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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 released after *in vitro* gastro-intestinal digestion of skimmed goat milk. The experimental approach 3 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 the antihypertensive tripeptides valine-proline-proline and isoleucine-proline-proline were released 10 from goat milk proteins during *in vitro* gastro-intestinal digestion at concentrations of $1829.8 \pm$ 11 216.4 and 141.4 \pm 15.1 µg L⁻¹, respectively. This research underlines the suitability of the 12 13 harmonized digestive model system to study the release of short bioactive peptides during gastrointestinal transit. 14

15 **1. Introduction**

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

27 Goat milk is one of the most important nutritional foods around the world, especially in Asia, Africa and many European countries. It has been found to be a good substitute of human milk in 28 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 concentration in sheep milk is higher than cow, camel and goat (Park, Juárez, Ramos, & Haenlein, 31 2007). Goat, camel and sheep milk have different proportions of the four major caseins (α S1, α S2, 32 β , κ) compared to cow counterparts. It has been reported that β -case in is the major component of 33 sheep and goat milk casein (from 18 to 24 g L⁻¹) (Ruprichová et al., 2015). The major component of 34 cow's milk case in is α S1 (~12 g L⁻¹) followed by β -case in (~11 g L⁻¹) (Omar, Harbourne, Oruna-35 Concha, 2016). Camel milk contains β -casein amounts comparable to cow's milk (~12 g L⁻¹) but 36 lower level of α S1-casein (~3 g L⁻¹) (Omar et al., 2016). Level of κ -casein is relatively higher in 37 cow, sheep and goat milk (~4-5 g L^{-1}) respect to camel milk (~2 g L^{-1}) (Omar et al., 2016; 38 Ruprichová et al., 2015). 39

Several studies have been done to investigate the bioactivity of goat milk protein hydrolysate and 40 41 the release of ACE-inhibitory and antioxidant peptides with individual proteases such as thermolysin, trypsin, subtilisin, papain and pepsin or their combinations (Ahmed, El-Bassiony, 42 Elmalt, & Ibrahim, 2015; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Hernández-43 Ledesma, Recio, Ramos, & Amigo, 2002). Espejo-Carpio et al. (2013) and De Gobba et al. (2014a) 44 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 contained tyrosine in their sequence and had antioxidant and ACE-inhibitory activities. ACE-47 inhibitory peptides represent sources of health-enhancing compounds of special interest, as 48 49 hypertension is a major independent risk factor for cardiovascular and related diseases. ACE is a dipeptidyl carboxypeptidase that catalyzes, in vivo, the conversion of the plasmatic peptide 50 angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE plays an important 51 52 role in the regulation of blood pressure and drugs that inhibit ACE are commonly prescribed for the treatment of hypertension or related cardiovascular diseases (Acharya et al., 2003). 53 54 Furthermore, antioxidant peptides are particularly interesting because they can potentially prevent or delay oxidative stress associated chronic diseases (Willcox et al. 2004). In this sense, milk 55 proteins have been considered as a carrier for the delivery of antioxidant peptides in the gastro-56 57 intestinal tract where they may exert direct protective effects by scavenging reactive oxygen species and reducing the oxidative stress (Tagliazucchi, Helal, Verzelloni, & Conte, 2016b). 58 Recently, several DPPI-IV-inhibitory peptides have been isolated and characterized from goat milk 59 proteins hydrolysed with a combination of trypsin and chymotrypsin (Zhang, Chen, Ma, & Chen, 60 61 2015). DPP-IV inhibitors can be used to reduce DPP-IV activity and increase the lifetime of incretins. These are peptidic gut hormones, which stimulate insulin secretion and β-cell-62 proliferation but that are quickly degraded by brush-border intestinal DPP-IV (Sebokova, Christ, 63 Boehringer, & Mizrahi, 2007). DPP-IV inhibitors can be employed in the management of type 2 64 diabetes, reducing DPP-IV activity and increasing the lifetime of incretins (Sebokova et al., 2007). 65

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*

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

87 2.2. Chemical analysis of skimmed goat milk

Raw goat milk was pasteurized for 15 seconds after the central temperature reached 72°C and
immediately defatted by centrifugation at 2000*g* for 20 min at room temperature to obtain the
skimmed goat milk. This last was stored at -80°C until further analysis. Skimmed milk sample was
analysed for pH, total solids, fat, and lactose by phenol-sulphuric acid method, and total nitrogen,
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

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-

Bolanz et al. (2012). To simulate the oral phase, 9 mL of skimmed goat milk were mixed with 12

