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# **Angiotensin-converting enzyme inhibitory peptides from goat milk released by *in vitro* gastro-intestinal digestion**

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## 1    **Abstract**

2    The aim of this study was to identify the angiotensin-converting enzyme inhibitory peptides  
3    released after *in vitro* gastro-intestinal digestion of skimmed goat milk. The experimental approach  
4    combined the recently developed harmonized static *in vitro* digestion model and mass spectrometry  
5    to identify bioactive peptides. Peptides in the post-pancreatic digested were extracted by  
6    ultrafiltration and isolated by reversed-phase high-performance liquid chromatography following  
7    mass spectrometry identification. Among the identified sequences, eighteen were identical to  
8    known bioactive peptides with ACE-inhibitory activity. Peptides with dipeptidyl peptidase IV-  
9    inhibitory and antioxidant activities were also identified. This is the first report demonstrating that  
10   the antihypertensive tripeptides valine-proline-proline and isoleucine-proline-proline were released  
11   from goat milk proteins during *in vitro* gastro-intestinal digestion at concentrations of  $1829.8 \pm$   
12    $216.4$  and  $141.4 \pm 15.1 \mu\text{g L}^{-1}$ , respectively. This research underlines the suitability of the  
13   harmonized digestive model system to study the release of short bioactive peptides during gastro-  
14   intestinal transit.

## 15    **1. Introduction**

16    Bioactive peptides are short sequences, encrypted in the parent proteins, which should be released  
17    to exert their activity in the human body (Nongonierma, & FitzGerald, 2015). Proteins from milk,  
18    especially from cow's milk, are the best source of bioactive peptides with various physiological  
19    activities including antioxidant, antibacterial, immune-modulating, dipeptidyl peptidase IV (DPP-  
20    IV) and angiotensin-converting enzyme (ACE) inhibition (Nongonierma, & FitzGerald, 2015). The  
21    biological activity of these peptides is based on the amino acid composition and sequence. The size  
22    of these bioactive peptide sequences playing multifunctional activities may vary from two to twenty  
23    amino acid residues (Nongonierma, & FitzGerald, 2015). Recently, also milk from other species,  
24    such as camel and goat have been exploited for the release of bioactive peptides (De Gobba,  
25    Espejo-Carpio, Skibsted, & Otte, 2014a; Espejo-Carpio, et al., 2016; Tagliazucchi, Shamsia, &  
26    Conte, 2016a).

27    Goat milk is one of the most important nutritional foods around the world, especially in Asia,  
28    Africa and many European countries. It has been found to be a good substitute of human milk in  
29    infant formulas especially for children who suffer allergic reactions to cow milk (Yadav, Singh, &  
30    Yadav, 2016). Caseins are, quantitatively, the most important proteins in milk. The caseins  
31    concentration in sheep milk is higher than cow, camel and goat (Park, Juárez, Ramos, & Haenlein,  
32    2007). Goat, camel and sheep milk have different proportions of the four major caseins ( $\alpha$ S1,  $\alpha$ S2,  
33     $\beta$ ,  $\kappa$ ) compared to cow counterparts. It has been reported that  $\beta$ -casein is the major component of  
34    sheep and goat milk casein (from 18 to 24 g L<sup>-1</sup>) (Ruprichová et al., 2015). The major component of  
35    cow's milk casein is  $\alpha$ S1 (~12 g L<sup>-1</sup>) followed by  $\beta$ -casein (~11 g L<sup>-1</sup>) (Omar, Harbourne, Oruna-  
36    Concha, 2016). Camel milk contains  $\beta$ -casein amounts comparable to cow's milk (~12 g L<sup>-1</sup>) but  
37    lower level of  $\alpha$ S1-casein (~3 g L<sup>-1</sup>) (Omar et al., 2016). Level of  $\kappa$ -casein is relatively higher in  
38    cow, sheep and goat milk (~4-5 g L<sup>-1</sup>) respect to camel milk (~2 g L<sup>-1</sup>) (Omar et al., 2016;  
39    Ruprichová et al., 2015).

40 Several studies have been done to investigate the bioactivity of goat milk protein hydrolysate and  
41 the release of ACE-inhibitory and antioxidant peptides with individual proteases such as  
42 thermolysin, trypsin, subtilisin, papain and pepsin or their combinations (Ahmed, El-Bassiony,  
43 Elmalt, & Ibrahim, 2015; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Hernández-  
44 Ledesma, Recio, Ramos, & Amigo, 2002). Espejo-Carpio et al. (2013) and De Gobba et al. (2014a)  
45 identified many casein-derived peptides from hydrolysed proteins of goat milk, which were  
46 enzymatically liberated by a combination of subtilisin and trypsin. Among them, many peptides  
47 contained tyrosine in their sequence and had antioxidant and ACE-inhibitory activities. ACE-  
48 inhibitory peptides represent sources of health-enhancing compounds of special interest, as  
49 hypertension is a major independent risk factor for cardiovascular and related diseases. ACE is a  
50 dipeptidyl carboxypeptidase that catalyzes, *in vivo*, the conversion of the plasmatic peptide  
51 angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE plays an important  
52 role in the regulation of blood pressure and drugs that inhibit ACE are commonly prescribed for the  
53 treatment of hypertension or related cardiovascular diseases (Acharya et al., 2003).

