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

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Abstract

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 combined the recently developed harmonized static *in vitro* digestion model and mass spectrometry to identify bioactive peptides. Peptides in the post-pancreatic digested were extracted by ultrafiltration and isolated by reversed-phase high-performance liquid chromatography following mass spectrometry identification. Among the identified sequences, eighteen were identical to known bioactive peptides with ACE-inhibitory activity. Peptides with dipeptidyl peptidase IV-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 from goat milk proteins during *in vitro* gastro-intestinal digestion at concentrations of 1829.8 ± 216.4 and 141.4 ± 15.1 μg L^{-1}, respectively. This research underlines the suitability of the harmonized digestive model system to study the release of short bioactive peptides during gastro-intestinal transit.

1. Introduction

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, especially from cow's milk, are the best source of bioactive peptides with various physiological activities including antioxidant, antibacterial, immune-modulating, dipeptidyl peptidase IV (DPP-IV) and angiotensin-converting enzyme (ACE) inhibition (Nongonierma, & FitzGerald, 2015). The biological activity of these peptides is based on the amino acid composition and sequence. The size 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, 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, & Conte, 2016a).

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 infant formulas especially for children who suffer allergic reactions to cow milk (Yadav, Singh, & 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, 2007). Goat, camel and sheep milk have different proportions of the four major caseins (αS1, αS2, β, κ) compared to cow counterparts. It has been reported that β-casein is the major component of sheep and goat milk casein (from 18 to 24 g L^{-1}) (Ruprichová et al., 2015). The major component of cow's milk casein is αS1 (~12 g L^{-1}) followed by β-casein (~11 g L^{-1}) (Omar, Harbourne, Oruna-Concha, 2016). Camel milk contains β-casein amounts comparable to cow's milk (~12 g L^{-1}) but lower level of αS1-casein (~3 g L^{-1}) (Omar et al., 2016). Level of κ-casein is relatively higher in cow, sheep and goat milk (~4-5 g L^{-1}) respect to camel milk (~2 g L^{-1}) (Omar et al., 2016; Ruprichová et al., 2015).

Several studies have been done to investigate the bioactivity of goat milk protein hydrolysate and 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, Elmalt, & Ibrahim, 2015; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Hernández-Ledesma, Recio, Ramos, & Amigo, 2002). Espejo-Carpio et al. (2013) and De Gobba et al. (2014a) identified many casein-derived peptides from hydrolysed proteins of goat milk, which were 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-inhibitory peptides represent sources of health-enhancing compounds of special interest, as 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 angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE plays an important 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).
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 proteins have been considered as a carrier for the delivery of antioxidant peptides in the gastro-intestinal tract where they may exert direct protective effects by scavenging reactive oxygen species and reducing the oxidative stress (Tagliazucchi, Helal, Verzelloni, & Conte, 2016b).
Recently, several DPPI-IV-inhibitory peptides have been isolated and characterized from goat milk proteins hydrolysed with a combination of trypsin and chymotrypsin (Zhang, Chen, Ma, & Chen, 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-proliferation but that are quickly degraded by brush-border intestinal DPP-IV (Sebokova, Christ, Boehringer, & Mizrahi, 2007). DPP-IV inhibitors can be employed in the management of type 2 diabetes, reducing DPP-IV activity and increasing the lifetime of incretins (Sebokova et al., 2007).

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

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

2. Materials and methods

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).

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).

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

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 minor modification for adaptation to milk (Tagliazucchi, Helal, Verzelloni, Bellesia, & Conte, 2016c). The protocol consisted of three consecutive steps: oral, gastric and intestinal phases. 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 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 with porcine pepsin (1115 U mL^{-1} of simulated gastric fluid). The gastric bolus was then incubated for 120 min at 37°C on a rotating wheel (10 rpm). A lower amount of pepsin was chosen in respect to the INFOGEST protocol since, in a previous work (Tagliazucchi et al., 2016c), the digestion of cow milk with a ratio of ~3000U of pepsin per mL of milk, as that used in the current study, resulted in a peptides pattern at the end of the digestion similar to the results obtained *in vivo* by Boutrou et al. (2013) from jejunal effluents of healthy humans after ingestion of caseins and whey proteins separately. The intestinal digestion was carried out by adding to the gastric bolus 24 mL of pancreatic fluid and 12 mL of bile fluid, adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was further incubated for 120 min at 37°C on a rotating wheel (10 rpm). The digested samples were immediately cooled on ice and frozen at –80°C for further analysis. The digestions were performed in triplicate. In addition, a control digestion, which included only the gastro-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.

