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Bovine milk antioxidant properties: effect of *in vitro* digestion and identification of antioxidant compounds

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Short title: **Antioxidant properties of digested milk**

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1 **Abstract**

2 Milk proteins contained encrypted in their sequence biologically active components that can
3 be released by enzymatic hydrolysis. Among the biological activities recognised in milk
4 components, the antioxidant activity is of great interest. The objective of the present study
5 was to analyse the antioxidant properties of whole, semi-skimmed and skimmed milk during
6 simulated gastro-intestinal digestion and to identify the compounds responsible for the
7 antioxidant activity. Simulated digestion increased the ABTS^{•+} radical scavenging activity of
8 milk. In digested whole milk, the main contribution to ABTS^{•+} radical scavenging activity was
9 due to high molecular weight fraction (>3 kg·mol⁻¹). For semi-skimmed and skimmed milk,
10 the main contribution was due to low molecular weight fraction (<3 kg·mol⁻¹). Twelve major
11 peaks were collected from low molecular weight fraction of digested skimmed milk by
12 reversed-phase high-performance liquid chromatography and evaluated for their ABTS^{•+}
13 radical scavenging activity. Among the different fractions, three (F2, F3 and F5) showed high
14 ABTS^{•+} and hydroxyl radical scavenging activity and lipid peroxidation inhibitory capacity.
15 The compounds (free amino acids and peptides) present in these fractions were identified with
16 nanoLC-QTOF MS/MS analysis. The amino acids tryptophan and tyrosine seemed
17 fundamental in the ABTS^{•+} and hydroxyl radical scavenging capacities whereas the amino
18 acids phenylalanine and histidine played an important role in the lipid peroxidation inhibitory
19 activity of the peptides. The results reported in this study suggested that milk proteins could
20 act as a carrier for the delivery of antioxidant compounds in the gastro-intestinal tract possibly
21 protecting the gastro-intestinal tract itself from the oxidative damage.

22 **Keywords:** *in vitro* gastro-intestinal digestion; antioxidant activity; mass spectrometry;
23 peptides, aromatic aminoacids.

24 **1. Introduction**

25 Antioxidant compounds are considered important for human health thanks to their ability to
26 scavenge free radicals and contribute to prevent chronic diseases, such as cancers, coronary
27 heart diseases, and neurodegenerative disorders (Del Rio et al. 2013). Dairy product showed
28 antioxidant activity and have to be considered as important dietary components that contribute
29 to the total intake of antioxidants. In milk, proteins (especially caseins) are the most important
30 radical scavenger compounds (Clausen et al. 2009).

31 Most bovine milk proteins, mainly β -lactoglobulin and α S1-casein, are also potential
32 allergens and cow's milk protein allergy is the most prevalent in infancy, reaching an
33 incidence of about 2% to 7.5% (Bu et al. 2013). Reduction of milk protein allergenicity by
34 technological processing such as heat treatment, lactic fermentation and enzymatic hydrolysis
35 is a topic of major relevance to develop hypoallergenic milk products (Bu et al. 2013).

36 Enzymatic hydrolysis is an effective means to generate bioactive peptides from intact protein
37 sequences (Pihlanto 2006). The biological activities of these peptides include antimicrobial,
38 anti-hypertensive, antithrombotic and antioxidative activities (Pihlanto 2006; Power-Grant et
39 al. 2013). Antioxidant peptides and amino acids are particularly interesting for their possible
40 contribution to health promotion and disease prevention (Power-Grant et al. 2013).

41 The composition and the biological properties of the peptide contained in milk hydrolysates
42 depend on the substrate, the proteolytic enzymes, the enzyme to substrate ratio and
43 physicochemical conditions (pH, hydrolysis time and temperature of reaction) (del Mar
44 Contreras et al. 2011). A variety of proteolytic enzymes has been used to generate milk
45 protein hydrolysates with differing degrees of hydrolysis, containing a diverse assortment of
46 peptides and different antioxidant activity. Digestive enzymes and combinations of different
47 proteinases such as alcalase and thermolysin have been utilized to successfully generate
48 antioxidant peptides from various milk proteins (Pihlanto 2006; Power-Grant et al. 2013). For

49 example, a peptic digest of bovine caseins produced the α S1-casein-derived peptide YFYPEL
50 with a strong superoxide anion scavenging activity (Suetsuna et al. 2000). One potent
51 antioxidant peptide (WYSLAMAASDI) was purified from bovine β -lactoglobulin hydrolysed
52 with Corolase PP (Hernández-Ledesma et al. 2005). Thermolysin was utilized to generate two
53 antioxidant peptides (LQKW and LDTDYKK) from β -lactoglobulin (del Mar Contreras et al.
54 2011).

55 Milk proteins are deeply transformed in the human gastro-intestinal tract because of the
56 presence of different proteases. Peptides generated in the gastro-intestinal may have different
57 biological properties such as antimicrobial, antioxidant, antihypertensive, etc. (Boutrou et al.
58 2015). However, till now, little studies have been carried on the production of antioxidant
59 compounds during the *in vitro* digestion of milk proteins and, above all, the nature of
60 bioactive antioxidant compounds released during digestion has not been revealed. *In vitro*
61 gastro-intestinal digestion enhance the antioxidant activity of a bovine milk-
62 based protein matrix (Power-Grant et al. 2016) as well as of a κ -casein and β -casein
63 preparation (Petrat-Melin et al. 2015; Petrat-Melin et al. 2016). Some antioxidant peptides
64 (such as WSVPPQPK and ISELGW) and free amino acids have been generated after *in vitro*
65 gastro-intestinal hydrolysis of human milk or infant formula (Raikos and Diassos 2014).
66 At present, *in vitro* digestion studies focused on single isolated proteins without considering
67 the influence of other components present in dairy products such as fat. Therefore, our *in vitro*
68 digestion study was targeted on the identification of antioxidant compounds released after the
69 *in vitro* digestion from complex food matrices represented by whole, semi-skimmed and
70 skimmed bovine milk.

71

72 **2. Materials and methods**

73 *2.1. Materials*

74 All electrophoresis, HPLC and MS/MS reagents were from Biorad (Hercules CA, U.S.A.),
75 whereas the remaining chemicals were purchased from Sigma-Aldrich (Milan, Italy) unless
76 otherwise stated. Amicon Ultra-4 regenerated cellulose $3 \text{ kg}\cdot\text{mol}^{-1}$ were supplied by Millipore
77 (Billerica MA, USA). The homogenized bovine milk (whole, semi-skimmed and skimmed
78 milk), belonging to the same batch of raw milk, were obtained from a local producer. The
79 different types of milk had the same total proteins ($3.1 \text{ g}\cdot\text{100mL}^{-1}$), caseins ($2.6 \text{ g}\cdot\text{100mL}^{-1}$),
80 carbohydrates ($4.8 \text{ g}\cdot\text{100mL}^{-1}$) and calcium ($120 \text{ mg}\cdot\text{100mL}^{-1}$) content but differ for the fat
81 content ($3.60 \text{ g}\cdot\text{100mL}^{-1}$, $1.55 \text{ g}\cdot\text{100mL}^{-1}$ and $0.05 \text{ g}\cdot\text{100mL}^{-1}$ in whole, semi-skimmed and
82 skimmed milk, respectively). The absorbance was read using a Jasco V-550 UV/Vis
83 spectrophotometer (Orlando FL, U.S.A.).

