction and utilization of proteins from many
42 diverse biomasses. In the food and feed industry, the most common technique used to extract proteins involves
43 solubilisation in water under neutral, acidic, or basic conditions (Qin et al., 2018), together with heating at
44 medium to high temperatures to increase the solubilisation, and/or possibly the use of specific enzymes. In
45 recent studies, acid or basic conditions combined with the addition of specific enzymes (mainly proteases) are
46 often used to hydrolyse proteins into peptides and free amino acids, and consequently increase the extraction
47 yield (Jung, 2009; Sari et al., 2015). Alternatively, the modification of the pH around the value of the isoelectric
48 point (e.g., 4-5 for caseins) represents an alternative technique to purify proteins after their precipitation (as in
49 the case of the dairy industry) (Borad et al., 2017).

50 Alkaline treatments, on the other side, are among the most used methods for protein extraction, highly valued
51 for their low cost and high extraction yield (Momen et al., 2021; Roberts et al., 1985). The parameters of the
52 extraction conditions, such as type and concentration of the solvent, pH, time, and temperature, play an
53 important role in this type of processing. Frequently, the alkaline extractions are obtained by using NaOH in
54 water at a concentration of 0.1-1 M (Sari et al., 2015). Next to the solvent concentration, also the time-
55 temperature combination represents a key factor (Awuah et al., 2007; van Boekel et al., 2010). Most processes
56 use a processing temperature between 25°C and 90°C, for a time varying between 10 and 120 minutes (Sari et
57 al., 2015). In addition, a combination with other processes/treatments is often performed. Samples are
58 commonly pre-treated by flaking, extrusion, de-oiling, ammonia fibre expansion, lyophilization in order to
59 eliminate the non-protein fractions (Sari et al., 2015). Severe conditions such as long processing times, heat,
60 extreme pH increase the extraction yield and are therefore widely used in the food and feed industry (Cui et
61 al., 2017; Zhang et al., 2014). On the other hand, they can cause adverse effects on the integrity, quality, and
62 subsequent digestibility of proteins (Meade et al., 2005; van Lieshout et al., 2020; Wu, W.;Hettiarachchy,
63 N.S.;Kalapathy, 1998). For this reason, more attention needs to be paid to these aspects in the field of food
64 processing (Meade et al., 2005).

65 A detailed understanding of the molecular changes that occur during extraction and how different extraction
66 methods affect the digestibility of proteins would be important to ensure the production of high nutritional
67 value proteins. Food processing conditions, as heat treatments and pH alteration, indeed affect proteins and
68 their constituents, leading to structural and chemical modifications. These changes generally involve all
69 proteins, of both animal and plant origin, and alter their digestibility and absorption (Sun-Waterhouse et al.,
70 2014). Anyway, in depth molecular studies linking specific protein modification occurring during
71 technological processing and digestion are very limited. Thus, to gain detailed molecular insights on how
72 different methods of processing can modify protein structure, and how this, in turn, affect protein digestibility,
73 whey proteins are used in this work as a model protein system to check the effect of processing on protein
74 modifications and hence digestibility. To do this, commercial whey proteins isolate are subjected to different
75 processing conditions for 3 hours, studying the effect of pH (between 2 and 13) and heat (30°C, 60°C, 90°C),
76 Aggregation, degradation, solubility, glycation, and racemization are determined on the processed proteins at
77 an unprecedented level of molecular details using state-of-the art chromatographic and spectrometric

78 techniques. Then, on the digestate, the degree of hydrolysis of proteins and the amount and the type of the
79 different peptides released are determined. In this way, the link between processing conditions, protein
80 structure modification and protein digestibility is clearly outlined.

81

82 **2 MATERIALS AND METHODS**

83 **2.1 Reagents and solvents**

84 Commercial whey protein, α -lactalbumin and β -lactoglobulin from Aptonia (UK) were used in the present
85 study. 20 \times XT MES running buffer, 20 \times XT reducing agent, 4 \times XT sample buffer, Coomassie Brilliant Blu
86 protein stain powder R-250, Criterion Bis-Tris Precast Gels (12% Bis-Tris, 13.3 \times 8.7 \times 0.1 cm), and Precision
87 Plus Protein Standards were from Bio-Rad (Hercules, CA, USA). Quant-iT™ Protein Assay Kit was purchased
88 from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Defoamer for Kjeldahl analysis was
89 purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, Kjeldahl catalyst tablets (3.5 g/tablet), and
90 sulfuric acid (96%), were purchased from Merck Millipore (Darmstadt, Germany). Ultrapure water was
91 obtained with Milli-Q® system (Merck Millipore, Darmstadt, Germany). The standards of β -lactoglobulin
92 (98% purity) and α -lactalbumin (92% purity), ammonium bicarbonate (NH₄HCO₃), ammonium carbonate
93 ((NH₄)₂CO₃), aspartic acid, boric acid, bovine bile, cysteic acid, DL-dithiothreitol (DTT), formic acid (>95%),
94 hydrochloric acid (37%, HCl), L-isoleucine, methionine sulphone, NAC (N-acetyl-cysteine), DL-norleucine,
95 OPA (o-phthaldialdehyde), pancreatin powder from porcine pancreas (8 \times USP), pepsin from porcine gastric
96 mucosa (757 U/mg), SDS (sodium dodecyl sulphate), sodium chloride (NaCl), disodium phosphate
97 (Na₂HPO₄), monobasic sodium phosphate (NaH₂PO₄), and tryptophan were purchased from Sigma Aldrich
98 (St. Louis, MO, USA). Amino Acid Standard Mixture (2.5 mM) was purchased from Thermo Scientific
99 (Waltham, MA, USA). Kj-tabs VS antifoam was purchased from VELP Scientifica (Usmate Velate, MB,
100 Italy). Acetonitrile (CH₃CN) and copper (II) oxide were purchased from VWR Chemicals (Radnor, PA, USA).
101 The AccQ•Fluor Reagent Kit for Amino Acid Analysis was purchased from Waters (Milford, MA, USA).
102 Calcium chloride (CaCl₂), magnesium chloride hexahydrate (MgCl₂(H₂O)₆), monobasic potassium phosphate
103 (KH₂PO₄), potassium chloride (KCl), sodium bicarbonate (NaHCO₃), and sodium hydroxide (NaOH) were
104 purchased from Carlo Erba (Milan, Italy).

105 **2.2 Induction of thermal and pH stress on whey protein samples**

106 Whey proteins (10 g) were solubilised in 100 mL of demineralized water and the pH was then adjusted to the
107 desired value (pH 2, 7, 9, 11, 13) using 0.1 M HCl or 0.5-5 M NaOH. The samples were then placed into oil
108 bath under stirring and heated for 3 hours to three different controlled temperatures, (30°C, 60°C and 90°C)
109 for each different pH value.

110 The processed whey samples were then lyophilized (freeze drier Lio 5P, 5 Pa, Milan, Italy) and stored at -
111 20°C until analysis.