54 Furthermore, antioxidant peptides are particularly interesting because they can potentially prevent  
55 or delay oxidative stress associated chronic diseases (Willcox et al. 2004). In this sense, milk  
56 proteins have been considered as a carrier for the delivery of antioxidant peptides in the gastro-  
57 intestinal tract where they may exert direct protective effects by scavenging reactive oxygen species  
58 and reducing the oxidative stress (Tagliazucchi, Helal, Verzelloni, & Conte, 2016b).

59 Recently, several DPPIV-inhibitory peptides have been isolated and characterized from goat milk  
60 proteins hydrolysed with a combination of trypsin and chymotrypsin (Zhang, Chen, Ma, & Chen,  
61 2015). DPP-IV inhibitors can be used to reduce DPP-IV activity and increase the lifetime of  
62 incretins. These are peptidic gut hormones, which stimulate insulin secretion and  $\beta$ -cell-  
63 proliferation but that are quickly degraded by brush-border intestinal DPP-IV (Sebokova, Christ,  
64 Boehringer, & Mizrahi, 2007). DPP-IV inhibitors can be employed in the management of type 2  
65 diabetes, reducing DPP-IV activity and increasing the lifetime of incretins (Sebokova et al., 2007).

66 However, despite many reports about the release of bioactive peptides from goat milk proteins after  
67 enzymatic treatment, there is also a lack of information on the identification of peptides released  
68 after *in vitro* gastrointestinal digestion of goat milk. Our research group recently employed a  
69 harmonized basic static *in vitro* digestive model, simulating human digestion and developed within  
70 the COST action INFOGEST (Minekus et al., 2014) to study the release and fate of short ACE-  
71 inhibitory peptides from camel and cow milk as well as from vegetable substrates such as pinto  
72 beans (Rutella, Solieri, Martini, & Tagliazucchi, 2016; Tagliazucchi, Martini, Bellesia, & Conte,  
73 2015; Tagliazucchi et al., 2016a).

74 The aim of this study was to investigate the release of ACE-inhibitory peptides during simulated  
75 gastro-intestinal digestion of skimmed goat milk. The digested samples were characterized for their  
76 ACE-inhibitory activity and the bioactive peptides identified using mass spectrometry analysis.

## 77    **2. Materials and methods**

### 78    *2.1. Materials*

79    All MS/MS reagents were obtained from Biorad (Hercules, CA, U.S.A.), whereas the chemicals and  
80    enzymes for the digestion procedure, ACE assay and degree of hydrolysis determination were  
81    purchased from Sigma-Aldrich (Milan, Italy). Amicon Ultra-0.5 regenerated cellulose filters with a  
82    molecular weight (MW) cut-off of 3 kDa were supplied by Millipore (Milan, Italy). The whole goat  
83    milk was obtained from farms at El-Alamin and Sidi-Barani areas around Alexandria (Egypt).  
84    Valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) peptides (95% purity) were  
85    synthesized by DBA (Milan, Italy). All the other reagents were from Carlo Erba (Milan, Italy).

86

### 87    *2.2. Chemical analysis of skimmed goat milk*

88    Raw goat milk was pasteurized for 15 seconds after the central temperature reached 72°C and  
89    immediately defatted by centrifugation at 2000g for 20 min at room temperature to obtain the  
90    skimmed goat milk. This last was stored at -80°C until further analysis. Skimmed milk sample was  
91    analysed for pH, total solids, fat, and lactose by phenol-sulphuric acid method, and total nitrogen,  
92    non-casein nitrogen by micro-Kjeldahl as reported by Tagliazucchi et al. (2016a).

93

### 94    *2.3. In vitro gastro-intestinal digestion of skimmed goat milk using the harmonized protocol*

95    For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST  
96    and further validated for milk was followed (Kopf-Bolanz et al., 2012; Minekus et al., 2014) with a  
97    minor modification for adaptation to milk (Tagliazucchi, Helal, Verzelloni, Bellesia, & Conte,  
98    2016c). The protocol consisted of three consecutive steps: oral, gastric and intestinal phases.  
99    Simulated salivary, simulated gastric, pancreatic and bile fluids were prepared according to Kopf-  
100    Bolanz et al. (2012). To simulate the oral phase, 9 mL of skimmed goat milk were mixed with 12  
101    mL of simulated salivary fluid containing 150 U mL<sup>-1</sup> of porcine  $\alpha$ -amylase and incubated for 5 min  
102    at 37°C on a rotating wheel (10 rpm). The gastric phase was carried out by adding to the bolus 24

103 mL of simulated gastric fluid. The pH was adjusted to 2.0 with HCl 6 mol L<sup>-1</sup> and supplemented  
104 with porcine pepsin (1115 U mL<sup>-1</sup> of simulated gastric fluid). The gastric bolus was then incubated  
105 for 120 min at 37°C on a rotating wheel (10 rpm). A lower amount of pepsin was chosen in respect  
106 to the INFOGEST protocol since, in a previous work (Tagliazucchi et al., 2016c), the digestion of  
107 cow milk with a ratio of ~3000U of pepsin per mL of milk, as that used in the current study,  
108 resulted in a peptides pattern at the end of the digestion similar to the results obtained *in vivo* by  
109 Boutrou et al. (2013) from jejunal effluents of healthy humans after ingestion of caseins and whey  
110 proteins separately. The intestinal digestion was carried out by adding to the gastric bolus 24 mL of  
111 pancreatic fluid and 12 mL of bile fluid, adjusting the pH to 7.0 and supplemented with pancreatin.  
112 The chyme was further incubated for 120 min at 37°C on a rotating wheel (10 rpm). The digested  
113 samples were immediately cooled on ice and frozen at -80°C for further analysis. The digestions  
114 were performed in triplicate. In addition, a control digestion, which included only the gastro-  
115 intestinal juices and enzymes, and water in place of milk, was carried out to consider the possible  
116 impact of the digestive enzymes in the subsequent analysis.