84

85 *2.2. In vitro gastro-intestinal digestion*

86 The two-stage *in vitro* digestive model was adapted from Helal et al. (2014). Aliquots (50
87 mL) of milk were mixed with 50 mL of water. The samples were then brought to pH 2.5 with
88 concentrated HCl and the gastric digestion was started by the addition to the 100 mL of
89 overall digestion media of 0.2 g of NaCl and 31500 U of pepsin. The samples were incubated
90 at 37°C in a shaking bath for 2h to simulate the gastric phase of digestion. At the end of the
91 gastric digestion, the pH was brought to 7.5 with NaHCO_3 , before adding $0.8 \text{ g}\cdot\text{L}^{-1}$ pancreatin
92 and $5 \text{ mg}\cdot\text{mL}^{-1}$ bile salts. The solution was then incubated at 37°C in a shaking bath for
93 further 2h to simulate the intestinal phase of digestion. The enzymes were inactivated by
94 heating at 95°C for 15 min, followed by cooling to room temperature. Aliquots of the samples
95 were withdrawn after mixing milk with water (after mixing pH 6.8), after acidification to pH

96 2.5, at the end of the gastric digestion, after alkalization to pH 7.5 and at the end of the
97 intestinal digestion. Each sample was digested in triplicate.

98

99 *2.3. Determination of the degree of hydrolysis*

100 The determination of the degree of hydrolysis of the digested samples was carried out as
101 reported by Adler-Nissen (1979). The hydrolysis degree was calculated as reported in
102 equation (1):

$$103 \mathbf{DH} = (\mathbf{h}/\mathbf{h}_{\text{tot}}) \cdot 100 \quad (1)$$

104 where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents·g⁻¹ of
105 protein of α-amino groups formed at the different stages of the simulated digestion, and **h_{tot}** is
106 the hydrolysis equivalent at complete hydrolysis to amino acids (calculated by summing the
107 contents of the individual amino acids in 1 g of protein and considering caseins as the only
108 proteins in milk). According to Adler-Nissen (1979), the **h_{tot}** value was fixed at 8 that is the
109 value calculated for caseins.

110

111 *2.4. SDS-PAGE Electrophoresis*

112 Samples of different types of un-fractionated milk taken at different times of digestion were
113 subjected to SDS-PAGE electrophoresis using 17% polyacrylamide separating gel as reported
114 in Helal et al. (2014). Three SDS gels were run of each milk sample.

115

116 *2.5. Fractionation of digested samples*

117 Samples from *in vitro* simulated digestion (4 mL of 5 times water-diluted sample) were
118 subjected to ultrafiltration with Amicon Ultra-4 nominal cutoff 3 kg·mol⁻¹ (Millipore, Italy),
119 at 7500g for 120 min at 4°C. At the end of the separation, two fractions were obtained: the
120 retentate containing mainly high molecular weight compounds (HMW fraction; > 3 kg·mol⁻¹)

121 and the permeate containing low molecular weight compounds (LMW fraction; $< 3 \text{ kg}\cdot\text{mol}^{-1}$).
122 The two fractions were filled up to 4 mL with a solution of HCl $0.01 \text{ mol}\cdot\text{L}^{-1}$ for the sample
123 collected during the gastric phase of the digestion or potassium phosphate buffer ($0.1 \text{ mol}\cdot\text{L}^{-1}$;
124 pH 7) for the sample collected during the intestinal phase.

125

126 *2.6. Reversed-phase high performance liquid chromatography (HPLC) analysis of peptides*

127 HPLC separation of the low molecular weight fractions of digested milk collected at the end
128 of the pancreatic digestion was performed with a Jasco HPLC system equipped with a
129 reversed phase column Hamilton HxSil C18 (Hamilton, Reno, Nevada; $250\text{mm} \times 4.6\text{mm}$, 5
130 μm , 100 \AA) as described in Tagliazucchi et al. (2015). The two solvents were: solvent A
131 mixture of water-trifluoroacetic acid (0.037%) and solvent B acetonitrile-trifluoroacetic acid
132 (0.027%). A linear gradient of solvent B in A ranging from 0% to 45% in 115 min with a flow
133 rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$ was used to separate the peptides contained in the low molecular
134 fractions of digested milk. The photodiode array (PDA) detector was set at 214 nm. Twelve
135 fractions from digested skimmed milk were collected and freeze-dried. These fractions were
136 re-filled to the original volume with a potassium phosphate buffer ($0.1 \text{ mol}\cdot\text{L}^{-1}$; pH 7) and
137 then analysed for their antioxidant activity.

138

139 *2.7. ABTS⁺ radical scavenging activity of digested samples and HPLC fractions*

140 The antioxidant activity of the sample collected during the *in vitro* digestion procedure and
141 from HPLC separation was determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-
142 sulphonic acid) method as described in Re et al. (1999) both on un-fractionated and
143 fractionated samples. The ABTS⁺ scavenging capacity was expressed as milligrams of
144 vitamin C per L of milk, by means of a calibration curve obtained with vitamin C (ranging
145 from 1 to $150 \text{ mg}\cdot\text{L}^{-1}$), in the same assay conditions.

146

147 *2.8 Antioxidant properties of selected HPLC fractions and amino acids*

148 The antioxidant properties of the selected HPLC fractions and amino acids were evaluated
149 using three different assays.

150 The ABTS assay was carried out as described in the paragraph 2.7.

151 The capacity to scavenge hydroxyl radicals was evaluated according to a method reported by
152 Ajibola et al. (2011) with some modifications. The assay consisted of mixing 50 μL of 3
153 $\text{mmol}\cdot\text{L}^{-1}$ TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) dissolved in HCl 50 $\text{mmol}\cdot\text{L}^{-1}$, 50 μL of 3
154 $\text{mmol}\cdot\text{L}^{-1}$ FeSO_4 , 50 μL of sample or vitamin C (at concentration ranging from 1 to 10
155 $\text{mmol}\cdot\text{L}^{-1}$), and 50 μL of 0.01% (v/v) hydrogen peroxide, in a clear bottom 96-well plate. The
156 mixture was incubated for 1h at 37°C and the absorbance was measured at 540 nm using a
157 microplate reader.

158 The ABTS and hydroxyl radical scavenging capacities were expressed as milligrams of
159 vitamin C per L of milk or, in the case of the amino acids as mg vitamin C per mmol of amino
160 acid.

161 The ability to inhibit lipid peroxidation was carried out using a linoleic acid emulsion system
162 (Ajibola et al. 2011). For that purpose, 200 μL of sample at concentration of 2 $\text{mmol}\cdot\text{L}^{-1}$, 200
163 μL of 99.5% ethanol and 2.6 μL of linoleic acid were mixed and the total volume was
164 adjusted to 500 μL with sodium phosphate buffer, 50 $\text{mmol}\cdot\text{L}^{-1}$, and pH 7.0. The mixture was
165 incubated at 40°C in the dark for seven days. The amount of generated lipid hydroperoxide
166 was measured by the FOX assay as reported by Tagliazucchi et al. (2010). The lipid
167 peroxidation inhibitory activity of the samples was expressed as percentage of inhibition
168 respect to a control reaction without the sample.

169

170 *2.9. Peptide profile determination with nanoflow liquid chromatography accurate mass*
171 *quadrupole time-of-flight mass spectrometry with electrospray ionization (LC-ESI-QTOF MS)*

172 The fractions with the highest antioxidant activity were subjected to QTOF MS/MS analysis
173 for peptide identification. Nano LC/MS and tandem MS experiments were performed on a
174 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-
175 Mass Q-TOF LC/MS via a Chip Cube Interface (Agilent Technologies). Chromatographic
176 separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment
177 column and a 43 mm × 75 µm analytical column, both packed with a Zorbax 300SB 5 µm
178 C18 phase (Agilent Technologies). The mobile phases composition and the gradient were the
179 same as reported by Tagliacruzchi et al. (2015). The mass spectrometer was tuned, calibrated
180 and set with the same parameters as reported by Dei Più et al. (2014).

181 For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de*
182 *novo* peptide sequencing was performed using Peptov software
183 (<http://proteomics.ucsd.edu/ProteoSAFe/>). The following parameters were considered:
184 enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da;
185 variable modification, oxidation (M) and phosphorylation (ST); maximal number of PTMs
186 permitted in a single peptide 3.