112 **2.3 Characterization of the protein fraction**

113 **2.3.1 Characterization of intact proteins**

114 The processed whey proteins were characterized by electrophoresis (SDS-PAGE) under reducing conditions
115 as previously described by Gasparini et al., 2020 except for preliminary sample preparation and the amount of
116 protein loaded. The processed whey proteins, in lyophilized form, were solubilized in DTT/Urea/AmBic buffer
117 (5 mM, 4 M, 100 mM) under stirring for 30 minutes. The solution was then centrifuged at 4°C, 3220g for 10
118 minutes (5810R Eppendorf, Hamburg, Germany) and the supernatant was used for the analysis. The amount
119 of protein loaded into each well was 10 µg (quantified by QuBit fluorometer, Invitrogen, Thermo Fisher
120 Scientific, Waltham, MA, USA). 20× reducing agent and 4× sample buffer (suitably diluted) were added to
121 each sample. The sample was then incubated at 95°C for 5 minutes, and then rapidly refrigerated for 5 minutes.
122 The samples were loaded into precast 10% gel (Criterion XT Bis-Tris, Bio-Rad, Hercules, CA, USA).
123 Electrophoretic run (SDS-PAGE) was performed at a constant voltage of 150 V using a Criterion
124 electrophoretic chamber (Bio-Rad, Hercules, CA, USA). Protein bands were visualized on the gel by
125 Coomassie Blue staining. Molecular marker standards (Precision Plus Protein unstained standard, Bio-Rad,
126 Hercules, CA, USA) were used to determine the molecular weight of the proteins in the sample.

127 The whey proteins were also characterized by using UPLC-MS (Acquity Waters with a single quadrupole mass
128 spectrometer) according to a previously published procedure (Gasparini et al., 2020). Briefly, the processed
129 whey proteins, in lyophilized form, were dissolved in water (100 mg/mL), mixed and centrifuged at 4°C, 3220g
130 for 10 minutes. Subsequently, the supernatant was filtered through a 0.45 µm filter membrane and injected
131 into UPLC-MS system using a RP column (ACQUITY UPLC BEH 300 C4 1.7 µm 2.1×150 mm, Waters,

132 Milford, MA, USA) and a gradient elution. Eluent A was H₂O with 0.1% formic acid, eluent B was acetonitrile
133 with 0.1% formic acid; gradient: 0–7 min 69% A, 7–20 min from 69% A to 64.5% A; flow: 0.2 mL/min;
134 column temperature: 35°C; sample temperature: 18°C; injection volume: 4 µL. The samples were analysed in
135 the Full Scan mode; ionization type: positive ions; scan range: 100–2000 m/z; capillary voltage: 3.2 kV; cone
136 voltage: 30 V; source temperature: 150 °C; desolvation temperature: 300 °C; cone gas flow: 100 L/h;
137 desolvation gas flow: 650 L/h. The identification was performed using external standards of pure bovine α-
138 lactalbumin and β-lactoglobulin. The characteristic ions of α-lactalbumin and β-lactoglobulin were extracted
139 and the chromatographic peaks corresponding to the native (unglycated) forms of whey proteins (α-
140 lactalbumin and β-lactoglobulin isoform A and B) and their glycosylated forms, i.e., mono and dilactosylated, were
141 integrated (when detected) in order to determine the relative percentage of protein glycation.

142 **2.3.2 Determination of soluble and insoluble nitrogen fraction**

143 The soluble nitrogen content was determined using the official Kjeldahl method according to EC 152/2009
144 (The Commission of The European Communities, 2009). The processed whey protein was first resuspended
145 in water (10 mg/mL), mixed with a shaker for 30 minutes, and then centrifuged at 4 °C, 3220g for 30 minutes.
146 Supernatant was analysed by Kjeldahl and the content of soluble proteins was determined by applying the
147 conversion factor for milk and dairy, 6.38 (Food and Agriculture Organisation (FAO) and World Health
148 Organization (WHO), 1973). The insoluble fraction was then estimated from difference of the soluble protein
149 fraction.

150 **2.3.3 Molecular size distribution of the soluble nitrogen fraction**

151 The molecular size distribution of the soluble protein fraction was determined by size exclusion
152 chromatography (SEC), using an Agilent 1260 Infinity II HPLC system equipped with a Refractive Index
153 Detector (Agilent Technologies, Santa Clara, CA, USA). For the analysis, 100 mg of processed whey protein
154 was solubilized in 10 mL of demineralized water. Then, the samples were mixed with an overhead shaker for
155 30 min at room temperature and centrifuged for 30 min at 3220g and 4°C. The supernatant was separated from
156 the pellet and subsequently filtered through a 0.45 µm nylon filter membrane into HPLC vials. The column
157 used for the analysis was a PL aquagel-OH mixed-M (7.5 × 300 mm, 8 µm particle size, Agilent Technologies,
158 Santa Clara, CA, USA) with a molecular mass range between 1 kDa and 500 kDa. The separation was

159 performed under isocratic conditions at a flow rate of 1 mL/min with milli-Q water as the mobile phase,
160 keeping the column temperature set at 30°C. Some samples, in which proper protein identification was
161 dubious, were also spiked with a known amount of β -lactoglobulin in order to confirm the retention time of
162 the native whey proteins, avoiding matrix effects leading to misidentification of the peaks. Peaks having
163 molecular mass higher than whey proteins were considered aggregated proteins. Peaks having molecular mass
164 lower than whey proteins were considered degraded proteins. For every sample, the relative percentage of the
165 different forms (aggregated, native, degraded) was obtained starting from the integrated areas of the
166 corresponding peaks compared to the total area of all detected peaks.

167

168 **2.4 Amino acid profile**

169 **2.4.1 Determination of total amino acids**

170 The total amino acids content was analysed using a previously published procedure (Leni et al., 2019) with
171 some modifications. 100 mg of treated sample were subjected to acidic hydrolysis with 6 M HCl for 23 hours
172 at 110°C. Once the acid hydrolysis was completed, the samples were brought back to room temperature and
173 7.5 mL of 5 mM water-solubilized norleucine was added. Subsequently, the samples were filtered and made
174 up to volume in 250 mL volumetric flasks.

175 A calibration curve for amino acid quantification was prepared by mixing 2.5 mM amino acid standard H
176 (Thermo Scientific, Rockford, IL, USA) and 2.5 mM of norleucine, cysteic acid, methionine-sulphone in 0.1
177 M HCl solution. Dilutions were performed to obtain the following concentrations: 0.625 mM, 0.3125 mM,
178 0.156 mM, 0.078 mM. Then, the standards, as well as the samples, were derivatized for their composition
179 using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) according to the manufacturer's
180 instructions.

181 Finally, total amino acids were detected and quantified by UPLC-MS (UPLC Acquity with a single quadrupole
182 detector; Waters, Milford, MA, USA) as previously reported by Buhler et al. (2019). Chromatographic
183 separation was performed using a C18 RP column (ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7
184 μm , 2.1 mm \times 150 mm, Waters, Milford, MA, USA). H₂O+0.1% formic acid was used as eluent A, while

185 acetonitrile with 0.1% formic acid was used as eluent B. The flow rate was 0.2 mL/min. Gradient: 0–7 min
186 100% A, 7–30 min from 100% A to 73.3% A; flow: 0.2 mL/min; column temperature: 35°C; sample
187 temperature: 18°C; injection volume: 10 µL. The samples were analysed in the SIR Scan mode; ionization
188 type: positive ions; capillary voltage: 3.2 kV; cone voltage: 30 V; source temperature: 150 °C; desolvation
189 temperature: 300 °C; cone gas flow: 100 L/h; desolvation gas flow: 650 L/h.