117 For each digestion, aliquots were taken after 0 and 5 minutes of salivary digestion, after 30, 60 ,90  
118 and 120 minutes of gastric digestion and after 30, 60 ,90 and 120 minutes of intestinal digestion.

119

#### 120 *2.4. Assessment of protein hydrolysis during the digestion and fractionation of the post-pancreatic* 121 *sample*

122 The peptide concentration was used as an indication of protein hydrolysis in the un-fractionated  
123 samples collected during the salivary, the gastric and the pancreatic phases of the digestion and  
124 measured by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay using leucine as standard amino  
125 acid (Adler-Nissen, 1979).

126 The hydrolysis degree (DH) was calculated as reported in equation (1):

127 **DH= (h/h<sub>tot</sub>) • 100 (1)**



128 Where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein  
129 of  $\alpha$ -amino groups formed at the different stages of the simulated digestion, and **h<sub>tot</sub>** is the  
130 hydrolysis equivalent (total number of amino groups) at complete hydrolysis to amino acids. The  
131 total number of amino groups was determined by hydrolysing skimmed goat milk in 6 mol L<sup>-1</sup> HCl  
132 at 110°C for 24 h. The h<sub>tot</sub> value was calculated resulting in 8.6 milliequivalents per gram of  
133 protein. DH data were subtracted with the data obtained in the control digestion.

134 Low molecular weight peptides were extracted by ultrafiltration from the post-pancreatic digested  
135 samples (corresponding to the aliquots collected after 120 min of pancreatic digestion). Briefly, 4  
136 mL of sample were loaded on an Amicon Ultra-4 nominal filter (cut-off 3 kDa) and centrifuged at  
137 7500g for 120 min at 4°C using a Hermle Z383K refrigerated centrifuge (HERMLE Labortechnik  
138 GmbH, Wehingen, Germany). The filtrates, containing low molecular weight peptides, were  
139 collected and freeze-dried. The peptide content of the filtrates was determined by using the TNBS  
140 method as described above and expressing the results as mg of leucine equivalent mL<sup>-1</sup>.

141 The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer (Orlando FL, U.S.A.).

142

#### 143 *2.5. Measurements of ACE-inhibitory activity*

144 ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983)  
145 using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate.  
146 The reaction was monitored at 345 nm for 10 min. For the calculation of the IC<sub>50</sub> value, the ACE  
147 assay was carried out in presence of different amounts of the un-fractionated samples collected  
148 during the gastric and pancreatic phases of the digestion and of the lower and higher of 3 kDa  
149 fractions of the post-pancreatic sample. IC<sub>50</sub> was defined as the concentration of peptides required  
150 to inhibit 50% of the enzymatic activity.

151 The absorbance was read using the same Jasco V-550 UV/Vis spectrophotometer as reported in  
152 paragraph 2.4.

153

154 *2.6. High-performance liquid chromatography separation of the peptide fraction*

155 High-performance liquid chromatography (HPLC) separation of the peptide fractions (< 3 kDa)  
156 obtained from the post-pancreatic sample after ultrafiltration was performed with a Jasco HPLC  
157 system equipped with a 250mm x 4.6mm reversed phase column Hamilton HxSil C18 (Hamilton,  
158 Reno, NV, USA) as described by Tagliazucchi et al. (2015). A linear gradient of solvent B  
159 (acetonitrile-trifluoroacetic acid 0.027%) in A (water-trifluoroacetic acid 0.037%) ranging from 0%  
160 to 45% in 115 min with a flow rate of 0.5 mL min<sup>-1</sup> was used to separate the peptides contained in  
161 the low molecular weight fractions of digested milk. The PDA detector was set at 214 and 280 nm.  
162 Nine major fractions were collected. These fractions were characterized for their peptide content  
163 (paragraph 2.4) and ACE-inhibitory activity (paragraph 2.5). The yield of the collected fractions  
164 was calculated as follows: peptides concentration in the fraction\*100/peptides concentration in the  
165 post-pancreatic < 3 kDa permeate.

166

167 *2.7. Nanoflow liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry*  
168 *with electrospray ionization (LC-ESI-QTOF MS) analysis of the HPLC fractions*

169 The fractions with the highest ACE-inhibitory activity collected from HPLC were subjected to  
170 QTOF MS/MS analysis for peptide identification. Nano LC/MS and tandem MS experiments were  
171 performed on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520  
172 Accurate-Mass Q-TOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA,  
173 USA). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL  
174 enrichment column and a 43mm × 75µm analytical column, both packed with a Zorbax 300SB 5  
175 µm C18 phase (Agilent Technologies).

176 For peptide identification, a non-targeted approach was applied as reported by Tagliazucchi et al.  
177 (2015). The mass spectrometer was tuned, calibrated and set with the same parameters as reported  
178 by Dei Più et al. (2014). For peptide identification and sequencing, MS/MS spectra were converted  
179 to .mgf and *de novo* peptide sequencing was performed using Pepnovo software

180 (<http://proteomics.ucsd.edu/ProteoSAFe/>). The following parameters were considered: enzyme,  
181 none; peptide mass tolerance,  $\pm 40$  ppm; fragment mass tolerance,  $\pm 0.12$  Da; variable  
182 modifications, oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a  
183 single peptide, 3.