187 A search for the biological activity of peptides identified was carried out through the BIOPEP
188 database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php). Confirmation of
189 peptides sequence in bovine milk proteins was performed using Peptide Match
190 (<http://research.bioinformatics.udel.edu/peptidematch/index.jsp>).

191

192 *2.10. HPLC analysis of tyrosine and tryptophan*

193 The amount of tyrosine in F2 and tryptophan in F5 was determined according to Frank and
194 Powers (2007). Derivatization was carried out by mixing 50 µL of sample with 50 µL of OPA

195 (ortho-phthalaldehyde) solution (consisting of 7.45 mmol·L⁻¹ of OPA and 11.4 mmol·L⁻¹ of 3-
196 mercaptopropionic acid in potassium tetraborate buffer 0.2 mmol·L⁻¹ pH 9.5). The HPLC
197 separation of the derivatized amino acids was carried out with the same C18 column as
198 reported in paragraph 2.6 using a binary gradient of mobile phase A (30 mmol·L⁻¹ potassium
199 phosphate buffer with 0.4% tetrahydrofuran pH 7.0) and mobile phase B (50% acetonitrile
200 and 50% water). The gradient started at 0% B for 0.5 min then linearly ramped up to 48% B
201 in 22 min. The mobile phase composition was raised up to 60% B in 12 min, then 100% B in
202 1 min and maintained for 4 min in order to wash the column. Flow rate was 1 mL·min⁻¹. The
203 detection was performed at 340 nm.

204

205 *2.11. Statistical analysis*

206 All data are presented as mean ± SD for three independent *in vitro* digestion experiments
207 performed on the same milk sample. Two-way univariate analysis of variance (ANOVA) with
208 Tukey post-hoc test was applied to determine significant differences ($P < 0.05$). Correlations
209 between variables were assessed using Pearson's method. All analyses were performed with
210 GraphPad Prism version 6.00 (GraphPad software, San Diego, CA).

211

212 3. Results and discussion

213

214 3.1 Assessment of protein hydrolysis during simulated digestion of whole, semi-skimmed and 215 skimmed milk

216 Simulated gastro-intestinal digestion of whole, semi-skimmed and skimmed milk resulted in
217 the partial hydrolysis of the milk proteins with formation of peptides with a molecular weight
218 lower than $10 \text{ kg}\cdot\text{mol}^{-1}$ as showed by electrophoresis (data not shown). As detailed in **Table 1**,
219 the degree of hydrolysis (DH) of the different types of milk at time 0h was similar and not
220 statistically different (average DH value 2.9 ± 0.3) regardless of milk type. DH increased
221 significantly ($P < 0.05$) during the peptic digestion for all the samples but with some
222 differences. The DH after peptic digestion was higher for samples with low fat content
223 (skimmed > semi-skimmed > whole milk). The pancreatic digestion produced a high and
224 significant ($P < 0.05$) increase in DH for all the digested samples. The degree of protein
225 hydrolysis was different considering the various types of milk and in particular was higher for
226 milk poor in fat respect to the milk rich in fat, despite having the same protein content.
227 Results showed that the presence of fat reduces the proteolysis both at gastric and intestinal
228 level. The exact mechanism is not known and currently under investigation. It could be
229 expected that, since surface plays a very important part in enzyme action, fats may reduce
230 surface tension and so lower surface energy, hence retard protein digestion.
231 The DH value measured after gastro-intestinal digestion with our model was lower than those
232 determined by Picariello et al. (2015) which found a degree of hydrolysis for skimmed milk
233 between 34.5 and 58 depending on the substrate to digestive enzyme ratio. Simulated
234 digestion of isolated κ - and β -casein resulted in a hydrolysis degree between 40 and 55
235 (Petrat-Melin et al. 2015; Petrat-Melin et al. 2016).

236

237 *3.2. ABTS^{•+} radical scavenging activity of digested whole, semi-skimmed and skimmed milk*

238 All three types of milk showed ABTS^{•+} radical scavenging activity before the digestion
239 (**Table 2**), but with some differences. Skimmed milk had a significant minor radical
240 scavenging activity respect to whole milk ($P < 0.05$). The higher value of ABTS^{•+} radical
241 scavenging activity in samples with more fats can be due to the reactivity of lipid soluble
242 antioxidants, such as α -tocopherol and carotenoids (Re et al., 1999), and fat globule
243 membrane proteins with ABTS^{•+} radical. More than 90% of the antioxidant activity in all the
244 analysed types of milk was in the $> 3 \text{ kg}\cdot\text{mol}^{-1}$ high molecular weight fraction (HMW)
245 underlining the role of protein in the total radical scavenging activity of milk. Clausen et al.
246 (2009) found that caseins are quantitatively the highest radical scavengers in milk whereas the
247 lower contribution of the low molecular weight fraction (LMW) is due to ascorbate and
248 especially urate. Caseins have a high content of antioxidative amino acids such as tyrosine,
249 tryptophan and phosphoserine, and quenching of free radicals by oxidation of these amino
250 acids was proposed as the explanation (Clausen et al. 2009; Cervato et al. 1999).

251 After acidification of the milk samples to pH 2.5 (corresponding to the time zero of the gastric
252 digestion), the ABTS^{•+} radical scavenging activity decreased significantly in all three types of
253 milk. This decrease was caused by a drop in the ABTS^{•+} value of the HMW fraction whereas
254 the value in the LMW fraction was unaffected. The gastric pH value (pH 2.5) is near to the
255 isoelectric point of casein (pI 4.6) and this determines changes in casein aggregation with a
256 masking of antioxidant sequences of amino acids, which can explain the decrease in ABTS^{•+}
257 radical scavenging activity after acidification. After 120 min of peptic digestion, the total
258 ABTS^{•+} value increased non-significantly respect to the time zero of gastric digestion in all
259 the milk types. However, the ABTS^{•+} radical scavenging activity was always lower than the
260 original value found in the different types of milk before the digestion except than in the
261 LMW fractions. The main contribution to ABTS^{•+} value, for all the types of milk beverages

262 after peptic digestion, was due to HMW fraction. The HMW fraction ABTS^{•+} radical
263 scavenging activity decreased, from the milk richest to the milk poorest in fats.

264 The passage into the alkaline media, from 120 min of the gastric digestion (pH 2.5) to time 0
265 min of the pancreatic digestion (pH 7.5), led to an increase in the ABTS^{•+} radical scavenging
266 activity in total and LMW and HMW fractions of all the types of milk beverages.

267 After 120 min of pancreatic digestion there was a high increase in the ABTS^{•+} value, for all
268 the three types of milk beverages. Whole milk showed the highest increase whereas there
269 were no statistically differences between semi-skimmed and skimmed milk.

270 The distribution of the radical scavenging activity between the LMW and HMW fractions was
271 different considering the diverse types of milk. In digested whole milk, the main contribution
272 to ABTS^{•+} radical scavenging activity was due to HMW fraction. For semi-skimmed and
273 skimmed milk, the main contribution was due to LMW fraction. During *in vitro* gastro-
274 intestinal digestion of bovine milk, protein hydrolysis determines the formation of low
275 molecular weight peptides with ABTS^{•+} radical scavenging activity probably due to
276 unmasking and liberation of some amino acids sequences with antioxidant activities that are
277 buried or inactive in the intact proteins. Hydrolysates obtained after peptic, tryptic and
278 chymotryptic hydrolysis of milk proteins showed radical scavenging activity (Pihlanto 2006;
279 Hernández-Ledesma et al. 2005). There is a clear correlation (Pearson coefficient $r=0.928$;
280 $P < 0.05$) between the DH and the amount of ABTS^{•+} radical scavenging activity found in
281 LMW fractions. The whole milk sample showed the lowest DH after pancreatic digestion and
282 only 38% of ABTS^{•+} radical scavenging activity was found in the LMW fraction. The
283 percentage of ABTS^{•+} radical scavenging activity in the LMW fraction increased to 79% and
284 90% in semi-skimmed and skimmed milk samples, respectively, according to the increase in
285 the hydrolysis degree. The fat content negatively influenced the LMW ABTS^{•+} radical
286 scavenging activity at the end of the digestion. Indeed, the presence of fat may lead to

287 peroxidative phenomena during gastro-intestinal digestion causing depletion of antioxidant
288 compounds. This fact may results in a lower ABTS^{•+} reactivity in the samples richest in fat.
289

290 *3.3. Antioxidant properties of the HPLC fractions from the permeate of digested skimmed* 291 *milk*

292 The peptides in the LMW fractions of digested milk were separated in the HPLC C18 column
293 and detected at 214 nm with PDA. As reported in **Figure 1**, the LMW fractions of the three
294 different types of milk showed the same HPLC pattern with the only difference in the
295 intensity of the peaks.