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

2.4. Assessment of protein hydrolysis during the digestion and fractionation of the post-pancreatic 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).

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

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

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 $\mathbf{h_{tot}}$ 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 samples (corresponding to the aliquots collected after 120 min of pancreatic digestion). Briefly, 4 mL of sample were loaded on an Amicon Ultra-4 nominal filter (cut-off 3 kDa) and centrifuged at 7500*g* 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 collected and freeze-dried. The peptide content of the filtrates was determined by using the TNBS 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.).

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 IC_{50} 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. IC_{50} 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 in paragraph 2.4.

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^{-1} 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, ± 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 μg L^{-1} of hydrolysates
197
198 *2.9. Statistical analysis*
199 All data are presented as mean ± 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 $P < 0.05$.

3. Results and Discussion

3.1. Assessment of protein hydrolysis during simulated digestion

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 pH was 6.64. Composition of goat milk can be very different depending on the diet, breed, feeding, 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, & Erdem, 2012). Wide variability can also be observed for total caseins (2.33-4.63%) and whey 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; Yuksel et al., 2012). Due to the highest content in calcium, vitamins (such as vitamin A, C and D), 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 also important for human health. Enzymatic hydrolysis is known to improve functional properties 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 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 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 hydrolysis degree. Un-digested goat milk showed a DH of 3.9 ± 0.2%, which was 4.3 ± 0.4% after 5 minutes of salivary digestion ($P > 0.05$). After 30 min of the gastric digestion, the DH sharply increased to 22.5 ± 1.8% (**Figure 1**). After that, the peptic activity resulted in a further slight increase in DH during the 120 minutes of gastric digestion. The transition from gastric to pancreatic treatment increased DH values significantly from 28.4 ± 2.3% at the end of gastric digestion to 65.1

± 1.0% after 30 min of pancreatic digestion (P<0.0001) (**Figure 1**). Subsequently, the DH rose gradually reaching the maximum value of 82.7 ± 1.2% after 120 min of the intestinal incubation. A comparison with previously reported data (Rutella et al., 2016; Tagliazucchi et al., 2016a) 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-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; Tagliazucchi et al., 2016a). The highest digestibility of goat milk proteins seems related to the higher susceptibility of these to peptic digestion compared to camel and cow milk proteins. The DH value measured after peptic hydrolysis of cow, camel and goat milk proteins, digested with the same harmonized digestion model, was 12.2%, 20.5% and 28.4%, respectively (Rutella et al., 2016; Tagliazucchi et al., 2016a).

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.

During the pepsin-catalysed part of the simulated physiological digestion, the IC_{50} 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 passage from gastric to pancreatic treatment increased the IC_{50} value significantly from 313.7 ± 26.5 µg of peptides mL^{-1} at the end of the gastric digestion to 704.5 ± 49.0 µg of peptides mL^{-1} after 30 min of the pancreatic digestion (P < 0.0001). Then, the IC_{50} value sharply rose as the digestion with pancreatic enzymes progressed reaching the highest value of 1808.8 ± 68.3 µg of peptides mL^{-}

255 [1] at the end of the digestion (**Figure 2**). As a result, at the end of the *in vitro* physiological digestion,
256 the ACE-inhibitory activity was lower than that observed after peptic digestion. Results reported in
257 **Figure 2** suggested that the ACE-inhibitory activity of goat milk hydrolysates decreased as the
258 peptides length decreased. Peptic hydrolysis can generate ACE-inhibitory peptides whereas further
259 degradation of the peptides into much smaller fragments by pancreatic proteases resulted in a
260 decrease in the ACE-inhibitory activity.
261 Because it has been suggested that small peptides are mainly responsible for the ACE-inhibitory
262 activity of digested milk (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Tagliazucchi et al.,
263 2016a) and short peptide sequences are good candidates to play a physiological antihypertensive
264 role *in vivo*, ACE-inhibitory activity and the peptide content of permeate, obtained following
265 ultrafiltration of the post-pancreatic sample, were also assayed. The peptides in the post-pancreatic
266 digested sample were extracted by ultrafiltration with a cut-off of 3 kDa. The < 3 kDa fraction of
267 the samples obtained at the end of the simulated digestion procedure contained 22.79 ± 0.83 mg
268 mL^{-1} of peptides and showed a IC_{50} value towards ACE activity of 1156.3 ± 10.5 µg of peptides
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 ACE-
271 inhibitory activity of the digested goat milk.
272 Espejo-Carpio et al., (2016) obtained IC_{50} values from 230 to 269 µg mL^{-1} for goat milk hydrolysed
273 with trypsin, subtilisin or a combination of these enzymes. In our experiments, IC_{50} values observed
274 during goat milk digestion with pepsin (gastric phase) are similar to those reported by Espejo-
275 Carpio et al., (2016). As the digestion with pancreatic enzymes proceed, peptides are cut in ever-
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 IC_{50} value 4 and 5 time higher than the value reported in the Espejo-Carpio
279 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 µg 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 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%.

