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# **Biological activities and peptidomic profile of in vitro-digested cow, camel, goat and sheep milks**

Davide Tagliazucchi<sup>1\*</sup>, Serena Martini<sup>1</sup>, Sherif Shamsia<sup>2</sup>, Ahmed Helal<sup>2</sup>, Angela Conte<sup>1</sup>

<sup>1</sup>Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2 - Pad. Besta, 42100 Reggio Emilia, Italy

<sup>2</sup>Department of Food and Dairy Sciences and Technology, Damanhour University, 22516 Damanhour, Egypt

1 **Abstract**

2 The present study was designed to compare *in vitro* digestibility, selected biological activities  
3 (antioxidant, angiotensin-converting enzyme (ACE)-inhibitory and dipeptidyl-peptidase-IV (DPP-  
4 IV)-inhibitory activities) and digested products of proteins from skimmed cow, camel, goat and  
5 sheep milks. The experimental approach combined the recently developed harmonized *in vitro*  
6 INFOGEST digestion model and mass spectrometry to identify peptides. Goat milk had the highest  
7 digestibility, while sheep milk showed the highest ACE-inhibitory activity after digestion. Cow  
8 milk was found to have the highest DPP-IV-inhibitory activity. A total of 522 peptides were  
9 identified after *in vitro* digestion of milks. Goat and sheep milk showed the highest similarity in  
10 peptide sequence with 151 common peptides. Thirteen, forty-three and twenty peptides with  
11 previously demonstrated antioxidant, ACE-inhibitory and DPP-IV-inhibitory activities were found  
12 in digested milks. Nineteen bioactive peptides in common were released from the different milks.  
13 Despite the limitations related to the analysis of one sample of milk for each species, possible  
14 differences in physiological functions after the ingestion of milk from different species are  
15 suggested by our results, however this requires confirmation by *in vivo* testing.

## 16 **1. Introduction**

17 Bioactive peptides have been defined as specific protein fragments that have a positive impact on  
18 body functions or conditions and may ultimately influence health (Rizzello et al., 2016). These  
19 peptides are inactive within the sequence of the parent protein and can be released under proteolytic  
20 conditions such as those in the gastro-intestinal tract or during food processing. These bioactive  
21 peptides potentially carry out their activity in the human body after the digestion process and once  
22 they are released from their original structure, and may act as regulatory compounds with hormone-  
23 like activity (Nongonierma & FitzGerald, 2015). The beneficial health effects of bioactive peptides  
24 include antimicrobial, antioxidative, dipeptidyl peptidase-IV (DPP-IV) and angiotensin-converting  
25 enzyme (ACE) inhibition, antihypertensive and immunomodulatory activities (Nongonierma &  
26 FitzGerald, 2015; Rizzello et al., 2016). Today, milk proteins are considered an important source of  
27 bioactive peptides and an increasing number of them have been identified in milk protein  
28 hydrolysates and fermented dairy products (Hernández-Ledesma, García-Nebot, Fernández-Tomé,  
29 Amigo, & Recio, 2014; FitzGerald, Murray, & Walsh, 2004; Nongonierma & FitzGerald, 2015;  
30 Egger, & Ménard, 2017).

31 Besides the well-known and most commonly consumed cow milk, a high consumption of milk of  
32 different origins (e.g. camel, goat and sheep milk) can be observed in other areas such as Asia,  
33 Africa and many eastern European countries. These alternative milks show high biological values,  
34 similar to those of cow milk, and are also used in the production of infant formulas or as a milk  
35 allergy-alternatives for those who suffer allergic reactions to cow milk (El-Agamy, Nawar,  
36 Shamsia, Awad, & Haenlein, 2009; Yadav, Singh, & Yadav, 2016).