296 Due to its high ABTS^{•+} radical scavenging activity, the LMW fraction of skimmed milk was
297 selected for MS/MS experiments with the aim to identify the compounds responsible for the
298 activity.

299 Twelve fractions from skimmed milk permeate (**Figure 1C**) were collected, freeze dried and
300 evaluated for their ABTS^{•+} radical scavenging activity. Only seven fractions were found to
301 have a considerable ABTS^{•+} radical scavenging activity (**Figure 2**).

302 Three fractions (F2, F3 and F5) were found to be the major contributors on the ABTS^{•+}
303 radical scavenging activity of the skimmed milk LMW fraction. These three fractions were
304 further characterized for their ability to scavenge hydroxyl radical and to inhibit lipid
305 peroxidation. All of the three fractions exhibited a certain degree of hydroxyl scavenging
306 activity. Fraction F2 was the most active against hydroxyl radical whereas fraction F3 showed
307 the highest lipid peroxidation inhibitory activity (**Table 3**).

308 The compounds responsible for the antioxidant activity of these three fractions were
309 tentatively identified with mass spectrometry.

310

311 *3.4. NanoLC-ESI-QTOF-MS/MS analysis of the HPLC collected fractions*

312 **Figure 3** shows the full MS spectra of the fractions F2, F3 and F5. Each peak was selected for
313 peptide identification by MS/MS ion scan using de novo sequencing software. Results from
314 peptide identification were subjected to a manual evaluation, and the validated peptide
315 sequences explained most of the major peaks in the MS spectra.

316 In the lowest part of the MS spectra of fraction F2 (**Figure 3A**) the most intense signals were
317 identified as the amino acids (iso)leucine (Lx; $m/z=132.1037$) and tyrosine (Y;
318 $m/z=182.0851$), and the dipeptide GP ($m/z=173.0840$). Additional intense signals were
319 identified as the dipeptide GLx ($m/z=189.1246$) and the tripeptides VVD ($m/z=332.1821$) and
320 LSH ($m/z= 356.1945$). The list of compounds identified in fraction F2 is shown in **Table 4**
321 together with the MS data, the protein precursor and the potential bioactivity.

322 In the lowest part of the MS spectra of fraction F3 (**Figure 3B**) the most intense signals were
323 identified as the amino acid phenylalanine (F; $m/z=166.1055$) and the dipeptide GP
324 ($m/z=173.0819$). An additional signal at m/z of 120.0859 was assigned to the amino acid
325 threonine. In the peptidic part of the spectra the most intense signals corresponded to the
326 dipeptides VLx (m/z 231.1736), ALx ($m/z= 203.1415$) and QLx ($m/z=260.1639$) and the
327 tripeptide SLxT ($m/z= 320.1849$). The list of compounds identified in fraction F3 is shown in
328 **Table 4** together with the MS data, the protein precursor and the potential bioactivity.

329 An additional free aromatic amino acid, tryptophan (W; $m/z=205.2208$), gave an intense
330 signal in the fraction F5 (**Figure 3C**). The most intense signals in the peptidic part of the MS
331 spectra of fraction F5 were identified as the tetrapeptide SAPL ($m/z=387.2272$) from β -
332 lactoglobulin (f36-39) and the α S1-casein-derived (f8-13) peptide HQGLPQ ($m/z=340.1831$;
333 double-charged ion). Additional high signals were attributed to the peptide TKIPA from β -
334 lactoglobulin (f76-80) present both as double-charged ($m/z=265.1741$) and mono-charged
335 ($m/z=529.3435$) ions and the α S2-casein-derived peptides ITVDDK (f71-76) as double-
336 charged ion ($m/z=345.6897$) and FPQ (f92-94) with m/z value of 391.2055. The peptide

337 AMEDIK (α S1-casein f53-58) was present in the spectra both in reduced and oxidized (at
338 methionine level) forms with m/z values of 353.6766 and 361.6770, respectively (**Table 5**).

339

340 *3.5. Identification of antioxidant compounds in HPLC fractions F2, F3 and F5*

341 To identify the amino acids and peptides with the most potential antioxidant activity, the
342 antioxidant properties of the peptides constitutive amino acids was determined (**Table 6**).
343 Tryptophan was the amino acid with the highest ABTS^{•+} value followed by cysteine and
344 tyrosine. The rest of the amino acids analysed did not exhibit antioxidant activity with this
345 method at 2 mmol·L⁻¹ concentration. Tyrosine and cysteine were also the amino acids with the
346 highest hydroxyl radical scavenging activity followed by methionine and tryptophan. The
347 amino acids phenylalanine and histidine showed the highest ability to inhibit lipid
348 peroxidation. The amino acid tryptophan appeared to be the most effective as antioxidant
349 since it showed high activity in all the assays.

350 Therefore, only the peptides containing the amino acids with antioxidant properties as well as
351 the corresponding free amino acids were considered as potential radical scavengers.

352 Among the different compounds identified in fraction F2, tyrosine seemed fundamental in the
353 ABTS^{•+} and hydroxyl radical scavenging capacities of the fraction, and to play a role in the
354 inhibition of formation of lipid hydroperoxide. Tyrosine was further quantified in the fraction
355 resulting in a concentration of 2.2 ± 0.1 mmol·L⁻¹ of milk. The free tyrosine standard showed
356 an ABTS^{•+} radical scavenging activity of 124.7 ± 12.3 mg of vitamin C·mmol⁻¹ of amino acid,
357 which resulted in an ABTS^{•+} value of 274.3 mg of vitamin C per 2.2 mmol of tyrosine.

358 Considering that the ABTS^{•+} radical scavenging activity of the fraction F2 was 303.1 mg of
359 vitamin C·L⁻¹ of milk, we concluded that the 90% of the ABTS^{•+} radical scavenging activity
360 of this fraction is due to the presence of free tyrosine. Free tyrosine also accounted for the
361 36.5% of the total hydroxyl radical scavenging activity of this fraction. Tyrosine is an

362 aromatic amino acid, which is known for its antioxidant activity. The antioxidant properties of
363 tyrosine is due to the presence of the phenolic moiety (aromatic ring with a hydroxyl group),
364 which makes tyrosine a good scavenger of free radicals and metal chelator (Pihlanto 2006).
365 Two additional peptides (LSH and GP), which contained amino acids able to scavenge
366 hydroxyl radical may account for the remaining scavenging capacity and lipid peroxidation
367 inhibitory activity of the fraction F2. The peptide LSH contained the amino acids serine and
368 histidine, which displayed hydroxyl radical scavenging capacity and strong lipid peroxidation
369 inhibitory activity (**Table 6**). The dipeptide GP is of particular interest because it displayed
370 multifunctional properties (**Table 4**).