190 **2.4.2 Determination of free amino acids**

191 For the determination of free amino acids, 50 mg of lyophilized sample was added with 10 mL of 0.1 M HCl
192 and 100 µL of 5 mM norleucine, mixed and centrifuged at 3220g for 10 min at room temperature. The free
193 amino acids (10 µL) were derivatized and quantified by UPLC-MS system as reported in the previous
194 paragraph.

195 **2.4.3 Determination of the enantiomeric purity of amino acids**

196 The enantiomeric purity of the amino acids was determined following the previously described procedure
197 (Prandi et al., 2019). 40 mL of acid hydrolysed samples (as described above in the determination of total amino
198 acids) were dried and derivatized before injection into an Agilent Technologies 7820° gas-chromatograph
199 coupled to an Agilent Technologies 5977B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA),
200 equipped with a chiral capillary column (CHIR-L-VAL, 25 m x 0.25 mm I.D., film thickness: 0.12 µm
201 Varian, Agilent Technologies, Santa Clara, CA, USA) for separation of amino acid enantiomers. The
202 percentage of D-amino acid formed after different processing conditions was calculated as D/D+L % for Ala,
203 Asp, Phe, Glu, and Lys.

204 **2.5 *In vitro* peptic digestion and simulated gastrointestinal digestion**

205 **2.5.1 Digestion with pepsin**

206 250 mg of each processed whey protein sample was solubilized in 5 mL of 0.01 M HCl, and the pH value was
207 instantly adjusted to 2. The samples were added with 12.5 mg of pepsin (727 U/mg), in an enzyme to substrate
208 ratio 1:20 (López-Fandiño et al., 2010), and were incubated under shaking for 2 h at 37°C. Subsequently, the
209 pH was raised to 7 and the samples were heated for 15 minutes at 90°C to completely inactivate the enzyme.

210 The supernatant was separated from the pellet by centrifugation at 4°C, 3220g for 10 min and stored at -20°C
211 for further analysis.

212 **2.5.2 *In vitro* simulated gastrointestinal digestion**

213 Processed whey proteins were submitted to *in vitro* gastrointestinal digestion according to the previously
214 published INFOGEST static model system (Brodkorb et al., 2019). Using this procedure, three digestive
215 compartments are simulated *in vitro*: mouth, stomach and duodenum. 75 mg of processed whey protein was
216 added to 0.5 mL milli-Q water and immediately underwent simulated digestion. The sample was mixed with
217 simulated saliva fluid (1:1 v/v) and incubated at 37°C for 2 minutes. Then, the simulated bolus was added with
218 simulated gastric fluid (1:1 v/v), pepsin (2000 U/mL) and mixed for 120 min at 37°C, pH 3. Finally, the gastric
219 chyme was added with simulated intestinal fluid (1:1 v/v), pancreatin (trypsin activity of 100 U/ mL) and bile
220 salts (10 mM) and mixed for 120 min at 37°C, pH 7. At the end of the duodenal phase, the samples were heated
221 at 90°C for 15 minutes to stop the enzymatic reaction, the supernatant was separated from the pellet by
222 centrifugation at 4°C (3220g for 45 min) and stored at -20°C until analysis.

223 **2.5.3 Determination of the degree of hydrolysis**

224 The degree of hydrolysis (DH%) was determined in each sample after peptic digestion and gastrointestinal
225 digestion using the previously reported OPA method (Leni et al., 2019; Spellman et al., 2003) with some
226 modifications. The OPA/NAC mixture solution was stirred for 1 h, protected from light, by mixing 50 mM
227 OPA solubilized in methanol, 50 mM NAC solubilized in water, 20% (w/v) SDS and 0.1 M borate buffer at
228 pH 9.5. The OPA assay was performed by adding 3 µL of the digested (or digestion blank) sample to 17 µL of
229 milli-Q water and 2.4 mL of OPA/NAC reagent. The absorbance was measured on the UV-Vis-
230 spectrophotometer at 340 nm (B530 JASCO, Oklahoma City, OK, USA). Degree of hydrolysis was obtained
231 by calculating the relative percentage between moles of free nitrogen groups determined by OPA assay, and
232 moles of bound nitrogen groups, the latter calculated from the protein content.

233 **2.5.4 Peptide analysis**

234 Peptide analysis was performed on pepsin digested and gastrointestinal digested samples. For the analysis,
235 each digested sample was filtered through a 0.45 µm filter membrane then a 1 mM solution of (L,L)-

236 phenylalanyl-phenylalanine (Phe-Phe) was added, used as an internal standard for semiquantification, at a final
237 concentration of 50 μ M in vial.

238 Samples were analysed in low resolution using the UPLC system coupled with ESI and MS (UPLC Acquity
239 with a single quadrupole detector; Waters, Milford, MA, USA). Chromatographic separation was performed
240 using a reversed phase column (ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7 μ m, 2.1 mm \times 150
241 mm, Waters, Milford, MA, USA). Eluent A was Milli-Q H₂O + 0.1% HCOOH, eluent B was CH₃CN + 0.1%
242 HCOOH. The analysis parameters were as follows: flow rate was maintained at 0.2 mL/min; the applied
243 gradient was: 0–7 min, 100% A; 7–50 min, from 100% A to 50% A; 50–52.6 min, 50% A; 52.6–53 min,
244 from 50% A to 0% A; 53–58.2 min, 0% A; 58.2–59 min, from 0% A to 100% A; 59–72 min, 100% A. The
245 run time was 72 min; the column temperature was 35°C; the autosampler temperature was set at 18°C; the
246 injection volume was 4 μ L. The following parameters were used for the detection: type of ionization, positive
247 ion mode; capillary voltage 3.20 kV; cone voltage 30 V; source temperature 150°C; desolvation temperature
248 300°C; cone gas 100 L/h; desolvation gas 650 L/h. Acquisition parameters: acquisition time, 7-58.2 min with
249 a scan range from 100 to 2000 m/z.

250 Finally, the samples were also analysed by UHPLC/ESI-MS/MS using a UHPLC Dionex Ultimate 3000
251 (Sunnyvale, CA, USA) coupled to a TSQ Vantage triple quadrupole (Thermo Scientific, Waltham, MA, USA),
252 using a method previously described (Prandi et al., 2017). The experimental molecular masses were obtained
253 by low-resolution mass spectrometry and the peptide sequences were identified using online sources (Uniprot,
254 Expasy - FindPept, and Proteomics Toolkit – Fragment Ion Calculator).

255 The samples were also analysed with high-resolution mass spectrometry. Detection was obtained by VIon IMS
256 QToF Mass Spectrometer (Waters, Milford, MA, USA), following the chromatographic parameters described
257 above (except the injection volume, which was 1 μ L and the autosampler temperature that was set at 10°C)
258 and with the following acquisition parameters: Experiment type, peptide map (IMS); MSe; source type, ESI;
259 polarity, positive; analyser mode, sensitivity; mode, standard transmission; capillary, 3.00 kV; sample cone
260 voltage, 40 V; source offset voltage, 80 V; source temperature, 120°C; desolvation temperature, 450°C; cone
261 gas, 50 L/h; desolvation gas, 800 L/h. MSe mode, high definition MSe; acquisition time, 7-58.2 min; scan
262 range, 100-2000 m/z; scan time, 0.4 s; low collision energy, 6V; high collision energy ramp, 20 to 45 V;

263 automatic lock correction (leucine enkephalin). Data processing was performed using UNIFI software (Waters,
264 Milford, MA, USA). The list of expected components includes the following protein accession numbers
265 (Uniprot): P00711, P07254, P02662, P02663, P02666, P02668, P24627. The permitted variable amino acid
266 modifications are deamidation (N, Q), oxidation (M), pyroglutamic acid N-term (E, Q), phosphorylation (S,
267 T, Y). Digestion reagent: nonspecific; minimum sequence length: 3 amino acids. To obtain the final list of
268 peptides, data filtering was performed using the following criteria: i) peptides with more than 10% matched
269 ions; ii) at least 2 first generation fragment ions; iii) elimination from the list of peptides which were visually
270 identified as in source fragments and have been instead erroneously identified by the software as independent
271 peptides.