37 Casein concentration is different between the different types of milk, whereas sheep milk has the  
38 highest concentration among cow, camel and goat milk (Park, Juárez, Ramos, & Haenlein, 2007).  
39 Moreover, the incidence of the four major caseins ( $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ -, and  $\kappa$ -caseins) is also different and  
40 related to the milk type (Tagliazucchi, Shamsia, Helal, & Conte, 2017). Divergence in the primary

41 structure of milk proteins across species may have an impact on the potential bioactivities of the  
42 released peptides.

43 The main bioactive peptides studied are those with antioxidant, ACE-inhibitory and DPP-IV  
44 inhibitory activities (Nongonierma & FitzGerald, 2015; Hernández-Ledesma, García-Nebot,  
45 Fernández-Tomé, Amigo, & Recio, 2014). In most cases, the active peptides were released by  
46 hydrolysis with individual proteases, such as pepsin, trypsin, papain, thermolysin or combination, or  
47 through the action of microbial enzymes during milk fermentation (Rizzello et al., 2016; Abd El-  
48 Salam, & El-Shibiny, 2017). Some recent studies addressed the release of bioactive peptides after *in*  
49 *vitro* digestion (Rutella, Solieri, Martini, Tagliazucchi, 2016; Tagliazucchi, Shamsia, & Conte,  
50 2016a; Egger, & Ménard, 2017; Tagliazucchi et al., 2017); however, there is a lack of information  
51 about the comparison between the bioactivities and the release of bioactive peptides from milks of  
52 different species after *in vitro* digestion. In addition, studies found in literature were focused on the  
53 release of ACE-inhibitory peptides and on determination of ACE-inhibitory activity of digested  
54 milks. For example, two recent studies applied the harmonized *in vitro* digestive system to study the  
55 release and fate of some ACE-inhibitory peptides, such as VPP, IPP, VY, HLPLPL during cow  
56 milk digestion (Kopf-Bolanz et al., 2014; Rutella et al., 2016). In two additional studies, 17 and 20  
57 bioactive peptides with ACE-inhibitory activity were found in camel and goat milk, respectively,  
58 subjected to the harmonized *in vitro* digestion (Tagliazucchi et al., 2016a and 2017). Moreover, a  
59 comparative analysis of the peptidomic profile of peptides released during *in vitro* digestion of  
60 different milk has never been reported until now.

61 Therefore, the present study was designed to compare *in vitro* digestibility, biological activities  
62 (antioxidant, ACE-inhibitory and DPP-IV-inhibitory activities) and digested products of proteins  
63 from skimmed cow, camel, goat and sheep milk employing a harmonized basic static *in vitro*  
64 digestive model, simulating human digestion and developed within the COST Action INFOGEST.

## 65 **2. Materials and methods**

### 66 *2.1. Materials*

67 All MS/MS reagents were from Bio-Rad (Hercules, CA, U.S.A.). Chemicals and enzymes for the  
68 digestion procedure, ACE and DPP-IV assays, antioxidant activity measurements and degree of  
69 hydrolysis determination were purchased from Sigma-Aldrich (Milan, Italy). Amicon Ultra-4  
70 regenerated cellulose filters with a molecular weight cut-off of 3 kDa were supplied by Millipore  
71 (Milan, Italy). The whole milk from camel, goat and sheep were obtained from farms at El-Alamin  
72 and Sidi-Barani areas around Alexandria (Egypt). Cow whole milk was obtained from a local  
73 producer (Reggio Emilia, Italy). All the other reagents were from Carlo Erba (Milan, Italy).

74

### 75 *2.2. Chemical analysis of skimmed cow, camel, goat and sheep milks*

76 Skimmed cow, camel, goat and sheep milks were prepared as reported in Tagliazucchi et al. (2017)  
77 and analysed for pH, fat, and lactose by phenol-sulphuric acid method, and total nitrogen, non-  
78 casein nitrogen by micro-Kjeldahl (Tagliazucchi et al. 2016a). Three analytical replicate for each  
79 milk sample were run for each assay.