371 Fraction F3 contained some peptides with previously demonstrated radical scavenging
372 activity, which can explain the high value of ABTS^{•+} and hydroxyl radical scavenging activity
373 found in this fraction. The α S1-casein-derived peptide YPEL (146-149) demonstrated radical
374 scavenger activity against DPPH, superoxide anion and hydroxyl radicals (Suetsuna et al.
375 2000). The presence of the tyrosine residue seems to be very important for the antioxidant
376 properties of the peptide YPEL since its deletion from the sequence halves the radical
377 scavenging activity (Suetsuna et al. 2000). This peptide also gave an intense signal in the MS
378 spectra suggesting that it may be present in high amounts in the fraction F3. The tetrapeptide
379 VRYL (α S2-casein 205-208) forms part of the antioxidant peptide PYVRYL, derived from
380 ovine casein hydrolysate (López-Expósito et al. 2007). The sequence RYL played an
381 important role in the activity since it still showed antioxidant activity (De Gobba et al. 2014a).

382 The peptide AVPYYPQ (β -casein 177-182) is a precursor of two well-known antioxidant
383 peptides, namely VPYPQ and PYPQ, identified in human milk submitted to gastro-intestinal
384 digestion (Raikos and Dassios 2014; Hernández-Ledesma et al. 2007). The domain PYPQ is
385 primary in determining their antioxidant properties. However, the peptide VPYPQ showed a
386 higher antioxidant activity than the peptide PYPQ.

387 This fraction also showed the best lipid peroxidation inhibitory activity. It contained the free
388 amino acid phenylalanine, which had strong inhibitory activity towards lipid peroxidation
389 (**Table 6**). This compound is therefore expected to be the primary contributor to the lipid
390 peroxidation inhibitory activity of fraction F3.

391 Tryptophan is a potent radical scavenger, which contains an indole group that is involved in
392 the stabilization of the tryptophan radical through resonance or delocalization of the unpaired
393 electron (Pihlanto 2006). Tryptophan in fraction F5 was quantified resulting in a value of 1.3
394 ± 0.1 mmol·L⁻¹ of milk. The free tryptophan standard showed an ABTS^{•+} radical scavenging
395 activity of 219.2 ± 16.1 mg of vitamin C·mmol⁻¹ of amino acid, which corresponded to a
396 value of 285 mg vitamin C per 1.3 mmol of tryptophan. Considering that, the ABTS^{•+} radical
397 scavenging activity of the fraction F5 was 450 mg of vitamin C·L⁻¹ of milk, tryptophan
398 accounted for the 63.3% of the ABTS^{•+} radical scavenging activity in this fraction. Based on
399 the data in **Table 3** and **6** free tryptophan also accounted for the 59.9% of the hydroxyl radical
400 scavenging activity of fraction F5. Additional peptides with potential radical scavenging
401 activity were found in the fraction F5. For example the peptides DAYPSGA (α S1-casein 157-
402 163) and DAYPS (α S1-casein 157-163) are precursors of the antioxidant peptide AYPS (De
403 Gobba et al. 2014b). Interestingly, this last peptide was identified, after casein hydrolysis, in a
404 fraction with high antioxidant activity together with the peptides RYPS and SRYPS,
405 suggesting that the sequence YPS could be primary for the antioxidant properties of these
406 peptides. This peptides (DAYPSGA and DAYPS) contained the amino acid tyrosine which
407 displayed strong ABTS and hydroxyl radical scavenging capacities and the amino acids
408 proline and serine active against the hydroxyl radical (**Table 6**). Several antioxidative
409 peptides contain the sequence YL or YI in their structure such as the tripeptides YYL, YLY,
410 YYI, YIY (Saito et al. 2003) and RYL (De Gobba et al., 2014a) as well as the longer peptides
411 YIPIQY, FALPQYLK, GYLEQ, YLKT and PYVRYL (De Gobba et al., 2014b; López-

412 Expósito et al. 2007). The amino acids phenylalanine and histidine played an important role in
413 the lipid peroxidation inhibitory activity of the peptides (De Gobba et al. 2014a). Therefore,
414 the peptides present in fraction F5 containing these amino acids could be considered the major
415 contributor to the lipid peroxidation inhibitory activity of this fraction. The peptides FPQ
416 (α S1-casein 92-94) and HQGLPQ (α S1-casein 8-13) also gave very intense peak in the MS
417 spectra (**Figure 3**), suggesting that they could be present at high concentration in fraction F5.

418

419 *3.6. Milk proteins as a carrier for the delivery of antioxidant compounds in the gastro-* 420 *intestinal tract*

421 Various evidence suggests that oxidative stress is closely associated with the onset and
422 progression of several chronic diseases (Willcox et al. 2004). Therefore, it is generally
423 speculated that antioxidants in the diet can be helpful in counteracting the onset of these
424 diseases. However, the link between *in vitro* and *in vivo* antioxidant capacities has not been
425 clearly established. With regard to this, despite the large number of *in vitro* studies reporting
426 the antioxidant activity of bioactive peptides, the *in vivo* effect of milk-derived antioxidant
427 peptides on human health remains unclear (Power-Grant et al., 2013).

428 The gastrointestinal tract is constantly exposed to reactive oxygen species, from the diet or
429 generated in the gastro-intestinal tract itself. Reactive radical species can derive from dietary
430 iron, which in the gastric environment (i.e. in presence of oxygen, acidic pH and H₂O₂) may
431 promote Fenton reaction generating superoxide anion and hydroxyl radicals (Halliwell et al.
432 2000). Reactive oxygen species in the gut can initiate, in presence of transition metals, the
433 lipid peroxidation of dietary poly-unsaturated fatty acids, resulting in the production of lipid
434 hydroperoxydes and advanced lipoxidation end products, which can be further absorbed and
435 involved in the pathogenesis of some cardiovascular diseases (Tagliazucchi et al. 2010). In
436 addition, dietary heme proteins are powerful pro-oxidant which can initiate gastric lipid

437 peroxidation (Tagliazucchi et al., 2010). Indeed diet can also be a source of lipid
438 hydroperoxide, lipo-oxidation end-products and hydrogen peroxide (Halliwell et al. 2000). An
439 additional source of free radical rise from the activation of immune cells naturally present in
440 the gastro-intestinal tract by diet-derived bacteria and toxins (Halliwell et al. 2000). Severe
441 oxidative stress in the gastrointestinal tract has been involved in the pathogenesis of colorectal
442 cancer and in inflammation-based gastro-intestinal tract diseases (Kim et al. 2012).
443 Bioactive peptides might exert direct protective effects in the gastro-intestinal tract by
444 scavenging reactive oxygen species and reducing the oxidative stress. The gastrointestinal
445 tract is in contact with digested food proteins and therefore, with a significant amount of food
446 derived peptides. With this view, milk proteins can be considered as a carrier for the delivery
447 of antioxidant compounds in the gastro-intestinal tract. In milk, antioxidant amino acids and
448 peptides are preserved from oxidation and degradation since they are encrypted in the protein
449 sequences. The simultaneous action of intestinal proteases determines a slow and continuous
450 release of antioxidant peptides and amino acids from the parent proteins protecting the gastro-
451 intestinal tract itself from the oxidative damage and the onset of oxidative diseases. The low
452 bioavailability of protein-derived bioactive peptides supports this hypothesis, suggesting that,
453 at least in part, the physiological effect of bioactive peptides on the organism could derive
454 from a biological effect in the gastro-intestinal tract.

455

456 **4. Conclusion**

457 Our results indicate that the amino acids tyrosine and tryptophan, released during *in vitro*
458 gastro-intestinal digestion and some identified tyrosine-containing peptides were the major
459 responsible for the radical scavenging activity of digested milk, whereas phenylalanine and
460 histidine-containing peptides played a crucial role in the lipid peroxidation inhibitory capacity
461 of digested milk . Many previous studies were carried out with the aim to identify antioxidant
462 peptides released from bovine milk after proteases treatment. Despite numerous antioxidant
463 peptides having been identified, it is likely that they lack a real physiological systemic effect
464 because they can be further degraded by membrane-bound amino-peptidase in the intestine or
465 they can be poorly absorbed due to their size and thus possibly are no longer available to elicit
466 a biological response. However, we propose that the biological activity of these antioxidant
467 compounds can be relevant for the gastro-intestinal tract. In our view, antioxidant compounds
468 can be slowly and continuously released from milk proteins protecting the gastro-intestinal
469 tract itself from oxidative damage.