184 A search for the biological activity of peptides identified was carried out through the BIOPEP  
185 database ([http://www.uwm.edu.pl/biochemia/biopep/start\\_biopep.php](http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php)). Confirmation of peptides  
186 sequence in goat milk proteins was performed using Peptide Match  
187 (<http://research.bioinformatics.udel.edu/peptidematch/index.jsp>).

188

## 189 *2.8. Quantification of valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) in HPLC* 190 *fractions*

191 For the quantification of VPP and IPP, a targeted approach as described by Rutella et al. (2016) was  
192 employed. Monoisotopic precursor selection was applied to identify the lactotripeptides. VPP and  
193 IPP were selectively fragmented using a mass to charge ratio of 312.18 and 326.21 (charge +1),  
194 respectively. The assignment process was complemented and validated by the manual inspection of  
195 MS/MS spectra. VPP and IPP were quantified using the method reported by Solieri, Rutella, and  
196 Tagliazucchi (2015) and their amount expressed as  $\mu\text{g L}^{-1}$  of hydrolysates

197

## 198 *2.9. Statistical analysis*

199 All data are presented as mean  $\pm$  standard deviation (SD) for three replicates for each prepared  
200 sample. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using  
201 Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered  
202 significant with  $P < 0.05$ .

### 203 3. Results and Discussion

204

#### 205 3.1. Assessment of protein hydrolysis during simulated digestion

206 The chemical composition of skimmed goat milk expressed as percentage (w/w) was: total solids  
207 14.5%, total proteins 3.78%, caseins 2.92%, whey proteins 0.86%, lactose 4.55%, fat <0.05%. The  
208 pH was 6.64. Composition of goat milk can be very different depending on the diet, breed, feeding,  
209 environmental conditions, stage of lactation and health status (Yadav et al., 2016). For example,  
210 total solids can range from 11 to 19% and total proteins from 1.9 to 5.3% (Yuksel, Avci, Uymaz, &  
211 Erdem, 2012). Wide variability can also be observed for total caseins (2.33-4.63%) and whey  
212 proteins (0.37-1.8%) (Yadav et al., 2016). Vice versa, low variability has been found for lactose  
213 (4.6-4.9%) and pH (6.47-6.82) (Yadav et al., 2016; Torres, Castro, Argüello, & Capote, 2013;  
214 Yuksel et al., 2012). Due to the highest content in calcium, vitamins (such as vitamin A, C and D),  
215 proteins and medium chain triglycerides, goat milk has some nutritional benefits in comparison  
216 with cow's milk (Park et al., 2007). Besides nutritional quality, functional properties of proteins are  
217 also important for human health. Enzymatic hydrolysis is known to improve functional properties  
218 of dietary protein without affecting its nutritive value by converting it into peptides with desired  
219 bioactivity. During digestion, the combined action of gastro-intestinal proteolytic enzymes may lead  
220 to the release of bioactive peptides from goat milk proteins.

221 The hydrolysis of goat milk proteins during the *in vitro* digestion was evaluated with the TNBS  
222 assay. **Figure 1** reports the resulting DH values, relative to the control condition, at various steps of  
223 the simulated gastro-intestinal transit. As expected, simulated salivary digestion did not affect the  
224 hydrolysis degree. Un-digested goat milk showed a DH of  $3.9 \pm 0.2\%$ , which was  $4.3 \pm 0.4\%$  after 5  
225 minutes of salivary digestion ( $P > 0.05$ ). After 30 min of the gastric digestion, the DH sharply  
226 increased to  $22.5 \pm 1.8\%$  (**Figure 1**). After that, the peptic activity resulted in a further slight  
227 increase in DH during the 120 minutes of gastric digestion. The transition from gastric to pancreatic  
228 treatment increased DH values significantly from  $28.4 \pm 2.3\%$  at the end of gastric digestion to  $65.1$

229  $\pm 1.0\%$  after 30 min of pancreatic digestion ( $P < 0.0001$ ) (**Figure 1**). Subsequently, the DH rose  
 230 gradually reaching the maximum value of  $82.7 \pm 1.2\%$  after 120 min of the intestinal incubation.  
 231 A comparison with previously reported data (Rutella et al., 2016; Tagliazucchi et al., 2016a)  
 232 showed that goat milk proteins were more prone to hydrolysis by gastro-intestinal proteases respect  
 233 to cow and camel milk digested with the same harmonized digestion model. *In vitro* gastro-  
 234 pancreatic digestion of cow and camel milk resulted in DH values of 57.4% and 69.6%,  
 235 respectively, in comparison to the value of 82.7% found in goat milk (Rutella et al., 2016;  
 236 Tagliazucchi et al., 2016a). The highest digestibility of goat milk proteins seems related to the  
 237 higher susceptibility of these to peptic digestion compared to camel and cow milk proteins. The DH  
 238 value measured after peptic hydrolysis of cow, camel and goat milk proteins, digested with the  
 239 same harmonized digestion model, was 12.2%, 20.5% and 28.4%, respectively (Rutella et al., 2016;  
 240 Tagliazucchi et al., 2016a).

241

### 242 *3.2. Evolution of ACE-inhibitory activity during in vitro digestion and RP-HPLC fractionation of <* 243 *3 kDa fraction of digested goat milk*

244 To evaluate the impact of the gastro-intestinal enzymes on the generation of ACE-inhibitory  
 245 peptides, samples withdrawn during the gastric and pancreatic phases of the digestion were  
 246 analysed for their ACE-inhibitory activity.