272 **2.6 Statistical analysis**

273 Data were analysed using SPSS version 26.0 software (SPSS Inc., Chicago, IL., USA). The data were subjected
274 to one-way analysis of variance (ANOVA), Tukey's b test and LSD test (when variance was homogeneous)
275 to determine significant differences between samples ($p < 0.05$). When the sample variance was not
276 homogeneous, a non-parametric test, the Kruskal-Wallis test and median test, were used. Spearman's test was
277 used to assess the correlation between samples and the effect of temperature and pH treatment ($p < 0.05$; $p <$
278 0.01). All experiments were performed in duplicate.

279

280 **3 RESULTS AND DISCUSSION**

281 **3.1 Heat treatment and pH stress on whey proteins**

282 The whey proteins in solution (10 g/100 mL) were subjected to different pH and thermal stresses, with
283 temperature ranging from low (30°C), to medium (60°C) to high (90°C), at different pHs (2, 7, 9, 11, and 13).
284 More pH conditions were evaluated in the basic range than in the acidic range, given the greater effects induced
285 on protein structures by the basic environment (van Lieshout et al., 2020). The longest time (3h) and the highest
286 temperature (90°C) were chosen considering the longest time and the highest temperature generally used for
287 protein extractions. The possibility that major structural modifications were already present in the commercial
288 protein could be ruled out by the absence of such modifications in the proteins treated at neutral pH and room

289 temperature (conditions that do not induce any major modifications). As an exception, as will be demonstrated
290 below, lactosylation appeared to emerge as the only already present modification. Thus, all the subsequent
291 observed modifications can be considered exclusively the consequence of the applied treatments.

292

293 **3.2 Chemical changes induced by heat/pH**

294 **3.2.1 Protein profile**

295 The SDS-PAGE of processed whey proteins, treated at three different temperatures (30°C, 60°C and 90°C)
296 and five different pH values (2, 7, 9, 11 and 13) is shown in Figure 1.

297 Neutral samples, samples treated in acidic conditions and samples treated in basic conditions at pH 9 and pH
298 11, at all tested temperatures, showed two well-defined bands at 14 and 18 kDa (Agyare & Damodaran, 2010),
299 corresponding to α -lactalbumin (α -LA) and β -lactoglobulin (β -LG), which therefore appeared to be stable
300 under these conditions. Instead, the intensities of the bands at pH 2/90°C, pH 11/60°C and pH 11/90°C were
301 lower, especially for α -LA, suggesting protein degradation and/or aggregation induced by the combination of
302 high temperature and extreme pH. Consistently, samples treated under basic conditions at pH 13, at all
303 temperatures, did not show defined bands suggesting a strong degradation/aggregation effect induced by the
304 basic pH.

305 Overall, as expected, these data indicate a higher propensity of whey proteins for molecular modifications at
306 basic pH, and a lower one at acid pH. Furthermore, according to the data reported by Galani and Apenten,
307 (1999) this propensity to molecular modifications increased by high temperatures.

308 << Insert Figure 1 >>

309 The same samples were analysed by UPLC/ESI-MS (Figure 2). The chromatographic method applied allowed
310 to distinctly identify the corresponding peaks for α -LA and β -LG, the latter in the two respective isoforms A
311 and B (Buhler et al., 2019). In perfect agreement with the SDS-PAGE results, all these peaks were observed
312 in all the samples treated in neutral and acidic conditions, and in samples treated in basic conditions at pH 9
313 and pH 11 at temperatures of 30°C and 60°C. In samples treated in extreme basic conditions (pH 13), at all
314 temperatures, and in samples treated at pH 9 and pH 11 at 90°C, no protein peak could be identified. These

315 results confirmed the degradation and/or aggregation induced by the extreme alkaline processing conditions
316 combined with high temperature.

317 <<Insert Figure 2>>

318 From the α -LA and β -LG mass spectra (the latter in the two respective isoforms), the glycosylated form can also
319 be seen in the multicharged pattern. In fact, the monolactosylated (+324 Da) and dilactosylated (+648 Da)
320 forms were quite evident, as shown in Figure 2B. Quite obviously, no mass spectra corresponding to whey
321 proteins were identified in the samples not showing any protein peak in the chromatogram. From the mass
322 spectra it was possible to calculate the relative percentage of the lactosylated forms, as compared to the native
323 unglycosylated proteins. Overall, the relative amount of the lactosylated and native forms, when detected, did not
324 show differences between the samples processed under different pH and heat conditions, indicating that
325 glycosylation was already present in the starting proteins, and was basically not affected by processing (data are
326 reported in Table S1a of the supplementary material). However, when extreme pH values were reached, both
327 forms were simply not detectable anymore.

328 **3.2.2 Solubility, aggregation and degradation of the protein fraction**

329 Protein solubility is an important property for evaluating whey protein denaturation (Kilara & Vaghela, 2018).
330 The percentage of the soluble and insoluble fraction in all samples was calculated as reported in the
331 experimental section. As far as the soluble fraction was concerned, aggregation and/or degradation of whey
332 proteins were evaluated by size exclusion chromatography (SEC, Figure 3) (Fekete et al., 2014).

333 The percentage distribution of insoluble and soluble proteins, and the distribution of soluble aggregated,
334 soluble native and soluble degraded proteins is reported in Figure 3C.

335 << Insert Figure 3 >>

336 According to Pelegri and Gasparetto, (2005) a positive correlation between temperature and protein
337 insolubility was observed on whey protein. In fact, insolubility increased after high temperature processing,
338 reaching its maximum near neutral pH, probably due to the proximity to the isoelectric point of whey proteins
339 (Ding et al., 2011; Lucy et al., 2008; Pelegri & Gasparetto, 2005). On the other hand, at acidic and basic pH,
340 the insolubilization induced by the process increased with the temperature in a much more limited way.

341 As far as the soluble fraction is concerned, SEC analysis indicated that degradation appears to be prevalent in
342 acid- and basic-treated samples. In addition, basic-treated samples also showed a consistent amount of

343 degraded proteins, and as a consequence a smaller amount of native protein. Despite the paucity of studies in
344 this field, our data are in agreement with other works that report the correlation between the formation of
345 soluble aggregates and heating (Le Bon et al., 1999; Nicolai et al., 2011; Pouzot et al., 2005).

346 Overall, the above data hint at very different effects on the protein structure according to pH. At neutral pH,
347 insolubilization is the most evident consequence of processing at high temperature. At acidic pH the most
348 important modification is degradation of the protein fraction, whereas at basic pH both degradation and the
349 formation of soluble aggregates are observed, beside insolubilization. Thus, in agreement with the previous
350 data, basic pH seems to have the most profound impact on the integrity of the protein fraction.