470 Further studies should be carried out to elucidate the *in vivo* contribution of these antioxidant
471 compounds to the antioxidant status of the gastro-intestinal tract after milk consumption.

Disclosure of Conflict of interest

Davide Tagliazucchi, Ahmed Helal, Elena Verzelloni, and Angela Conte declare that they have no conflict of interest

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Figure captions

Fig. 1 UV-chromatograms of the low molecular weight fractions ($< 3 \text{ kg}\cdot\text{mol}^{-1}$) obtained from the whole (A), semi-skimmed (B) and skimmed (C) bovine milk after *in vitro* gastro-intestinal digestion. Twelve fractions (from F1 to F12) were collected from the low molecular weight fraction of skimmed milk (see panel C). Detection was achieved at 214 nm. The showed chromatograms are representative of three independent experiments.

Fig. 2 Antioxidant activity of the high-performance liquid chromatography-collected fractions from permeate ($< 3 \text{ kg}\cdot\text{mol}^{-1}$) obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion. Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test.

Fig 3 Mass spectrum of high-performance liquid chromatography fraction F2 (A), F3 (B) and F5 (C) from nanoLC-qTOF MS/MS analysis of the permeate ($< 3 \text{ kg}\cdot\text{mol}^{-1}$) obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion. Identified compounds are reported in Tables 4 and 5. The showed mass spectra are representative of three independent experiments.

Table 1

Degree of hydrolysis (DH) of bovine milk proteins before and after *in vitro* gastro-intestinal digestion. Data are means \pm SD; n=3

| Sample | Whole milk | Semi-skimmed milk | Skimmed milk |
|----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Before digestion | 3.1 \pm 0.5 ^a | 2.8 \pm 0.5 ^a | 3.1 \pm 0.2 ^a |
| After peptic digestion | 7.2 \pm 0.6 ^b | 7.4 \pm 0.2 ^b | 8.8 \pm 0.7 ^c |
| After pancreatic digestion | 20.8 \pm 0.4 ^d | 24.3 \pm 0.3 ^e | 30.7 \pm 0.8 ^f |

^{a-f} significant differences are shown by different letters (Tukey's test, $P < 0.05$).

Table 2.

Changes in antioxidant activity determined with ABTS assay on the different types of milk during digestion (LMW: low molecular weight fraction, HMW: high molecular weight fraction). Results are expressed as mg of vitamin C·L⁻¹ of milk. Data are means ± SD; n=3

| Sample | Before digestion | Gastric digestion | | Pancreatic digestion | |
|---------------------------------|---------------------------|-------------------------------|-------------------------------|---------------------------------|-------------------------------------|
| | After mixing pH 6.8 | Time 0 min pH 2.5 | Time 120 min pH 2.5 | Time 0 min pH 7.5 | Time 120 min pH 7.5 |
| <i>Whole milk</i> | | | | | |
| Total | 622.3 ± 44.5 | 282.4 ± 19.3 ^a | 415.2 ± 9.1 ^a | 881.0 ± 39.9 ^{a,b,c} | 3374.3 ± 104.6 ^{a,b,c,c} |
| LMW (< 3 kg·mol ⁻¹) | 27.6 ± 4.7 | 13.6 ± 4.0 | 80.5 ± 9.5 | 180.6 ± 16.7 ^{a,b,c} | 1267.7 ± 100.2 ^{a,b,c,c} |
| HMW (> 3 kg·mol ⁻¹) | 597.2 ± 24.9 | 280.0 ± 21.8 ^a | 367.7 ± 30.5 ^a | 719.9 ± 22.4 ^{b,c} | 2044.6 ± 183.1 ^{a,b,c,c} |
| <i>Semi-skimmed milk</i> | | | | | |
| Total | 571.4 ± 22.4 | 221.7 ± 24.6 ^{a,e} | 268.6 ± 3.6 ^{a,e} | 642.7 ± 18.5 ^{a,b,c,e} | 2657.1 ± 39.6 ^{a,b,c,d,e} |
| LMW (< 3 kg·mol ⁻¹) | 39.4 ± 6.2 | 16.6 ± 3.2 | 62.5 ± 5.1 | 169.1 ± 14.2 ^{a,b,c} | 2104.6 ± 80.6 ^{a,b,c,d,e} |
| HMW (> 3 kg·mol ⁻¹) | 527.0 ± 38.1 | 203.1 ± 16.4 ^{a,e} | 198.1 ± 12.9 ^{a,e} | 484.8 ± 27.0 ^{a,b,c,e} | 489.6 ± 21.1 ^{a,b,c,e} |
| <i>Skimmed milk</i> | | | | | |
| Total | 515.3 ± 15.2 ^e | 176.5 ± 27.1 ^{a,e} | 229.6 ± 8.0 ^{a,e,f} | 592.7 ± 23.4 ^{a,b,c,e} | 2751.2 ± 46.9 ^{a,b,c,d,e} |
| LMW (< 3 kg·mol ⁻¹) | 42.5 ± 10.8 | 14.7 ± 5.2 | 76.3 ± 9.1 | 146.9 ± 12.1 ^{a,b,c} | 2481.4 ± 110.1 ^{a,b,c,d,e} |
| HMW (> 3 kg·mol ⁻¹) | 462.8 ± 34.7 ^e | 146.9 ± 23.4 ^{a,e,f} | 143.4 ± 11.6 ^{a,e,f} | 420.1 ± 19.6 ^{b,c,e,f} | 351.8 ± 38.6 ^{a,b,c,e} |

^a indicates significantly different respect to the same sample before the digestion (Tukey's test, $P < 0.05$).

^b indicates significantly different respect to the time 0 of gastric digestion (Tukey's test, $P < 0.05$).

^c indicates significantly different respect to the time 120 of gastric digestion (Tukey's test, $P < 0.05$).

^d indicates significantly different respect to the time 0 of pancreatic digestion (Tukey's test, $P < 0.05$).

^e indicates significantly different respect to whole milk at the same time and pH (Tukey's test, $P < 0.05$).

^f indicates significantly different respect to semi-skimmed milk at the same time and pH (Tukey's test, $P < 0.05$).

Table 3. Radical scavenging properties and lipid peroxidation inhibitory activity of the reversed phase-high performance liquid chromatography fractions F2, F3 and F5 of $< 3 \text{ kg}\cdot\text{mol}^{-1}$ permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion.

| | <i>ABTS radical scavenging</i> | <i>Hydroxyl radical scavenging</i> | <i>Inhibition of lipid peroxidation</i> |
|-----------|------------------------------------|------------------------------------|---|
| | <i>mg vitamin C·L⁻¹</i> | | <i>% of inhibition</i> |
| <i>F2</i> | 303.1 ± 12.8^a | 4643.1 ± 153.6^a | 21.0 ± 3.6^a |
| <i>F3</i> | 476.0 ± 27.7^b | 1084.4 ± 61.9^b | 97.4 ± 4.7^b |
| <i>F5</i> | 450.0 ± 25.3^b | 457.8 ± 28.1^c | 58.9 ± 7.3^c |

Data are means \pm SD ($n = 3$). Values in the same columns with different lowercase letter are significantly different (Tukey's test; $P < 0.05$).