80

### 81 *2.3. In vitro gastro-intestinal digestion of skimmed cow, camel, goat and sheep milks using the* 82 *harmonized protocol*

83 For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST  
84 was followed (Minekus et al., 2014) with minor modifications for adaptation to milk (Tagliazucchi,  
85 Helal, Verzelloni, Bellesia, & Conte, 2016b). The protocol consisted of three consecutive steps:  
86 oral, gastric and intestinal phases. The three steps were carried out in absence of light. Simulated  
87 salivary, gastric, and intestinal fluids (SSF, SGF and SIF) (Kopf-Bolanz et al., 2012) were  
88 employed for each step. First, oral digestion was performed by adding 12 mL of the stock SSF  
89 solution and 150 U mL<sup>-1</sup> of porcine  $\alpha$ -amylase to 9 mL of skimmed milk. The sample was shaken  
90 for 5 min at 37°C. Second, the gastric digestion step was carried out by adding to the bolus 24 mL

91 of SGF. The pH was adjusted to 2.0 with 6 mol L<sup>-1</sup> of HCl and supplemented with porcine pepsin  
92 (1115 U mL<sup>-1</sup> of simulated gastric fluid). After 2 h of incubation at 37°C, the final intestinal step  
93 was carried out by adding 36 mL of SIF (prepared by mixing 24 mL of pancreatic fluid and 12 mL  
94 of bile salts). Then, the pH was adjusted to 7.0, supplemented with pancreatin and the samples were  
95 incubated at 37°C for 2 h. All samples were immediately cooled on ice and frozen at -80°C for  
96 further analysis. The digestions were performed in triplicate. In addition, a control digestion, which  
97 included only the gastro-intestinal juices and enzymes, and water in place of milk, was carried out  
98 to consider the possible impact of the digestive enzymes in the subsequent analysis. For each  
99 digestion, aliquots were taken after 0 and 5 minutes of salivary digestion, after 30, 60 ,90 and 120  
100 minutes of gastric digestion and after 30, 60 ,90 and 120 minutes of intestinal digestion.

101

#### 102 *2.4. Assessment of protein hydrolysis during the digestion and preparation of the peptidic fractions* 103 *from digested cow, camel, goat and sheep milks*

104 Protein hydrolysis during the *in vitro* digestion was followed by measuring the amounts of released  
105 amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay and leucine as standard  
106 (Adler-Nissen, 1979). The obtained raw data were corrected by the contribution of the control  
107 digestion and normalised with respect to the initial content in proteins of the respective milk.

108 Data are expressed as mmol leucine equivalent g<sup>-1</sup> milk proteins and reported as a mean value and  
109 standard deviation from the three analytical replicates. Low molecular weight peptides were  
110 extracted by ultrafiltration (cut-off 3 kDa) from the post-pancreatic digested samples as described  
111 by Tagliazucchi et al. (2017). The peptide content in the peptidic fraction was determined by using  
112 the TNBS method as described above and expressing the results as mg of leucine equivalent mL<sup>-1</sup>.

113

#### 114 *2.5. Biological activities analysis*

115

##### 116 *2.5.1. Antioxidant activities analysis*

117 The antioxidant activity of the sample collected during the *in vitro* digestion procedure was  
118 determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) method as  
119 described in Re et al. (1999). The antioxidant properties of the peptidic fractions were evaluated  
120 using three different assays.

121 The ABTS assay was carried out as described above. The capacity to scavenge hydroxyl radicals  
122 was evaluated according to Tagliazucchi, Helal, Verzelloni, & Conte (2016c). In the assay, 50  $\mu\text{L}$  of  
123 appropriately diluted samples or standard (vitamin C) were mixed with 50  $\mu\text{L}$  of TPTZ (2,4,6-tri(2-  
124 pyridyl)-S-triazine) at a concentration of 3  $\text{mmol L}^{-1}$ , 50  $\mu\text{L}$  of 3  $\text{mmol L}^{-1}$   $\text{FeSO}_4$ , and 50  $\mu\text{L}$  of  
125 0.01% (v/v) hydrogen peroxide, in a clear bottom 96-well plate. The mixture was incubated for 1 h  
126 at 37°C, and the absorbance was measured at 540 nm using a microplate reader.