351 **3.2.4 Total content of amino acids and free amino acids**

352 The amount of total amino acids (Figure 4A), analysed as reported in the experimental section (detailed profile
353 reported in Table S1b of the Supplementary material), showed a marked decrease in the samples treated in
354 alkaline conditions compared to neutral and acidic conditions, supporting the hypothesis of protein degradation
355 due to the alkaline treatment. In particular, the loss in the samples treated under basic conditions mostly
356 concerned some specific amino acids (Lys, Arg, Thr, Ser), whose side chains are notoriously more reactive in
357 warm/basic conditions (Borad et al., 2017). Basic pH is also expected to promote hydrolysis of Asn and Gln
358 side chains to Asp and Glu respectively, but since those side chains are anyway hydrolysed during sample
359 preparation, this possible modification cannot be detected by standard amino acid analysis.

360 The amount of free amino acids was very low in the proteins treated in acidic and neutral environment, never
361 exceeding 0.33 % of the total amount of AA. This percentage increased to a still quite low 1.57 %, in a
362 temperature dependent manner, for alkaline treated proteins. This very low amount of free amino acids
363 observed for whey proteins, even those treated under basic conditions, indicates that simple hydrolysis of the
364 peptide bond is not the prevalent protein degradation process.

365 In general, also the amino acid content mostly showed a susceptibility to protein degradation mostly occurring
366 at basic pH.

367 **3.2.5 Enantiomeric purity of amino acids**

368 The percentage of the D-enantiomer of some amino acids was determined by chiral GC in order to evaluate
369 the racemization of whey proteins due to the heat treatment combined with the pH. The percentage of the D-
370 enantiomer for Ala, Asp, Phe, Glu and Lys, as shown in Figure 4B, clearly showed an increase because of the
371 combined exposure of whey proteins to extreme basic pH and heat. As expected, alkaline treated samples (pH
372 13) at 60°C and 90°C led to a percentage of D-enantiomers ranging between 25 and 46% (therefore almost
373 complete racemization). These results are in agreement with previous work suggesting that the racemization
374 mechanism is influenced by heating and alkali treatment (Friedman, 2010). In addition, a positive correlation
375 between Ala, Asp, Phe and temperature was observed by the Spearman correlation test.

376 Neutral samples, acid treated and basic treated samples from pH 9 to pH 11 showed the lowest percentage of
377 D-amino acids, with a percentage of D-enantiomers ranging from 3 to 10%. The very low quantity of free
378 amino acids in the basic samples (see previous paragraph 3.2.4) indicates that this racemization mostly happens
379 inside the proteins, when amino acids are still bound together through peptide bonds. The increase of D-amino
380 acids within proteins, in addition to generating amino acids that cannot be used anymore for protein synthesis
381 (thus leading to a decrease in the nutritional value), certainly hinders the digestibility of proteins by preventing
382 enzymatic cleavage, due to the “wrong” configuration which hampers protease recognition.

383 <<Insert Figure 4>>

384 **3.3 Evaluation of the digestibility of proteins in differently treated samples**

385 **3.3.1 Simulated digestion protocols**

386 Digestion of the processed samples was performed using two different protocols. A simplified digestion
387 procedure was first performed in order to estimate digestibility, by solubilizing processed whey proteins at pH
388 2 and then adding pepsin, followed by mixing at a constant temperature of 37°C for 2 hours. In addition to
389 that, a validated static consensus method of simulated gastrointestinal digestion *in vitro* was then secondly
390 used in order to assess the degree of hydrolysis of the proteins and the peptides released at the end of complete
391 digestion (end of the duodenal digestion tract) (Brodkorb et al., 2019). The latter model system is characterized
392 by the use of salts and other components which recreate simulated salivary fluid, simulated gastric fluid and
393 simulated intestinal fluid, specific digestive enzymes such as pepsin from porcine gastric mucosa, pancreatin
394 from porcine pancreas and bovine bile extract, and it is performed at controlled pH.

395 **3.3.2 Degree of hydrolysis after digestion with pepsin and after gastrointestinal digestion simulated *in***
396 ***vitro***

397 The degree of hydrolysis represents the percentage of the total number of peptide bonds in a protein that have
398 been cleaved during hydrolysis. The degree of hydrolysis, in percentage (DH%), was calculated with the OPA
399 method, both after digestion with pepsin and after total gastrointestinal digestion (GD), as detailed in the
400 experimental section. For all processed samples, as expected, DH% increased from the pepsin only digestion
401 to the full gastrointestinal digestion. The maximum DH% was reached in the samples treated at pH 7 and 90°C,
402 clearly indicating that the insolubilization of the proteins (see data above) did not hinder the digestibility of
403 the proteins. In fact, a gradual increase in DH% at pH 7 was observed in a temperature-dependent manner from
404 30°C to 90°C (Figure 5A), thus, despite the increased insolubilization, the temperature treatment seems to
405 favour the digestibility of the proteins. The same effect, albeit with slightly lower DH% values, was observed
406 at acidic pH (data not shown). The increase in digestibility observed for neutral- and acidic-treated samples
407 processed at high temperature might indicate that the loss of the tertiary structure can make the protein more
408 accessible to digestive enzymes, provided that marked protein aggregation or amino acid degradation is not
409 present

410 On the other hand, for basic-treated samples processed at high temperature we observed a marked decrease in
411 digestibility, as reported in Figure 5B. This indicates that the changes seen above induced by basic pH
412 (aggregation, protein degradation, amino acid racemization) strongly influence the accessibility of enzymes
413 and the subsequent digestibility. For proteins treated at pH 13, the apparent bioaccessibility observed,
414 measured by DH%, was less than 50% of the value observed for proteins treated at neutral or acidic pH,
415 suggesting a dramatic decrease of the digestibility in heavily processed protein at basic pH.

416 <<Insert Figure 5>>

417 **3.3.3 Peptidomic analysis after digestion with pepsin and after gastrointestinal digestion simulated *in***
418 ***vitro***

419 Peptidomic analysis was performed to investigate the molecular details of pepsin digestion and gastrointestinal
420 digestion performed on whey proteins stressed by heat and pH treatments. Using a non-targeted approach, a
421 peptidomic analysis was performed combining low- and high-resolution mass spectrometry. First, from the
422 chromatographic peaks and from each associated mass spectrum, the most abundant peptides were detected,

423 estimating their amount by comparison with the internal standard Phe-Phe. Then, the molecular masses
424 associated to those peptides were determined, and the possible sequences associated with those molecular
425 masses were defined inside the target proteins (α -LA and β -LG). Through MS/MS fragmentation, the
426 theoretical fragmentation pattern of every possible sequence having compatible mass was compared with the
427 observed one, allowing to have a peptide sequence lists for each sample including all and only the most
428 abundant peptides. Finally, the same samples were analysed in high resolution by Q-ToF Mass Spectrometry
429 in order to confirm and complete the list, also including peptides not previously identified and peptides
430 carrying modifications in the side chains. The peptide sequences identified for each sample are reported in
431 Table S2 of the Supplementary material. Some casein peptides were also identified, but they have not been
432 reported.

433 Global descriptive data for all analysed samples (number of peptides identified, percentage of peptides from
434 β -LG and α -LA, percentage of coverage of β -LG and α -LA, average length of identified peptides, hydrolysis
435 of the side chains of Asn and Gln, the cleavage site) are shown in Table 1.