247 During the pepsin-catalysed part of the simulated physiological digestion, the  $IC_{50}$  value for ACE-  
 248 inhibitory activity (defined as the peptide concentration required to inhibit 50% of the ACE  
 249 activity) slightly but not significantly decreased during the first 90 minutes of gastric digestion,  
 250 suggesting a modest increase in the ACE-inhibitory activity of the hydrolysates (**Figure 2**). The  
 251 passage from gastric to pancreatic treatment increased the  $IC_{50}$  value significantly from  $313.7 \pm$   
 252  $26.5 \mu\text{g of peptides mL}^{-1}$  at the end of the gastric digestion to  $704.5 \pm 49.0 \mu\text{g of peptides mL}^{-1}$  after  
 253 30 min of the pancreatic digestion ( $P < 0.0001$ ). Then, the  $IC_{50}$  value sharply rose as the digestion  
 254 with pancreatic enzymes progressed reaching the highest value of  $1808.8 \pm 68.3 \mu\text{g of peptides mL}^{-1}$

<sup>1</sup> at the end of the digestion (**Figure 2**). As a result, at the end of the *in vitro* physiological digestion, the ACE-inhibitory activity was lower than that observed after peptic digestion. Results reported in **Figure 2** suggested that the ACE-inhibitory activity of goat milk hydrolysates decreased as the peptides length decreased. Peptic hydrolysis can generate ACE-inhibitory peptides whereas further degradation of the peptides into much smaller fragments by pancreatic proteases resulted in a decrease in the ACE-inhibitory activity.

Because it has been suggested that small peptides are mainly responsible for the ACE-inhibitory activity of digested milk (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Tagliazucchi et al., 2016a) and short peptide sequences are good candidates to play a physiological antihypertensive role *in vivo*, ACE-inhibitory activity and the peptide content of permeate, obtained following ultrafiltration of the post-pancreatic sample, were also assayed. The peptides in the post-pancreatic digested sample were extracted by ultrafiltration with a cut-off of 3 kDa. The < 3 kDa fraction of the samples obtained at the end of the simulated digestion procedure contained  $22.79 \pm 0.83$  mg mL<sup>-1</sup> of peptides and showed a IC<sub>50</sub> value towards ACE activity of  $1156.3 \pm 10.5$  µg of peptides mL<sup>-1</sup> (**Table 1** and **Figure 2**). No activity was found in the > 3 kDa fraction of the post-pancreatic sample (**Figure 2**) suggesting that low molecular weight peptides were responsible for the ACE-inhibitory activity of the digested goat milk.

Espejo-Carpio et al., (2016) obtained IC<sub>50</sub> values from 230 to 269 µg mL<sup>-1</sup> for goat milk hydrolysed with trypsin, subtilisin or a combination of these enzymes. In our experiments, IC<sub>50</sub> values observed during goat milk digestion with pepsin (gastric phase) are similar to those reported by Espejo-Carpio et al., (2016). As the digestion with pancreatic enzymes proceed, peptides are cut in ever-smaller fragments and amino acids by pancreatic proteases. As a result, ACE-inhibitory activity declined due to the susceptibility of released peptides during the gastric digestion to pancreatic enzymes resulting in a IC<sub>50</sub> value 4 and 5 time higher than the value reported in the Espejo-Carpio study at the end of the digestion. In according to our data, Tagliazucchi et al., (2016a) also

found gastro-intestinal digestion strongly influenced the ACE-inhibitory activity of camel milk with  $IC_{50}$  value of  $1771.4 \mu\text{g mL}^{-1}$  at the end of the digestion. On the other hand, *in vitro* digestion of human and donkey milk in condition far from the harmonized IVD system (Bidasolo, Ramos and Gomez-Ruiz, 2012; Hernández-Ledesma et al., 2007) resulted in  $IC_{50}$  values 4-7 times lower than that found after digestion of camel and goat milk with the INFOGEST harmonized *in vitro* model (Tagliazucchi et al., 2016a and this study) underlining the importance to use *in vitro* models which accurately reflects the *in vivo* physiological conditions.

With the aim of identifying putative active peptides, the  $< 3$  kDa fraction of the samples obtained during simulated digestion was loaded onto the HPLC C18 column and peptides were detected at 214 and 280 nm with a photodiode array detector.

The chromatogram presented in **Figure 3** shows most peptides eluted in the first 60 minutes. As shown in **Figure 3**, nine fractions (F1–F9) were collected.

The peptide content of collected fractions ranged from 0.18 to  $4.99 \text{ mg mL}^{-1}$  (**Table 1**). The yield of the nine collected fractions was estimated (**Table 1**). Fractions from F1, F2 and F5 showed the highest yield ranging from 15.5 and 21.9%. The sum of the peptide concentration in the collected fractions resulted in  $19.68 \text{ mg mL}^{-1}$ , giving a total yield of 86.4%.

Most of the fractions exerted a weak or non-detectable ACE-inhibitory activity (**Table 1**). However, three fractions (F2, F3 and F5) showed low  $IC_{50}$  values, which means high ACE-inhibitory activity. The lowest  $IC_{50}$  values of  $14.1 \pm 0.8$  and  $17.9 \pm 0.8 \mu\text{g peptides mL}^{-1}$  were found in fractions F5 and F3, respectively.