Table 3. Compounds identified in the reversed phase-high performance liquid chromatography fractions F2 and F3 of $< 3 \text{ kg}\cdot\text{mol}^{-1}$ permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion

| <i>Fraction</i> | <i>Observed mass (m/z)</i> | <i>Calculated mass^a</i> | <i>Peptide sequence^b</i> | <i>Protein precursor</i> | <i>Bioactivity^c</i> |
|-----------------|----------------------------|------------------------------------|-------------------------------------|--|--|
| F2 | | | | | |
| | 132.1037 | 132.1025 | Lx | various proteins | / |
| | 182.0851 | 182.0812 | Y | various proteins | Antioxidant |
| | 173.0840 | 173.0921 | GP | various proteins | Peptide regulating the stomach mucosal membrane activity; DPP IV inhibitor; ACE inhibitor; PEP inhibitor |
| | 189.1246 | 189.1234 | GLx | various proteins | DPP IV inhibitor (GI); ACE inhibitor (GL/GI) |
| | 332.1821 | 332.1816 | VVD | Digestive enzymes (α -amylase, lipase) | / |
| | 356.1945 | 356.1928 | LSH | various proteins | / |
| F3 | | | | | |
| | 120.0859 | 120.0655 | T | various proteins | / |
| | 166.1055 | 166.0863 | F | various proteins | / |
| | 173.0819 | 173.0921 | GP | various proteins | Peptide regulating the stomach mucosal membrane activity; DPP IV inhibitor; ACE inhibitor; PEP inhibitor |
| | 203.1415 | 203.1309 | ALx | various proteins | DPP IV inhibitor (AL); ACE inhibitor (AI) |
| | 219.1533 | 219.1339 | SLx | various proteins | DPP IV inhibitor (SL/SI) |
| | 231.1736 | 231.1703 | VLx | various proteins | Glucose uptake stimulating peptide (VL); DPP IV inhibitor (VL/VI) |
| | 260.1639 | 260.1605 | QLx | various proteins | DPP IV inhibitor (QL/QI) |
| | 275.6693 | 550.3348 | VRYL | α S2-casein f (205-208) | ACE inhibitor; Fragment and precursor of |

| | | | | antioxidant peptide |
|----------|----------|---------|--|----------------------------------|
| 320.1849 | 320.1816 | SLxT | β -casein (various fragments) | / |
| 334.1987 | 334.1973 | TLT | β -casein f(126-128) | / |
| 485.3159 | 485.3082 | IQPK | α S2-casein f(194-197) | / |
| 521.2642 | 521.2606 | YPEL | α S1-casein f(146-149) | Antioxidant |
| 674.3555 | 674.3508 | AVPYYPQ | β -casein f(177-182) | Precursor of antioxidant peptide |

^aMonoisotopic mass

^bLx indicates leucine or isoleucine

^cPotential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV; PEP: Prolyl endopeptidase

Table 5. Compounds identified in the reversed phase-high performance liquid chromatography fraction F5 of $< 3 \text{ kg}\cdot\text{mol}^{-1}$ permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion

| <i>Fraction</i> | <i>Observed mass (m/z)</i> | <i>Calculated mass^a</i> | <i>Peptide sequence^b</i> | <i>Protein precursor</i> | <i>Bioactivity^c</i> |
|-----------------|----------------------------|------------------------------------|-------------------------------------|----------------------------------|---|
| F5 | | | | | |
| | 205.2208 | 205.2262 | W | various proteins | Antioxidant |
| | 231.1734 | 231.1703 | LxV | various proteins | Glucose uptake stimulat peptide (LV/IV); DPP IV inhibitor (LV) |
| | 231.1734 | 231.1703 | VLx | various proteins | Glucose uptake stimulat peptide (VL); DPP IV inhibitor (VL/V) |
| | 265.1741 | 529.3344 | TKIPA | β -lactoglobulin f (76-80) | / |
| | 295.1655 | 295.1652 | YLxx | various proteins | DPP IV inhibitor (YL/Y) ACE inhibitor (YL) Fragment of antioxidant peptides |
| | 317.6827 | 634.3923 | YKVPK | α S1-casein f (104-108) | / |
| | 340.1831 | 679.3522 | HQGLPQ | α S1-casein f (8-13) | / |
| | 345.6897 | 690.3668 | ITVDDK | α S2-casein f (71-76) | / |
| | 353.6766 | 706.3440 | AMEDIK | α S1-casein f (53-58) | / |
| | 387.2272 | 387.2165 | SAPL | β -lactoglobulin f (36-39) | / |
| | 391.2055 | 391.1976 | FPQ | α S2-casein f (92-94) | / |
| | 446.5737 | 1337.6808 | HIQKEDVPSER | α S1-casein f (80-90) | / |
| | 552.2430 | 552.2227 | DAYPS | α S1-casein f (157-161) | Precursor of antioxidant peptides |
| | 680.3035 | 680.2886 | DAYPSGA | α S1-casein f (157-163) | Precursor of antioxidant peptides |

^aMonoisotopic mass

^bLx indicates leucine or isoleucine

^cPotential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV

Table 6. Antioxidant properties of pure amino acids and the dipeptide glycine-proline.

| <i>Amino acids^a</i> | <i>ABTS radical scavenging</i> | <i>Hydroxyl radical scavenging</i> | <i>Inhibition of lipid peroxidation</i> |
|--------------------------------|--|------------------------------------|---|
| | <i>mg vitamin C·mmol⁻¹ amino acid</i> | | <i>% of inhibition^b</i> |
| <i>Trp</i> | 219.2 ± 5.9 ^a | 211.1 ± 3.1 ^a | 31.7 ± 1.1 ^a |
| <i>Tyr</i> | 124.7 ± 3.8 ^b | 769.7 ± 9.5 ^b | 4.9 ± 0.2 ^b |
| <i>Cys</i> | 162.5 ± 4.4 ^c | 766.4 ± 8.7 ^b | n.d. |
| <i>Met</i> | n.d. | 450.9 ± 6.4 ^c | 11.3 ± 0.8 ^c |
| <i>Thr</i> | n.d. | 64.1 ± 2.1 ^d | n.d. |
| <i>Pro</i> | n.d. | 59.1 ± 3.4 ^d | n.d. |
| <i>His</i> | n.d. | 55.3 ± 2.8 ^{d,e} | 80.3 ± 2.3 ^d |
| <i>Arg</i> | n.d. | 39.4 ± 1.4 ^e | 13.8 ± 1.1 ^c |
| <i>Lys</i> | n.d. | n.d. | n.d. |
| <i>Asp</i> | n.d. | n.d. | n.d. |
| <i>Glu</i> | n.d. | n.d. | n.d. |
| <i>Phe</i> | n.d. | 165.6 ± 8.3 ^f | 91.4 ± 5.6 ^e |
| <i>Leu</i> | n.d. | n.d. | n.d. |
| <i>Ile</i> | n.d. | n.d. | n.d. |
| <i>Gly</i> | n.d. | n.d. | n.d. |
| <i>Val</i> | n.d. | n.d. | n.d. |
| <i>Ala</i> | n.d. | n.d. | n.d. |
| <i>Ser</i> | n.d. | 188.1 ± 7.3 ^g | 50.3 ± 1.7 ^f |

^a Three letters code

^b % of inhibition referred to a control reaction without amino acids (set as 100% of peroxidation). Amino acids were tested at concentration of 2 mmol L⁻¹

n.d. not detected activity

Data are means ± SD (*n* = 3). Values in the same columns with different lowercase letter are significantly different (Tukey's test; *P* < 0.05).