436 <<Insert Table 1>>

437 The number of the identified peptides turned out to be quite variable, and with no clear relation with the
438 treatments. It is to be reminded that this number is generated by two complete opposite phenomena, since the
439 peptides are at the same time the product and the substrate of every enzymatic action. The actual sum of the
440 semiquantitative data in every sample, which gives a rough estimate of the peptide amount, indicated a
441 progressive increase associated with the temperature from 30 to 60 ° C according to degree of hydrolysis and
442 pH from 2 to 11 which is totally reversed starting from pH 11 at 90°C after pepsin digestion. The decreasing
443 trend continues also in the gastrointestinal phase, becoming drastically low at pH 13. The collapse of the sum
444 of the semiquantitative data of the identified peptides suggest a more bounded accessibility of the enzymes to
445 the cleavage sites in extremely basic condition.

446 For all the digested samples, more peptides derived from β -LG rather than α -LA were detected, which was
447 expected due to the higher abundance of β -LG in our samples (and in bovine milk; Layman et al., 2018).

448 Also expected, for all processed samples the average length of the identified peptides decreased going from
449 the pepsin digestion to the full gastrointestinal digestion, considering the more extensive enzymatic cleavage
450 taking place in the latter. Interestingly, the mean length after the full gastrointestinal digestion slightly

451 increased in basic digested samples (pH 9, 11, 13) if compared to the samples treated at neutral and acidic pH,
452 indicating a more limited accessibility of the enzymes to the cleavage sites, thus a decrease in digestibility, in
453 agreement with the data above reported on the degree of hydrolysis. Indeed, in basic treated samples, an
454 increase prevalence of longer peptides on the same sequence of peptides present in neutral/acidic samples,
455 were detected, indicating failed enzymatic cleavages. Moreover, the percentage of deamidation increased at
456 basic pH, indicating a strong deamidation of Asn and Gln at basic pH as a side effect.

457 The preferential cleavage sites of the released peptides were also evaluated considering the recurrent amino
458 acid at the C-terminus. Almost all samples after pepsin digestion contained mostly peptides with a Leu residue
459 at the C-terminus (from 18 to 40% of the total peptides identified in each sample), consistently with the known
460 pepsin specificity for lipophilic amino acids. Interestingly, and again indicating a definite change in enzyme
461 action, Lys was the preferred C-terminal residue (26% of the total number of peptides detected) at pH 13 at
462 90°C. Leu and Glu were the preferred cleavage sites after gastrointestinal digestion (which includes a gastric
463 phase with pepsin, followed by a duodenal phase with pancreatin). Again, in the sample processed at pH 13 at
464 90 °C, Lys was the most recurrent residue attached to the C-terminal. This analysis confirmed that the mode
465 of action of the digestive enzymes undergo some changes in basic-treated samples, likely due to the chemical
466 modifications induced on proteins, and also mirroring the decrease in digestibility observed above.

467

468 **4 CONCLUSIONS**

469 The reported in-depth molecular characterization of whey proteins, used as model system, stressed by heat and
470 pH treatments similar to the ones found in literature for protein extraction, and the link between the molecular
471 changes and protein digestibility, allow to draw some conclusions on the best methods for protein extraction,
472 having in mind not only the protein yield (as it is often the case), but also the nutritional value of the extracted
473 proteins.

474 A first conclusion is that the set of molecular modifications induced by the processing clearly changes
475 according to pH and temperature. In this sense, pH seems to trigger different set of modifications, whereas
476 temperature has the role of “enhancing” the molecular modifications typical for acid, neutral or basic
477 conditions.

478 Neutral pH conditions are clearly the least affecting, only inducing precipitation of the proteins in a temperature
479 dependent manner, without major protein and amino acid degradation or racemization, or peptide bond
480 hydrolysis, thus preserving the nutritional value of the proteins quite intact. Quite interestingly, the digestibility
481 of the proteins is not at all impaired by their precipitation, which appears then to be perfectly reversible, and
482 susceptibility to digestive enzymes is even improved in samples treated at high temperature.

483 At acidic pH also no extensive amino acid degradation or racemization was observed, thus again nutritional
484 quality was preserved, even if proteins showed a tendency to generate compounds having lower molecular
485 weight, also in a temperature dependent manner. The fact that the total amino acid amount was unchanged, but
486 also that free amino acids were not extensively present, can lead us to conclude that only a limited peptide
487 bond hydrolysis takes place in these conditions, partially generating large polypeptides. Accordingly, and quite
488 interestingly, digestibility seemed to be even better in these samples than in neutral-treated proteins.

489 At basic pH, with an increasing tendency according to the pH value, a full array of chemical modifications
490 appears in the protein structure. Formation of soluble aggregates, protein degradation, and racemization (which
491 reaches massive values at very basic pH and high temperatures) are mostly present. The total amino acid
492 amount drops down to a value corresponding to about 20-30% less than the original value, indicating that not
493 only peptide hydrolysis is responsible for protein degradation (the amount of free amino acids remains quite
494 limited), but also other forms of amino acid degradation are present, mostly affecting the most susceptible side
495 chains. These findings alone would immediately indicate a loss of the nutritional value, but linked to those,
496 and clearly caused by those, a significant drop in digestibility was also observed, with a strong reduction in
497 the degree of hydrolysis after simulated digestion (with a drop exceeding 50% in the worst case), and a change
498 in the composition, modifications, and length of the released peptides, suggesting a difficult
499 accessibility/recognition of the digestive enzymes to the modified proteins and a difficult cleavage of the
500 peptide bonds.

501 Taken all together, the above data indicate that neutral and acid treatments, even at very low pH and even at
502 very high temperature, can be safely applied for protein extraction without any major damage to the nutritional
503 value of the protein biomass to be extracted, and in case of the acidic extraction might even lead to an increased
504 digestibility. On the other side, basic extraction conditions should be applied with extreme caution, when the
505 preservation of the nutritional values is a target, and if needed by certainly avoiding extreme pH, high

506 temperatures, and long extraction times, given the quick propensity of protein at basic pH to undergo
507 modifications which impair the amino acid content, the amino acid integrity, and the processability by digestive
508 enzymes, ultimately resulting, in the most extreme cases, in a biomass having a highly degraded nutritional
509 value. Our data also indicate that the measurement of amino acid racemization can be used as fast molecular
510 marker of the damages induced to proteins, which can be easily linked to their integrity and digestibility.
511 As per the general validity of these results, some considerations can be made. It is certainly true that plant-
512 derived proteins are found to be subjected to extreme extraction conditions much more often than animal-based
513 proteins, and is certainly true that in some cases the effect might be expected to be different. Anyway, amino
514 acids in a soluble protein (belonging to the albumin or globulin class), being all proteins made of the same 20
515 amino acids, are expected to react to the external environmental stressors in a very similar way, irrespective of
516 the protein origin. The structural modifications here reported (aggregation, degradation, racemization, amino
517 acid destruction) are intrinsically linked to the amino acid chemistry.
518 On this ground, we believe that our results have a general validity that goes beyond the origin of the proteins
519 used, and that can be useful for every person working on proteins, also plant-based proteins. Anyway, they
520 will have certainly to be further confirmed on a larger range of proteins, also including plant-based proteins.
521 These future experiments will shed more lights on the general validity of the results here presented.
522

523 **SUPPLEMENTARY MATERIAL**

524 The supplementary material as reported in the text presents in detail:

525 Table S1a: Percentage of native and lactosylated proteins in the differently treated samples

526 Table S1b: Total amino acid content in the differently treated samples

527 Table S2: The peptide sequences identified after peptic (a, c) and gastrointestinal (b, d) digestion for alpha-
528 lactalbumin (a, b) and beta-lactoglobulin (c, d).