300

### 3.3. Identification of ACE-inhibitory and additional bioactive peptides in HPLC fractions F2, F3 and F5

Based on ACE-inhibitory activities of the collected HPLC fractions, F2, F3 and F5 were selected and analysed with nanoflow LC-ESI-QTOF MS to identify the peptides present in these fractions.

**Figure 4** shows the full MS spectra of the fractions F2, F3 and F5. Each peak was selected for

306 peptide identification by MS/MS ion scan using *de novo* sequencing software. Results from peptide  
 307 identification were subjected to a manual evaluation, and the validated peptide sequences were  
 308 responsible for the majority of the most intense peaks in the MS spectra (**Tables 2-4**).  
 309 Fraction F2 (**Table 2**) contained 16 peptides, most of them being dipeptides. Two peptides found in  
 310 fraction F2 showed very low IC<sub>50</sub> values against ACE. The dipeptide AI and the tripeptide IPP  
 311 demonstrated IC<sub>50</sub> values of 3.41 and 5 µmol L<sup>-1</sup>, respectively (Nakahara et al., 2010; Nakamura,  
 312 Yamamoto, Sakai, & Takano, 1995). The dipeptide AI has been found to be released from pinto  
 313 bean and camel milk proteins submitted to the same harmonized *in vitro* digestion model  
 314 (Tagliazucchi et al., 2015; Tagliazucchi et al., 2016a) and, here, gave an intense signal in the mass  
 315 spectra suggesting its presence at high concentration. In addition, the dipeptide AY showed low  
 316 IC<sub>50</sub> value of 19 µmol L<sup>-1</sup> (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Some tripeptides  
 317 found in F2 share the carboxy-terminal sequence with ACE-inhibitory dipeptides, a structural  
 318 feature of paramount importance for the inhibitory activity (Wu, Aluko, & Nakai, 2006). The  
 319 tripeptides AEK, GLN and ALN are precursors of the ACE-inhibitory dipeptides EK and LN,  
 320 previously isolated from cow's milk hydrolysed with lactic acid bacteria (van Platerink, Janssen, &  
 321 Haverkamp, 2008).  
 322 In fraction F3 (**Table 3**), 19 peptides, having 2–4 amino acid residues, were identified. The most  
 323 effective ACE-inhibitory peptide was GPV (IC<sub>50</sub>= 4.7 µmol L<sup>-1</sup>) followed by VY (IC<sub>50</sub>= 7.1 µmol  
 324 L<sup>-1</sup>) (Saito, Wanezaki, Kawato, & Imayasu, 1994; Wu et al., 2006). Four others peptides able to  
 325 inhibit ACE activity were detected in this fraction (IAE, IP, PL and AF) with IC<sub>50</sub> values ranging  
 326 between 35 and 330 µmol L<sup>-1</sup> for IAE and PL, respectively (Byun, & Kim, 2002; Cheung et al.,  
 327 1980; Murray, & FitzGerald, 2007). The dipeptide VY was also effective *in vivo* in mild  
 328 hypertensive subjects (Kawasaki et al., 2000). In addition, the β-casein derived tripeptide HLP and  
 329 the dipeptide VM are fragments of the ACE-inhibitor peptides LHLP and TEQESGVPVM  
 330 (Kohmura et al., 1989; Tanzadehpanah et al., 2016).



331 The range of peptide length in fraction F5 (**Table 4**) was between 2 and 8 amino acid residues.  
 332 Fraction F5, which showed the lowest IC<sub>50</sub> value, contained the tripeptides VPP and LxVL as well  
 333 as the dipeptide LxY, which showed IC<sub>50</sub> values between 2.1 and 18 μmol L<sup>-1</sup> (Nakamura et al.,  
 334 1995; Wu et al., 2006). Additional peptides with lower ACE-inhibitory activity were found in this  
 335 fraction such as PLW (IC<sub>50</sub>= 36 μmol L<sup>-1</sup>), DAYPSGAW (IC<sub>50</sub>=98 μmol L<sup>-1</sup>) and VAV (IC<sub>50</sub>= 260  
 336 μmol L<sup>-1</sup>) (FitzGerald, & Meisel, 2000). The peptides LRF and IIA are precursors of well-known  
 337 ACE-inhibitory peptides with whom they share the carboxy-terminal sequence, which is the most  
 338 important for the inhibitory activity (Saito et al., 1994).  
 339 In the fractions with the best inhibitory activity towards ACE, several novel peptides were  
 340 identified which may be involved in the ACE-inhibitory activity of these fractions.  
 341 In a study on inhibition of ACE by glycyl dipeptides with varying carboxy- or amino-terminal  
 342 residues (Cheung et al., 1980), it was found that the kind of amino acid at the carboxy-terminal was  
 343 more important in determining the ACE-inhibitory activity. The amino acids W, Y, P, F, I and L at  
 344 the carboxy-terminal were the most favourable residues for the ACE-inhibitory activity, whereas at  
 345 the amino-terminal, the amino acids V, I and R were the most effective for the inhibitory activity.  
 346 Based on this consideration, some dipeptides found in the three fractions (VV, SLx and NLx in F2;  
 347 VLx and TLx in F3; HV in F5) have the potential to inhibit the ACE activity. Especially, the  
 348 dipeptide VLx found in F3 meet both the structural requirements for ACE-inhibition. This dipeptide  
 349 also corresponds to the C-terminal residues of the potent ACE-inhibitory peptide LVL (IC<sub>50</sub> value  
 350 of 12 μmol L<sup>-1</sup>), previously isolated from porcine plasma (Hazato & Kase, 1986). Other peptides  
 351 that may be susceptible of being considered as ACE inhibitors were the sequences YTDAPSF  
 352 (fraction F5), APSF (fraction F2), LxSL (fraction F4), YQEPVL (fraction F5) and ISLL (fraction  
 353 F5) since the presence of amino acids F or L in the C-terminal position favours ACE-binding  
 354 (Cheung et al., 1980). Likewise, peptides terminating in K, such as the peptide GVPK identified in  
 355 fraction 3, are reported as good candidates to exert inhibitory activity (Bidasolo et al., 2012). Some  
 356 peptides, which gave intense peaks in the MS spectra, had amino acid sequences that are not in

357 accordance with the results of structure-activity correlation studies on ACE-inhibitory peptides.