Figure 1

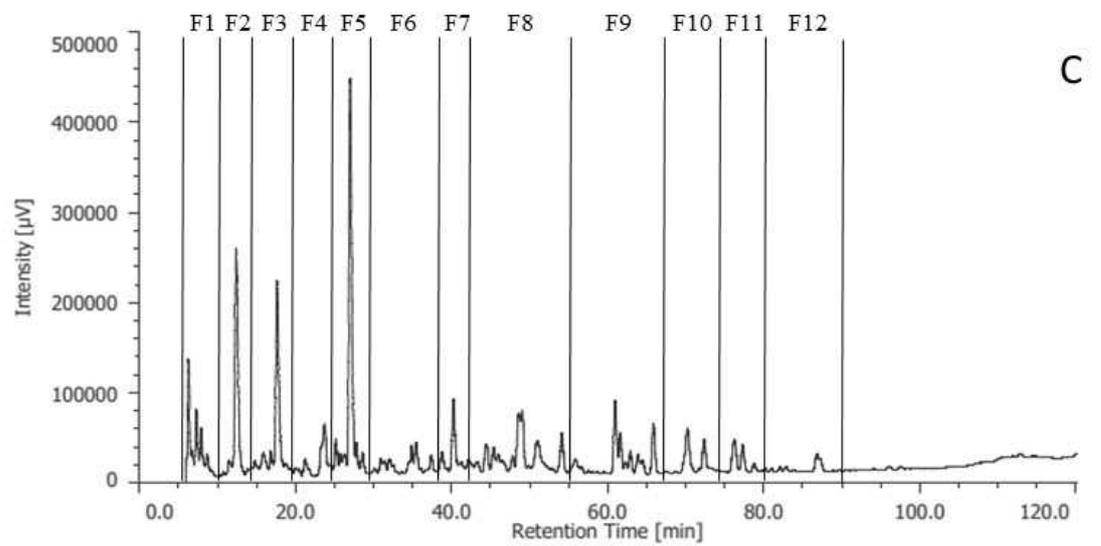
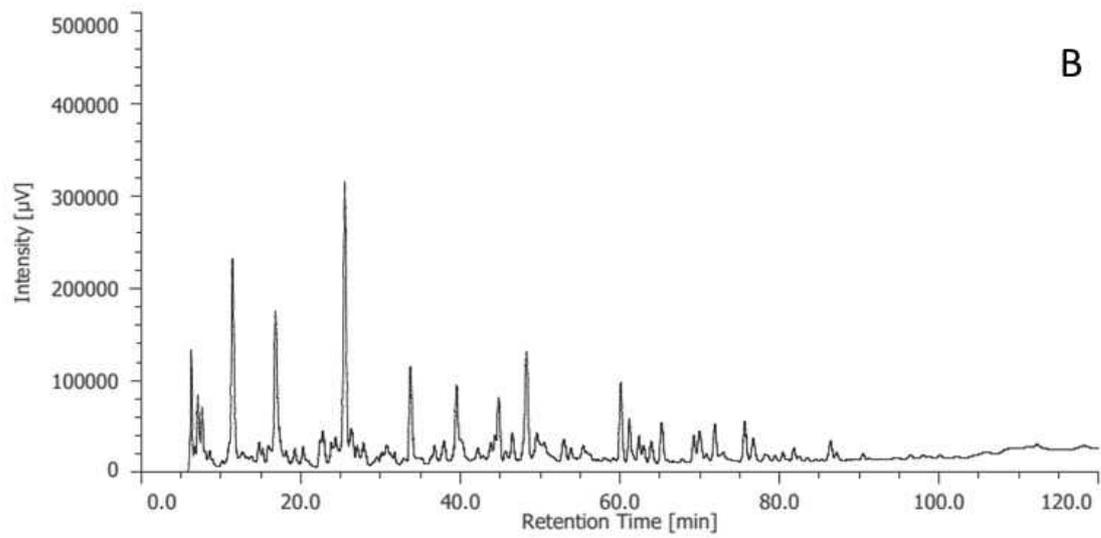
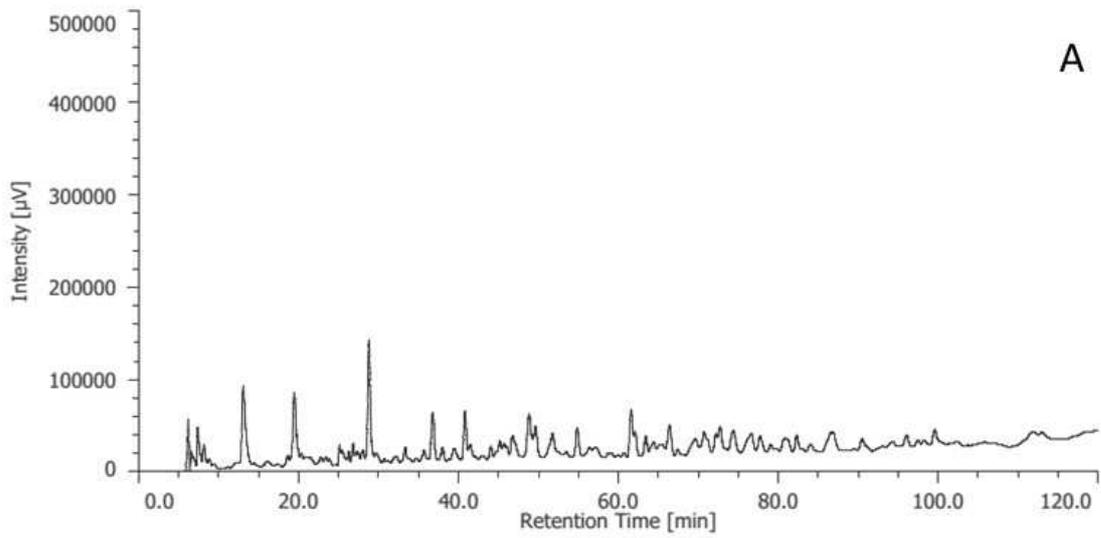


Figure 2

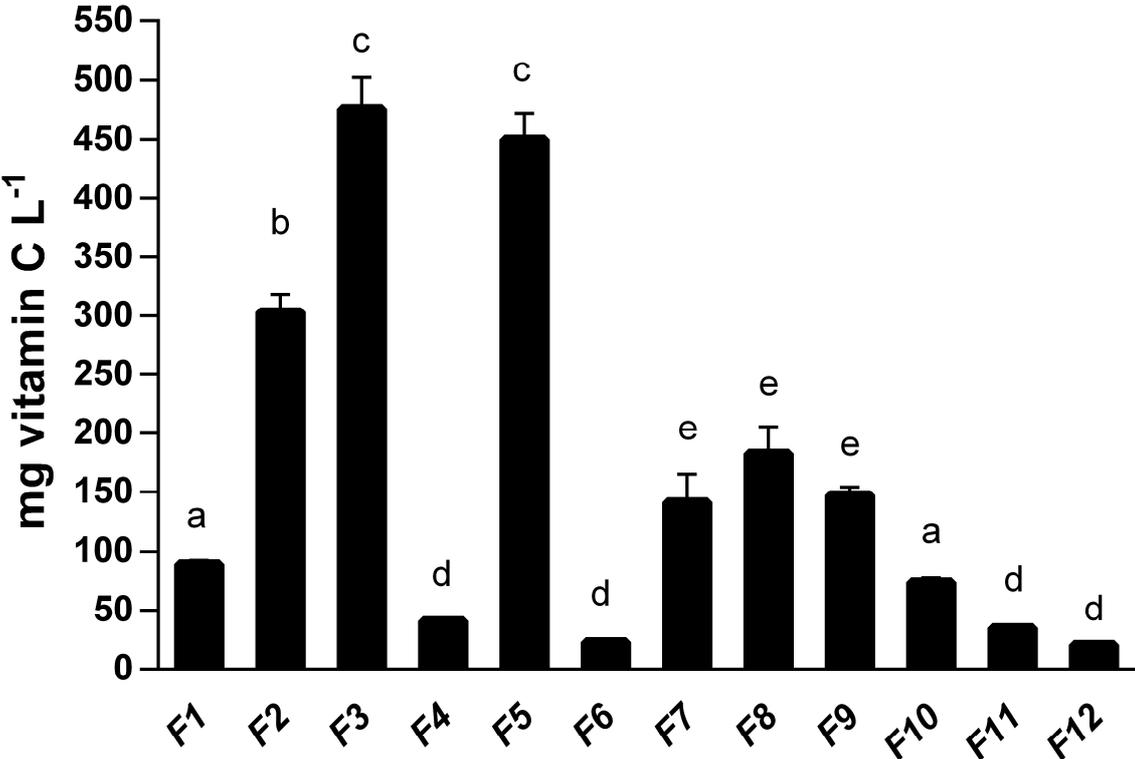


Figure 3