- mL of simulated salivary fluid containing 150 U mL⁻¹ of porcine α -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

mL of simulated gastric fluid. The pH was adjusted to 2.0 with HCl 6 mol L⁻¹ and supplemented 103 with porcine pepsin (1115 U mL⁻¹ of simulated gastric fluid). The gastric bolus was then incubated 104 for 120 min at 37°C on a rotating wheel (10 rpm). A lower amount of pepsin was chosen in respect 105 to the INFOGEST protocol since, in a previous work (Tagliazucchi et al., 2016c), the digestion of 106 cow milk with a ratio of ~3000U of pepsin per mL of milk, as that used in the current study, 107 resulted in a peptides pattern at the end of the digestion similar to the results obtained *in vivo* by 108 Boutrou et al. (2013) from jejunal effluents of healthy humans after ingestion of caseins and whey 109 proteins separately. The intestinal digestion was carried out by adding to the gastric bolus 24 mL of 110 pancreatic fluid and 12 mL of bile fluid, adjusting the pH to 7.0 and supplemented with pancreatin. 111 The chyme was further incubated for 120 min at 37°C on a rotating wheel (10 rpm). The digested 112 samples were immediately cooled on ice and frozen at -80°C for further analysis. The digestions 113 were performed in triplicate. In addition, a control digestion, which included only the gastro-114 115 intestinal juices and enzymes, and water in place of milk, was carried out to consider the possible impact of the digestive enzymes in the subsequent analysis. 116 117 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*

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

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

127 **DH**= $(h/h_{tot}) \cdot 100$ (1)

Where h is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein
of α-amino groups formed at the different stages of the simulated digestion, and htot is the
hydrolysis equivalent (total number of amino groups) at complete hydrolysis to amino acids. The
total number of amino groups was determined by hydrolysing skimmed goat milk in 6 mol L⁻¹ HCl
at 110°C for 24 h. The h_{tot} value was calculated resulting in 8.6 milliequivalents per gram of
protein. DH data were subtracted with the data obtained in the control digestion.

Low molecular weight peptides were extracted by ultrafiltration from the post-pancreatic digested 134 samples (corresponding to the aliquots collected after 120 min of pancreatic digestion). Briefly, 4 135 mL of sample were loaded on an Amicon Ultra-4 nominal filter (cut-off 3 kDa) and centrifuged at 136 137 7500g for 120 min at 4°C using a Hermle Z383K refrigerated centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany). The filtrates, containing low molecular weight peptides, were 138 collected and freeze-dried. The peptide content of the filtrates was determined by using the TNBS 139 140 method as described above and expressing the results as mg of leucine equivalent mL^{-1} . The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer (Orlando FL, U.S.A.). 141

142

143 2.5. Measurements of ACE-inhibitory activity

ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983) using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate. The reaction was monitored at 345 nm for 10 min. For the calculation of the IC₅₀ value, the ACE assay was carried out in presence of different amounts of the un-fractionated samples collected during the gastric and pancreatic phases of the digestion and of the lower and higher of 3 kDa fractions of the post-pancreatic sample. IC₅₀ was defined as the concentration of peptides required to inhibit 50% of the enzymatic activity.

The absorbance was read using the same Jasco V-550 UV/Vis spectrophotometer as reported inparagraph 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) obtained from the post-pancreatic sample after ultrafiltration was performed with a Jasco HPLC 156 157 system equipped with a 250mm x 4.6mm reversed phase column Hamilton HxSil C18 (Hamilton, Reno, NV, USA) as described by Tagliazucchi et al. (2015). A linear gradient of solvent B 158 (acetonitrile-trifluoroacetic acid 0.027%) in A (water-trifluoroacetic acid 0.037%) ranging from 0% 159 to 45% in 115 min with a flow rate of 0.5 mL min⁻¹ was used to separate the peptides contained in 160 the low molecular weight fractions of digested milk. The PDA detector was set at 214 and 280 nm. 161 Nine major fractions were collected. These fractions were characterized for their peptide content 162 163 (paragraph 2.4) and ACE-inhibitory activity (paragraph 2.5). The yield of the collected fractions was calculated as follows: peptides concentration in the fraction*100/peptides concentration in the 164 165 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