358 Interestingly, these peptides share the –PQ domain at the carboxy-terminal such as FPQ (fraction

359 F5), AVPQ and NVPQ (fraction F3). The amino acid Q at the carboxy-terminal is generally

360 considered negative for the ACE-inhibitory activity of peptides. However, recently, some non-

361 competitive inhibitors of ACE showed a Q residue (or in general an amide-containing amino acid)

362 at the carboxy-terminal (Ni, Li, Liu, & Hu, 2012; Tanzadehpanah, Asoodeh, Saberi, & Chamani,

363 2013). These peptides bind the N-domain of ACE, which is different from the FAPGG binding site

364 (C-domain), affecting the conformation change of the active site and resulting in loss of activity

365 (Acharya et al., 2003). These –PQ containing peptides and an additional peptide found in fraction 3

366 (LVQ), which contained Q at the carboxy-terminal, may be involved in ACE-inhibition by a non-

367 competitive mechanism. To the best of our knowledge, these peptides have never been found in any

368 active hydrolysate and thus represent novel bioactive peptides.

369 Some peptides with other biological activities have been found in the three analysed fractions. Most

370 of the observed dipeptides (18 out of 20) had dipeptidyl peptidase IV (DPP-IV) inhibitory activity.

371 Only the dipeptides LxY and FT lack this activity.

372 Some other peptides and amino acids with previously demonstrated radical scavenging activity

373 were found in the three fractions. The amino acid Y, found in fraction F2, exhibited high 2,2'-

374 azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydroxyl radical scavenging

375 activities (Tagliazucchi et al., 2016b). Three antioxidant dipeptides, AY, VY and LxY (identified in

376 fractions F2, F3 and F5 respectively), contain the amino acid Y at the carboxy-terminal which is

377 responsible for their radical scavenging activity (Beermann, Euler, Herzberg, & Stahl, 2009; Cheng,

378 Chen, & Xiong, 2010; Yokomizo, Takenaka, & Takenaka, 2002). The peptide VY seems to be

379 particularly interesting, behaving as a multifunctional bioactive peptide. Its release seems common

380 in milk from various species. It has been also identified after *in vitro* gastro-intestinal digestion of

381 camel and goat milk (Tagliazucchi et al., 2016a and this work) and it has been also found in human

382 plasma after consumption of a milk beverage, indicating that this peptide is released also from

383 cow's milk caseins and is bioavailable in humans (Foltz et al., 2007). Some other peptides are  
384 fragments or precursors of known antioxidant peptides, such as the peptides DAYPSGAW  
385 (identified in F5) (De Gobba, Tompa, & Otte, 2014b), YAKPV (identified in F2) (De Gobba,  
386 Tompa, & Otte, 2014b), YQEPVLGP (identified in F5) (Silva, Pihlanto, & Malcata, 2006) and  
387 PLW (identified in F5) (Liu et al., 2015).

388

### 389 3.4. Quantification of VPP and IPP

390 Among the milk-derived bioactive peptides, the anti-hypertensive lacto-tripeptides VPP and IPP  
391 have attracted particular attention in the last years (Nongonierma, & FitzGerald, 2015) because of  
392 the numerous *in vivo* clinical trials showing the blood pressure-reducing effect of the two  
393 lactotriptides in pre-hypertensive and mildly hypertensive patients (Fekete, Givens, & Lovegrove,  
394 2015; Turpeinen, Järvenpää, Kautiainen, Korpela, & Vapaatalo, 2013). Lactotriptides VPP and  
395 IPP are easily released from cow caseins by starter lactic acid bacterium *Lactobacillus helveticus* as  
396 well as non-starter strains of *Lactobacillus casei* and *Lactobacillus rhamnosus* (Nongonierma, &  
397 FitzGerald, 2015; Solieri et al., 2015). Recently, our research group demonstrated for the first time  
398 that gastro-intestinal proteolytic enzymes are able to release the antihypertensive tripeptides VPP  
399 and IPP from cow milk caseins and IPP from camel milk caseins (Rutella et al., 2016; Tagliazucchi  
400 et al., 2016a).

401 In goat milk, the lactotriptides are present in  $\beta$ -casein (VPP; fragment 84-86) and  $\kappa$ -casein (IPP;  
402 fragment 108-110). Digestion with the harmonized model resulted in the release of both tripeptides.  
403 At the end of simulated digestion, IPP and VPP amounts were  $141.4 \pm 15.1$  and  $1829.8 \pm 216.4$   $\mu\text{g}$   
404  $\text{L}^{-1}$  of hydrolysates, respectively. *In vitro* digestion of goat milk demonstrated that VPP is released  
405 from milk caseins at higher amounts than IPP. The higher release of VPP respect to IPP could be  
406 due to a higher amount of  $\beta$ -casein in goat milk respect to  $\kappa$ -casein. According to previously  
407 reported data, goat milk protein composition was 55-71% of  $\beta$ -casein and 15-20% of  $\kappa$ -casein  
408 (Ruprichová et al., 2015).