127 The ability to inhibit lipid peroxidation was carried out using a linoleic acid emulsion system  
128 (Tagliazucchi et al., 2016c). For that purpose, 200  $\mu\text{L}$  of sample (at a peptide concentration of 1 g  
129  $\text{L}^{-1}$ ) were added to 200  $\mu\text{L}$  of ethanol and 2.6  $\mu\text{L}$  of linoleic acid, and the total volume was adjusted  
130 to 500  $\mu\text{L}$  with sodium phosphate buffer, 50  $\text{mmol L}^{-1}$ , and pH 7.0. The mixture was incubated at  
131 40°C in the dark for a week. The amount of generated lipid hydroperoxide was measured by the  
132 FOX assay as reported by Tagliazucchi et al. (2010).

133 The obtained raw data were corrected by the contribution of the control digestion and normalised  
134 with respect to the initial content in proteins of the respective milk or to the peptide content in the  
135 peptidic fractions. ABTS scavenging capacity was expressed as  $\mu\text{mol}$  of vitamin C  $\text{g}^{-1}$  milk proteins  
136 or  $\mu\text{mol}$  vitamin C  $\text{g}^{-1}$  of peptides. Hydroxyl radical scavenging capacities was expressed as  $\mu\text{mol}$   
137 vitamin C  $\text{g}^{-1}$  of peptides. The lipid peroxidation inhibitory activity of the samples was expressed as  
138 percentage of inhibition with respect to a control reaction carried out in presence of the peptidic  
139 fraction of the control digestion.

140 Three analytical replicate were run for each sample in all the assays.

141

142 *2.5.2. Measurements of angiotensin-converting enzyme (ACE)-inhibitory activity*

143 ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983)  
144 using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate.  
145 For the calculation of the IC<sub>50</sub> value, the ACE assay was carried out in presence of different  
146 amounts of the milk peptidic fractions and the data were corrected for the contribution of the control  
147 digestion. IC<sub>50</sub> was defined as the concentration of peptides required to inhibit 50% of the  
148 enzymatic activity and expressed as µg of peptides mL<sup>-1</sup>. The IC<sub>50</sub> values were determined using  
149 nonlinear regression analysis and fitting the data with the log (inhibitor) vs. response model  
150 generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). For the enzymatic  
151 assay three analytical replicate were carried out.

152

153 *2.5.3. Measurements of dipeptidyl peptidase IV (DPP-IV)-inhibitory activity*

154 The enzyme DPP-IV was extracted from rat intestinal acetone powder. Namely, 100 mg of  
155 intestinal acetone powder was added to 3 mL of 0.1 mol L<sup>-1</sup> Tris-HCl pH 8.0 buffer and sonicated in  
156 a sonic bath (for 30 sec 4 times). After centrifugation at 10000g for 30 min, the resulting  
157 supernatant was directly analysed. For the calculation of the DPP-IV activity of the rat intestinal  
158 acetone extract, variable amounts of the extract (from 5 to 40 µL) were added to 5 µL of the  
159 substrate glycine-proline-p-nitroanilide (Gly-Pro-pNA 6.4 mmol L<sup>-1</sup>) and the 0.1 mol L<sup>-1</sup> Tris-HCl  
160 pH 8.0 buffer was added to reach 300 µL (final volume of the assay). After 10 min of incubation at  
161 37°C, the amount of release p-nitroanilide (pNA) was measured at 405 nm using a microplate  
162 reader. One unit of DPP-IV is defined as the quantity of enzyme that releases 1.0 µmol of pNA  
163 from Gly-Pro-pNA per minute at pH 8.0 at 37°C.