529

530 **References**

- 531 Agyare, K. K., & Damodaran, S. (2010). PH-stability and thermal properties of microbial transglutaminase-
 532 Treated whey protein isolate. *Journal of Agricultural and Food Chemistry*, 58(3), 1946–1953.
 533 <https://doi.org/10.1021/jf903530d>
- 534 Awuah, G. B., Ramaswamy, H. S., & Economides, A. (2007). Thermal processing and quality: Principles
 535 and overview. *Chemical Engineering and Processing: Process Intensification*, 46(6), 584–602.
 536 <https://doi.org/10.1016/j.cep.2006.08.004>
- 537 Borad, S. G., Kumar, A., & Singh, A. K. (2017). Effect of processing on nutritive values of milk protein.
 538 *Critical Reviews in Food Science and Nutrition*, 57(17), 3690–3702.
 539 <https://doi.org/10.1080/10408398.2016.1160361>
- 540 Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal,
 541 C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C.,
 542 Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., ... Recio, I. (2019). INFOGEST static in vitro
 543 simulation of gastrointestinal food digestion. *Nature Protocols*, 14(4), 991–1014.
 544 <https://doi.org/10.1038/s41596-018-0119-1>
- 545 Buhler, S., Solari, F., Gasparini, A., Montanari, R., Sforza, S., & Tedeschi, T. (2019). *LWT - Food Science*
 546 *and Technology* UV irradiation as a comparable method to thermal treatment for producing high quality
 547 stabilized milk whey. *105*(July 2018), 127–134. <https://doi.org/10.1016/j.lwt.2019.01.051>
- 548 Cui, Q., Ni, X., Zeng, L., Tu, Z., Li, J., Sun, K., Chen, X., & Li, X. (2017). Optimization of Protein
 549 Extraction and Decoloration Conditions for Tea Residues. *Horticultural Plant Journal*, 3(4), 172–176.
 550 <https://doi.org/10.1016/j.hpj.2017.06.003>
- 551 Ding, X., Yang, Y., Zhao, S., Li, Y., & Wang, Z. (2011). Analysis of α -lactalbumin, β -lactoglobulin A and B
 552 in whey protein powder, colostrum, raw milk, and infant formula by CE and LC. *Dairy Science and*
 553 *Technology*, 91(2), 213–225. <https://doi.org/10.1007/s13594-011-0006-9>
- 554 Food and Agriculture Organisation (FAO) and World Health Organization (WHO). (1973). Energy and
 555 protein requirements. https://apps.who.int/iris/bitstream/handle/10665/41042/WHO_TRS_522_eng.pdf
- 556 FAO. (2009). *How to Feed the World in 2050*.
 557 http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf
 558 f
- 559 Fekete, S., Beck, A., Veuthey, J. L., & Guillarme, D. (2014). Theory and practice of size exclusion
 560 chromatography for the analysis of protein aggregates. *Journal of Pharmaceutical and Biomedical*
 561 *Analysis*, 101, 161–173. <https://doi.org/10.1016/j.jpba.2014.04.011>
- 562 Friedman, M. (1996). Nutritional Value of Proteins from Different Food Sources. A Review. *Journal of*
 563 *Agricultural and Food Chemistry*, 44(1), 6–29. <https://doi.org/10.1021/jf9400167>
- 564 Friedman, M. (2010). Origin, Microbiology, Nutrition, and Pharmacology of d -Amino Acids. *Chemistry &*
 565 *Biodiversity*, 7, 1491–1530. <https://doi.org/10.1002/cbdv.200900225>
- 566 Galani, D., & Owusu Apenten, R. K. (1999). Heat-induced denaturation and aggregation of β -lactoglobulin:
 567 Kinetics of formation of hydrophobic and disulphide-linked aggregates. *International Journal of Food*
 568 *Science and Technology*, 34(5–6), 467–476. <https://doi.org/10.1046/j.1365-2621.1999.00314.x>
- 569 Gasparini, A., Van Gool, M. P., Bultsma, M., Cutroneo, S., Sforza, S., & Tedeschi, T. (2020). Modifications
 570 induced by controlled storage conditions on whey protein concentrates: Effects on whey protein

- 571 lactosylation and solubility. *International Dairy Journal*, 109, 104765.
572 <https://doi.org/10.1016/j.idairyj.2020.104765>
- 573 Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., & Tiwari, B. (2017). Future protein supply and
574 demand: Strategies and factors influencing a sustainable equilibrium. *Foods*, 6(7), 1–21.
575 <https://doi.org/10.3390/foods6070053>
- 576 Jung, S. (2009). Aqueous extraction of oil and protein from soybean and lupin: A comparative study. *Journal*
577 *of Food Processing and Preservation*, 33(4), 547–559. [https://doi.org/10.1111/j.1745-](https://doi.org/10.1111/j.1745-4549.2009.00400.x)
578 [4549.2009.00400.x](https://doi.org/10.1111/j.1745-4549.2009.00400.x)
- 579 Kilara, A., & Vaghela, M. N. (2018). Whey proteins. In Rickey Y. Yada (Ed.), *Proteins in Food Processing*
580 (Second ed). Elsevier Ltd. <https://doi.org/10.1016/B978-0-08-100722-8.00005-X>
- 581 Layman, D. K., Lönnerdal, B., & Fernstrom, J. D. (2018). Applications for α -lactalbumin in human nutrition.
582 *Nutrition Reviews*, 76(6), 444–460. <https://doi.org/10.1093/nutrit/nuy004>
- 583 Le Bon, C., Nicolai, T., & Durand, D. (1999). Growth and structure of aggregates of heat-denatured β -
584 lactoglobulin. *International Journal of Food Science and Technology*, 34(5–6), 451–465.
585 <https://doi.org/10.1046/j.1365-2621.1999.00310.x>
- 586 Leip, A., Weiss, F., Lesschen, J. P., & Westhoek, H. (2014). The nitrogen footprint of food products in the
587 European Union. *Journal of Agricultural Science*, 152(2014), S20–S33.
588 <https://doi.org/10.1017/S0021859613000786>
- 589 Leni, G., Caligiani, A., & Sforza, S. (2019). Killing method affects the browning and the quality of the
590 protein fraction of Black Soldier Fly (*Hermetia illucens*) prepupae: a metabolomics and proteomic
591 insight. *Food Research International*, 115(August 2018), 116–125.
592 <https://doi.org/10.1016/j.foodres.2018.08.021>
- 593 López-Fandiño, R., Martos, G., Contreras, P., & Molina, E. (2010). Egg white ovalbumin digestion
594 mimicking physiological conditions. *Journal of Agricultural and Food Chemistry*, 58(9), 5640–5648.
595 <https://doi.org/10.1021/jf904538w>
- 596 Lucy, C. A., MacDonald, A. M., & Gulcev, M. D. (2008). Non-covalent capillary coatings for protein
597 separations in capillary electrophoresis. *Journal of Chromatography A*, 1184(1–2), 81–105.
598 <https://doi.org/10.1016/j.chroma.2007.10.114>
- 599 Meade, S. J., Reid, E. A., & Gerrard, J. A. (2005). The impact of processing on the nutritional quality of food
600 proteins. *Journal of AOAC International*, 88(3), 904–922. <https://doi.org/10.1093/jaoac/88.3.904>
- 601 Mollea, C., Marmo, L., & Bosco, F. (2013). Valorisation of Cheese Whey, a By-Product from the Dairy
602 Industry. *Food Industry*. <https://doi.org/10.5772/53159>
- 603 Momen, S., Alavi, F., & Aider, M. (2021). Alkali-mediated treatments for extraction and functional
604 modification of proteins: Critical and application review. *Trends in Food Science and Technology*,
605 110(October 2020), 778–797. <https://doi.org/10.1016/j.tifs.2021.02.052>
- 606 Nicolai, T., Britten, M., & Schmitt, C. (2011). β -Lactoglobulin and WPI aggregates: Formation, structure
607 and applications. *Food Hydrocolloids*, 25(8), 1945–1962.
608 <https://doi.org/10.1016/j.foodhyd.2011.02.006>
- 609 Pelegri, D. H. G., & Gasparetto, C. A. (2005). Whey proteins solubility as function of temperature and pH.
610 *LWT - Food Science and Technology*, 38(1), 77–80. <https://doi.org/10.1016/j.lwt.2004.03.013>
- 611 Pouzot, M., Nicolai, T., Visschers, R. W., & Weijers, M. (2005). X-ray and light scattering study of the