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 ± 0.8 and 17.9 ± 0.8 µg peptides mL^{-1} were found in fractions F5 and F3, respectively.

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

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 responsible for the majority of the most intense peaks in the MS spectra (**Tables 2-4**).

Fraction F2 (**Table 2**) contained 16 peptides, most of them being dipeptides. Two peptides found in fraction F2 showed very low IC_{50} values against ACE. The dipeptide AI and the tripeptide IPP demonstrated IC_{50} values of 3.41 and 5 μmol L^{-1}, respectively (Nakahara et al., 2010; Nakamura, Yamamoto, Sakai, & Takano, 1995). The dipeptide AI has been found to be released from pinto bean and camel milk proteins submitted to the same harmonized *in vitro* digestion model (Tagliazucchi et al., 2015; Tagliazucchi et al., 2016a) and, here, gave an intense signal in the mass spectra suggesting its presence at high concentration. In addition, the dipeptide AY showed low IC_{50} value of 19 μmol L^{-1} (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Some tripeptides found in F2 share the carboxy-terminal sequence with ACE-inhibitory dipeptides, a structural 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, previously isolated from cow's milk hydrolysed with lactic acid bacteria (van Platerink, Janssen, & Haverkamp, 2008).

In fraction F3 (**Table 3**), 19 peptides, having 2–4 amino acid residues, were identified. The most effective ACE-inhibitory peptide was GPV (IC_{50}= 4.7 μmol L^{-1}) followed by VY (IC_{50}= 7.1 μmol 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 IC_{50} values ranging between 35 and 330 μmol L^{-1} for IAE and PL, respectively (Byun, & Kim, 2002; Cheung et al., 1980; Murray, & FitzGerald, 2007). The dipeptide VY was also effective *in vivo* in mild hypertensive subjects (Kawasaki et al., 2000). In addition, the β-casein derived tripeptide HLP and the dipeptide VM are fragments of the ACE-inhibitor peptides LHLP and TEQESGVPVM (Kohmura et al., 1989; Tanzadehpanah et al., 2016).

The range of peptide length in fraction F5 (**Table 4**) was between 2 and 8 amino acid residues. Fraction F5, which showed the lowest IC_{50} value, contained the tripeptides VPP and LxVL as well as the dipeptide LxY, which showed IC_{50} values between 2.1 and 18 μmol 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 (IC_{50}= 36 μmol L^{-1}), DAYPSGAW (IC_{50}=98 μmol L^{-1}) and VAV (IC_{50}= 260 μmol L^{-1}) (FitzGerald, & Meisel, 2000). The peptides LRF and IIA are precursors of well-known ACE-inhibitory peptides with whom they share the carboxy-terminal sequence, which is the most important for the inhibitory activity (Saito et al., 1994).

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 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; VLx and TLx in F3; HV in F5) have the potential to inhibit the ACE activity. Especially, the 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 (IC_{50} value of 12 μmol 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 (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 (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 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 lactotripeptides in pre-hypertensive and mildly hypertensive patients (Fekete, Givens, & Lovegrove,
394 2015; Turpeinen, Järvenpää, Kautiainen, Korpela, & Vapaatalo, 2013). Lactotripeptides 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 lactotripeptides are present in β-casein (VPP; fragment 84-86) and κ-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 ± 15.1 and 1829.8 ± 216.4 μg
404 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 β-casein in goat milk respect to κ-casein. According to previously
407 reported data, goat milk protein composition was 55-71% of β-casein and 15-20% of κ-casein
408 (Ruprichová et al., 2015).

409 Comparison with previously reported data pointed out that the release of the lactrotripeptides 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 β- and/or κ-casein. For example, because of the presence of IPP in
412 both cow and camel milk β- and κ-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 β-
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.

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