164 For the inhibition assay, in a 96-well plate 50 µL of diluted peptidic fractions, 235 µL of 0.1 mol L<sup>-1</sup>  
165 Tris-HCl pH 8.0 buffer and 10 µL of enzyme solution (0.1 U mL<sup>-1</sup>) were added. The reaction was  
166 initiated by the addition of 5 µL of substrate solution (Gly-Pro-pNA 6.4 mmol L<sup>-1</sup>). After 20 min of

167 incubation at 37°C, the amount of release p-nitroanilide (pNA) was measured at 405 nm using a  
168 microplate reader.

169 The concentration of peptides required to cause 50% inhibition of the DPP-IV activity (IC<sub>50</sub>) was  
170 determined by plotting the percentage of DPP-IV inhibition as a function of sample final  
171 concentration (natural logarithm). IC<sub>50</sub> values were expressed as mg of peptides mL<sup>-1</sup>. Data were  
172 corrected for the contribution of the control digestion. For the enzymatic assay three analytical  
173 replicate were carried out.

174

175 *2.6. Analysis of the peptidomic profile of peptidic fractions of cow, camel, goat and sheep milks by*  
176 *nanoflow liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with*  
177 *electrospray ionization (LC-ESI-QTOF MS)*

178 The peptidic fractions from digested cow, camel, goat and sheep milks were subjected to QTOF  
179 MS/MS analysis for peptide identification. Nano LC/MS and tandem MS experiments were  
180 performed on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520  
181 Accurate-Mass Q-TOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA,  
182 USA). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4 mm 40 nL  
183 enrichment column and a 43 mm × 75µm analytical column, both packed with a Zorbax 300SB 5  
184 µm C18 phase (Agilent Technologies).

185 For peptide identification, a non-targeted approach already optimized for the analysis of digested  
186 milk was applied as reported by Tagliacruzchi et al. (2016b). The mass spectrometer was tuned,  
187 calibrated and set with the same parameters as reported by Dei Più et al. (2014). This approach  
188 suffers of several limitations especially related to the detection and identification of small peptides  
189 and any peptide containing free cysteine (Fricker, 2015). Small peptides (<500 Da) are often  
190 inefficiently ionized giving a low intensity *m/z* signal which hampered the selection of precursor for  
191 successive MS/MS fragmentation. To overcome this problem, each digested milk was run twice by  
192 changing the range of precursor selection. In the first run MS/MS level experiments were acquired

193 using a 4 amu precursor selection width and  $m/z$  500–1700 scan range. To detect also small  
194 peptides, in the second run MS/MS level experiments were acquired using a 4 amu precursor  
195 selection width and  $m/z$  50–500 scan range. The database search approach also has its limitations.  
196 First, if the correct fragment is not derived from one of the proteins in the database, the search  
197 cannot provide the correct peptide identification. Secondly, the software commonly used for  
198 proteomic study and adapted for peptide identification, such as Mascot, have normally a  
199 minimum peptide length for identification of five residues and are not able to identify short peptides  
200 (Koskinen, Emery, Creasy, & Cottrell, 2011). Therefore, for the identification of peptides, we used  
201 a *de novo* sequencing software, which is able to identify also shorter peptide such as di- or tri-  
202 peptides.

203 For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de novo*  
204 peptide sequencing was performed using Pepnovo software  
205 (<http://proteomics.ucsd.edu/ProteoSAFe/>). The following parameters were considered: enzyme,  
206 none; peptide mass tolerance,  $\pm 40$  ppm; fragment mass tolerance,  $\pm 0.12$  Da; variable  
207 modifications, oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a  
208 single peptide, 3. A search for the biological activity of peptides identified was carried out through  
209 the BIOPEP and MBPDB databases (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008;  
210 Nielsen, Beverly, Qu, & Dallas, 2017).

211

## 212 2.7. Statistical analysis

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

### 217 **3. Results and discussion**

218

#### 219 *3.1 Comparison between the digestibility of cow, camel, goat and sheep milk proteins*

220 The chemical composition of skimmed cow, camel, goat and sheep milks is reported in **Table 1**.

221 Sheep milk contained significant higher ( $P<0.05$ ) amount of total proteins and caseins respect to the  
222 other milks. The content in total proteins and caseins was not significant different between cow,  
223 camel and goat milks. Indeed, no significant statistical differences were observed between the total  
224 whey proteins and lactose content as well as the pH value of the different milk.