- 612 structure of large protein aggregates at neutral pH. *Food Hydrocolloids*, 19(2), 231–238.
613 <https://doi.org/10.1016/j.foodhyd.2004.06.003>
- 614 Prandi, B., Faccini, A., Lambertini, F., Bencivenni, M., Jorba, M., Droogenbroek, B. Van, Bruggeman, G.,
615 Schöber, J., Petrusan, J., Elst, K., & Sforza, S. (2019). Food wastes from agrifood industry as possible
616 sources of proteins : A detailed molecular view on the composition of the nitrogen fraction , amino acid
617 profile and racemisation degree of 39 food waste streams. *Food Chemistry*, 286(December 2018),
618 567–575. <https://doi.org/10.1016/j.foodchem.2019.01.166>
- 619 Prandi, B., Lambertini, F., Faccini, A., Suman, M., Leporati, A., Tedeschi, T., & Sforza, S. (2017). Mass
620 spectrometry quantification of beef and pork meat in highly processed food: Application on Bolognese
621 sauce. *Food Control*, 74, 61–69. <https://doi.org/10.1016/j.foodcont.2016.11.032>
- 622 Qin, F., Johansen, A. Z., & Mussatto, S. I. (2018). Evaluation of different pretreatment strategies for protein
623 extraction from brewer’s spent grains. *Industrial Crops and Products*, 125(September), 443–453.
624 <https://doi.org/10.1016/j.indcrop.2018.09.017>
- 625 Roberts, P. J., Simmonds, D. H., Wootton, M., & Wrigley, C. W. (1985). Extraction of protein and solids
626 from wheat bran. *Journal of the Science of Food and Agriculture*, 36(1), 5–10.
627 <https://doi.org/10.1002/jsfa.2740360103>
- 628 Sari, Y. W., Mulder, W. J., Sanders, J. P. M., & Bruins, M. E. (2015). Towards plant protein refinery:
629 Review on protein extraction using alkali and potential enzymatic assistance. *Biotechnology Journal*,
630 10(8), 1138–1157. <https://doi.org/10.1002/biot.201400569>
- 631 Spellman, D., McEvoy, E., O’Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis
632 of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of
633 hydrolysis. *International Dairy Journal*. [https://doi.org/10.1016/S0958-6946\(03\)00053-0](https://doi.org/10.1016/S0958-6946(03)00053-0)
- 634 Sun-Waterhouse, D., Zhao, M., & Waterhouse, G. I. N. (2014). Protein Modification During Ingredient
635 Preparation and Food Processing: Approaches to Improve Food Processability and Nutrition. *Food and*
636 *Bioprocess Technology*, 7(7), 1853–1893. <https://doi.org/10.1007/s11947-014-1326-6>
- 637 The Commission of The European Communities. (2009). Commission Regulation (EC) No.152/2009.
638 *Official Journal of the European Union*, 52, 1–28. https://doi.org/10.3000/1725258X.L_2009.152.ita
- 639 Torres-León, C., Ramírez-Guzman, N., Londoño-Hernandez, L., Martínez-Medina, G. A., Díaz-Herrera, R.,
640 Navarro-Macias, V., Alvarez-Pérez, O. B., Picazo, B., Villarreal-Vázquez, M., Ascacio-Valdes, J., &
641 Aguilar, C. N. (2018). Food Waste and Byproducts: An Opportunity to Minimize Malnutrition and
642 Hunger in Developing Countries. *Frontiers in Sustainable Food Systems*, 2(September), 1–17.
643 <https://doi.org/10.3389/fsufs.2018.00052>
- 644 van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Jasti,
645 P. R., & Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Molecular*
646 *Nutrition and Food Research*, 54(9), 1215–1247. <https://doi.org/10.1002/mnfr.200900608>
- 647 van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E., & Hettinga, K. A. (2020). How processing may
648 affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical*
649 *Reviews in Food Science and Nutrition*, 60(14), 2422–2445.
650 <https://doi.org/10.1080/10408398.2019.1646703>
- 651 Wu, W.;Hettiarachchy, N.S.;Kalapathy, U. ;William. W. P. (1998). Functional properties and nutritional
652 quality of alkali- and heat-treated soy protein isolate. *Journal of Food Quality*, 22(501), 119–133.
653 <https://doi.org/https://doi.org/10.1111/j.1745-4557.1999.tb00545.x>
- 654 Zhang, C., Sanders, J. P. M., & Bruins, M. E. (2014). Critical parameters in cost-effective alkaline extraction

655 for high protein yield from leaves. *Biomass and Bioenergy*, 67(0), 466–472.
656 <https://doi.org/10.1016/j.biombioe.2014.05.020>
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658 **Figure captions:**

659 **Figure 1** Electrophoretic pattern of whey proteins after being treated at different pH/temperatures for three
660 hours.

661 **Figure 2** A) LC-MS chromatograms of protein samples after treatment at 30°C and pH 2 (top left), 7 (middle
662 left) and 13 (bottom left); B) MS spectra of α -lactalbumin, β -lactoglobulin A and β -lactoglobulin B taken
663 from the sample treated at pH 2, 30°C. The asterisks indicate the glycated forms of the proteins.

664 **Figure 3** SEC chromatogram of the samples treated at pH 2 at 30°C (A) and pH 11 at 60°C (B). The putative
665 distribution of aggregated, native and degraded protein is showed in figure. C) Putative distribution of the
666 insoluble and soluble fraction (native, aggregated and degraded protein).

667 **Figure 4** A) Total amino acid content in samples treated at different pH and temperature. Different letters
668 mean statistically different samples ($p < 0.05$) (one-way ANOVA, LSD test). B) Formation of D-amino acids
669 in samples treated at different pH and temperatures. The amino acid Ala, Asp, Phe, Glu and Lys were evaluated
670 for their significant differences in each sample, different letters mean statistically differences between samples
671 ($p < 0.05$) (one-way ANOVA, Tukey's b test).

672 **Figure 5** A) Effect of different temperature conditions at pH 7 on the DH% of samples subjected to peptic and
673 gastrointestinal digestion. B) Effect of the different pH on the DH% of samples subjected to peptic and
674 gastrointestinal digestion.

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