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

enrichment column and a 43mm \times 75 μ m 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

by Dei Più et al. (2014). For peptide identification and sequencing, MS/MS spectra were converted

to .mgf and *de novo* peptide sequencing was performed using Pepnovo software

180	(<u>http://proteomics.ucsd.edu/ProteoSAFe/</u>). The following parameters were considered: enzyme,
181	none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 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). 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 $\mu g L^{-1}$ 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 14.5%, total proteins 3.78%, caseins 2.92%, whey proteins 0.86%, lactose 4.55%, fat <0.05%. The 207 208 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, total solids can range from 11 to 19% and total proteins from 1.9 to 5.3% (Yuksel, Avci, Uymaz, & 210 Erdem, 2012). Wide variability can also be observed for total caseins (2.33-4.63%) and whey 211 212 proteins (0.37-1.8%) (Yadav et al., 2016). Vice versa, low variability has been found for lactose (4.6-4.9%) and pH (6.47-6.82) (Yadav et al., 2016; Torres, Castro, Argüello, & Capote, 2013; 213 214 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 with cow's milk (Park et al., 2007). Besides nutritional quality, functional properties of proteins are 216 217 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 bioactivity. During digestion, the combined action of gastro-intestinal proteolytic enzymes may lead 219 220 to the release of bioactive peptides from goat milk proteins.

The hydrolysis of goat milk proteins during the in vitro digestion was evaluated with the TNBS 221 222 assay. Figure 1 reports the resulting DH values, relative to the control condition, at various steps of the simulated gastro-intestinal transit. As expected, simulated salivary digestion did not affect the 223 224 hydrolysis degree. Un-digested goat milk showed a DH of $3.9 \pm 0.2\%$, which was $4.3 \pm 0.4\%$ after 5 minutes of salivary digestion (P > 0.05). After 30 min of the gastric digestion, the DH sharply 225 226 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

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

241

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

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

247 During the pepsin-catalysed part of the simulated physiological digestion, the IC₅₀ value for ACE-

inhibitory activity (defined as the peptide concentration required to inhibit 50% of the ACE

activity) slightly but not significantly decreased during the first 90 minutes of gastric digestion,

suggesting a modest increase in the ACE-inhibitory activity of the hydrolysates (Figure 2). The

251 passage from gastric to pancreatic treatment increased the IC₅₀ value significantly from $313.7 \pm$

252 26.5 μ g of peptides mL⁻¹ at the end of the gastric digestion to 704.5 \pm 49.0 μ g of peptides mL⁻¹ after

253 30 min of the pancreatic digestion (P < 0.0001). Then, the IC₅₀ value sharply rose as the digestion

with pancreatic enzymes progressed reaching the highest value of $1808.8 \pm 68.3 \,\mu g$ of peptides mL⁻

¹ 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.

261 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., 262 2016a) and short peptide sequences are good candidates to play a physiological antihypertensive 263 264 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 265 digested sample were extracted by ultrafiltration with a cut-off of 3 kDa. The < 3 kDa fraction of 266 267 the samples obtained at the end of the simulated digestion procedure contained 22.79 ± 0.83 mg mL⁻¹ of peptides and showed a IC₅₀ value towards ACE activity of 1156.3 \pm 10.5 µg of peptides 268 269 mL^{-1} (**Table 1** and **Figure 2**). No activity was found in the > 3 kDa fraction of the post-pancreatic 270 sample (Figure 2) suggesting that low molecular weight peptides were responsible for the ACEinhibitory activity of the digested goat milk. 271

Espejo-Carpio et al., (2016) obtained IC₅₀ values from 230 to 269 μ g mL⁻¹ for goat milk hydrolysed 272 273 with trypsin, subtilisin or a combination of these enzymes. In our experiments, IC₅₀ values observed during goat milk digestion with pepsin (gastric phase) are similar to those reported by Espejo-274 Carpio et al., (2016). As the digestion with pancreatic enzymes proceed, peptides are cut in ever-275 276 smaller fragments and amino acids by pancreatic proteases. As a result, ACE-inhibitory activity 277 declined due to the susceptibility of released peptides during the gastric digestion to pancreatic 278 enzymes resulting in a IC₅₀ 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 279

found gastro-intestinal digestion strongly influenced the ACE-inhibitory activity of camel milk with IC₅₀ value of 1771.4 μ 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 IC₅₀ 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

289 214 and 280 nm with a photodiode array detector.

290 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 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 mg mL⁻¹, giving a total yield of 86.4%.

296 Most of the fractions exerted a weak or non-detectable ACE-inhibitory activity (Table 1). However,

three fractions (F2, F3 and F5) showed low IC₅₀ values, which means high ACE-inhibitory activity.

The lowest IC₅₀ values of 14.1 \pm 0.8 and 17.9 \pm 0.8 μ g peptides mL⁻¹ were found in fractions F5 and F3, respectively.