409 Comparison with previously reported data pointed out that the release of the lactotripeptides during  
410 the digestion depends on the concentration and digestibility of casein in milk as well as on their  
411 presence in specific fragments of  $\beta$ - and/or  $\kappa$ -casein. For example, because of the presence of IPP in  
412 both cow and camel milk  $\beta$ - and  $\kappa$ -casein, it is released in greater amount during *in vitro* digestion  
413 of these milk respects to goat milk (Rutella et al., 2016; Tagliazucchi et al., 2016a). Furthermore,  
414 the higher release of VPP in goat milk respect to cow's milk may be related both to the highest  $\beta$ -  
415 casein concentration in goat milk and to the greater digestibility of goat milk caseins (Rutella et al.,  
416 2016). It is important to note that all the digestions were carried out using the same harmonized  
417 model, which means the same milk to digestive enzymes ratio. Considering the sum of VPP and  
418 IPP released from milk of different species at the end of the *in vitro* digestion, goat milk appeared to  
419 be the best source of the anti-hypertensive lactotripeptides. It would be expected that consumption  
420 of 200 mL of milk would result in a release of about 3.5, 2.4 and 0.5 mg of the lactotripeptides (sum  
421 of VPP and IPP) from goat, cow and camel milk, respectively (Rutella et al., 2016; Tagliazucchi et  
422 al., 2016a). Several clinical studies on hypertensive subjects showed that the administration of daily  
423 doses of VPP/IPP in the range of 2-10 mg were associated with a decrease of the systolic (4.0  
424 mmHg) and diastolic (1.9 mmHg) blood pressure in hypertensive patients (Turpeinen et al., 2013).

425

#### 426 **4. Conclusion**

427 *In vitro* gastro-intestinal digestion of goat skimmed milk resulted in the release of ACE-inhibitory  
428 peptides. Additional identified short peptides have demonstrated DPP-IV inhibitory or antioxidant  
429 activities. ACE-inhibitory activity was highest at the end of the peptic phase of the digestion while  
430 steadily declined during the pancreatic phase. Ultrafiltration of the post-pancreatic digested sample  
431 showed that the < 3 kDa fraction with small peptides was responsible for the ACE-inhibitory  
432 activity of the digested milk. Fractionation of the < 3 kDa fraction by HPLC resulted in 9 fractions,  
433 of which three fractions (F2, F3 and F5) displayed the highest ACE-inhibitory activity.

434 Peptide identification by LC-MS indicated that most of the peptides in the three fractions were  
435 identical to ACE-inhibitory peptides known from milk proteins. Several new peptides not  
436 previously reported to occur in any milk protein hydrolysates were identified in these active  
437 fractions having structural characteristic similar to reported ACE-inhibitory peptides. Moreover,  
438 three new identified peptides (FPQ, AVPQ and NVPQ) could act as non-competitive inhibitor of  
439 ACE and are also expected to contribute to the ACE-inhibitory activity of these fractions. This  
440 work also provides evidence, for the first time, of the fact that VPP and IPP may be released during  
441 the gastrointestinal digestion of goat milk  $\beta$ - and  $\kappa$ -casein. The amount of VPP and IPP released  
442 from goat milk was higher than that previously found after *in vitro* digestion of cow and camel milk.  
443 This study firstly demonstrated that peptides with potential bioactive effects included the  
444 lactotripeptides VPP and IPP are generated by *in vitro* gastro-intestinal digestion of goat milk.

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## Figure captions

**Figure 1.** Changes in hydrolysis degree (DH) of goat milk proteins during *in vitro* gastro-intestinal digestion. S0 and S5 mean time zero and 5 min of salivary digestion, respectively. G30, G60, G90 and G120 mean time 30, 60, 90 and 120 min of gastric digestion, respectively. P30, P60, P90 and P120 mean time 30, 60, 90 and 120 min of intestinal digestion, respectively. Values represent means  $\pm$  SD of triplicate digestions. Different letters indicate that the values are significantly different ( $P < 0.05$ ).

**Figure 2.** Evolution of the angiotensin-I converting enzyme (ACE)-inhibitory activity ( $IC_{50}$  values) during simulated gastrointestinal digestion of goat milk. Each column corresponds to an aliquot withdrawn during hydrolysis as described in **Figure 1**.  $IC_{50}$  is defined as the concentration of peptides required to inhibit 50% of the ACE activity. Different letters within the same column indicate that the values are significantly different ( $P < 0.05$ ).

**Figure 3.** UV-chromatogram of the low molecular weight peptidic fraction (<3 kDa) obtained from goat milk subjected to consecutive gastro-intestinal digestion. F1-F9 represent the collected fractions used for the analysis of the angiotensin-converting enzyme inhibitory activity. The black line represents the trace at 214 nm whereas the grey line represents the trace at 280 nm. The chromatogram shown is representative of three independent experiments.

**Figure 4.** Mass spectrum of peptide fractions F2 (A), F3 (B) and F5 (C) from nanoflow LC-ESI-QTOF MS/MS analysis. Identified peptides are reported in tables 2, 3 and 4. Asterisk indicate non-identified peaks. The mass spectra shown are representative of three independent experiments.