300

301 3.3. Identification of ACE-inhibitory and additional bioactive peptides in HPLC fractions F2, F3
302 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 identification were subjected to a manual evaluation, and the validated peptide sequences were 307 responsible for the majority of the most intense peaks in the MS spectra (Tables 2-4). 308 Fraction F2 (Table 2) contained 16 peptides, most of them being dipeptides. Two peptides found in 309 fraction F2 showed very low IC₅₀ values against ACE. The dipeptide AI and the tripeptide IPP 310 demonstrated IC₅₀ values of 3.41 and 5 μ mol L⁻¹, respectively (Nakahara et al., 2010; Nakamura, 311 Yamamoto, Sakai, & Takano, 1995). The dipeptide AI has been found to be released from pinto 312 bean and camel milk proteins submitted to the same harmonized in vitro digestion model 313 (Tagliazucchi et al., 2015; Tagliazucchi et al., 2016a) and, here, gave an intense signal in the mass 314 315 spectra suggesting its presence at high concentration. In addition, the dipeptide AY showed low IC₅₀ value of 19 µmol L⁻¹ (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Some tripeptides 316 found in F2 share the carboxy-terminal sequence with ACE-inhibitory dipeptides, a structural 317 318 feature of paramount importance for the inhibitory activity (Wu, Aluko, & Nakai, 2006). The tripeptides AEK, GLN and ALN are precursors of the ACE-inhibitory dipeptides EK and LN, 319 320 previously isolated from cow's milk hydrolysed with lactic acid bacteria (van Platerink, Janssen, & 321 Haverkamp, 2008). In fraction F3 (Table 3), 19 peptides, having 2–4 amino acid residues, were identified. The most 322 effective ACE-inhibitory peptide was GPV (IC₅₀= 4.7 μ mol L⁻¹) followed by VY (IC₅₀= 7.1 μ mol 323 324 L⁻¹) (Saito, Wanezaki, Kawato, & Imayasu, 1994; Wu et al., 2006). Four others peptides able to inhibit ACE activity were detected in this fraction (IAE, IP, PL and AF) with IC₅₀ values ranging 325 between 35 and 330 µmol L⁻¹ for IAE and PL, respectively (Byun, & Kim, 2002; Cheung et al., 326 1980; Murray, & FitzGerald, 2007). The dipeptide VY was also effective in vivo in mild 327 hypertensive subjects (Kawasaki et al., 2000). In addition, the β -case in derived tripeptide HLP and 328 329 the dipeptide VM are fragments of the ACE-inhibitor peptides LHLP and TEQESGVPVM (Kohmura et al., 1989; Tanzadehpanah et al., 2016). 330

The range of peptide length in fraction F5 (**Table 4**) was between 2 and 8 amino acid residues.

Fraction F5, which showed the lowest IC₅₀ value, contained the tripeptides VPP and LxVL as well

as the dipeptide LxY, which showed IC₅₀ values between 2.1 and 18 μ mol L⁻¹ (Nakamura et al.,

1995; Wu et al., 2006). Additional peptides with lower ACE-inhibitory activity were found in this

fraction such as PLW (IC₅₀= 36 μ mol L⁻¹), DAYPSGAW (IC₅₀=98 μ mol L⁻¹) and VAV (IC₅₀= 260

 μ mol L⁻¹) (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

important for the inhibitory activity (Saito et al., 1994).

339 In the fractions with the best inhibitory activity towards ACE, several novel peptides were

identified which may be involved in the ACE-inhibitory activity of these fractions.

In a study on inhibition of ACE by glycyl dipeptides with varying carboxy- or amino-terminal

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

the carboxy-terminal were the most favourable residues for the ACE-inhibitory activity, whereas at

the amino-terminal, the amino acids V, I and R were the most effective for the inhibitory activity.

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

also corresponds to the C-terminal residues of the potent ACE-inhibitory peptide LVL (IC₅₀ value

of 12 μ mol L⁻¹), previously isolated from porcine plasma (Hazato & Kase, 1986). Other peptides

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

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

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

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

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)

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

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 any368 active hydrolysate and thus represent novel bioactive peptides.

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

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

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

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

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

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

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

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

388

389 *3.4. Quantification of VPP and IPP*

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

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

18

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

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

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

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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 (IC₅₀ values) during simulated gastrointestinal digestion of goat milk. Each column corresponds to an aliquot withdrawn during hydrolysis as described in **Figure 1**. IC₅₀ 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 nonidentified peaks. The mass spectra shown are representative of three independent experiments.