225 The degradation of milk proteins by gastro-intestinal proteolytic enzymes was compared by  
226 measuring the amount of released free amino groups using TNBS assay (**Figure 1**). As expected,  
227 the amount of free amino groups before the digestion (corresponding to the time 0 of the salivary  
228 phase of digestion) was not significantly different between the different milk and remained constant  
229 during the 5 minutes of salivary incubation. An increase in the hydrolysis was observed for milk of  
230 different species during gastric digestion. After 30 minutes of gastric digestion the amount of free  
231 amino groups released from goat milk was significantly higher ( $P<0.001$ ) than that released from  
232 cow, camel and sheep milk. No significant statistical differences were observed between the milk  
233 from sheep and camel, whereas cow milk showed significantly less amino groups than the other  
234 milks ( $P>0.05$ ). The amount of released amino groups increased slightly but not significantly  
235 during the subsequent 90 minutes of peptic digestion in all the milks. The transition from gastric to  
236 pancreatic environment produced a significant increase in the amount of free amino groups in all  
237 the digested milks. Subsequently, the quantity of released amino groups showed a tendency to  
238 gradually increase during the entire pancreatic phase of the digestion. At the end of the digestion,  
239 goat milk showed a significant higher amount of released amino groups ( $P<0.001$ ) compared to  
240 camel, cow and sheep milks. No significant differences were found between the amount of free  
241 amino groups released from cow, camel and sheep milk ( $P>0.05$ ). These results showed that  
242 gastric and duodenal enzymes degraded goat milk proteins faster and more efficiently than camel,

243 cow and sheep milk. These conclusions are supported by comparison with previously published  
244 data. For example, Almaas et al. (2006) found that goat milk proteins were degraded faster than  
245 cow milk using human gastro-intestinal proteolytic enzymes. On the other hand, Salami et al.  
246 (2008) found that the extent of hydrolysis of camel caseins with pancreatic enzymes was greater  
247 than that of cow caseins. Digestion of camel, cow and goat milk with the same protocol used in this  
248 study resulted in a higher digestibility of goat milk respect to camel and cow milk (Rutella et al.,  
249 2016; Tagliazucchi, et al., 2016a; Tagliazucchi et al., 2017).

250 The different enzyme-to-substrate ratio during the digestion, especially in the case of sheep milk,  
251 which showed the highest initial protein content, may have had an impact on the hydrolysis of milk  
252 proteins. Espejo-Carpio, Pérez-Gálvez, Guadix and Guadix (2013) reported an increase in the  
253 digestibility of goat milk proteins as a function of the enzyme-to-substrate ratio. The lower  
254 digestibility of sheep milk proteins can be partially attributed to the lower enzyme-to-substrate ratio  
255 respect to the other digested milks.

256

### 257 *3.2 Evolution of antioxidant activity during in vitro digestion and antioxidant properties of the post-* 258 *pancreatic peptidic fractions*

259 The variation in antioxidant activity during the digestion of the different milk was followed by the  
260 ABTS assay and reported in **Figure 2**.

261 All the studied milk showed ABTS radical scavenging activity before the digestion (corresponding  
262 to the time 0 of the salivary phase of digestion), but with some differences (**Figure 2**). Sheep milk  
263 had a significant higher ABTS radical scavenging activity with respect to the other milks ( $P<0.05$ ),  
264 whereas cow milk showed the lowest ABTS radical scavenging activity. Clausen, Skibsted, &  
265 Stagsted (2009) found that caseins are quantitatively the highest radical scavengers in milk whereas  
266 the lower contribution of the low molecular weight compounds is due to ascorbate and especially  
267 urate. Caseins have a high content of antioxidative amino acids such as tyrosine, tryptophan and  
268 phosphoserine, and quenching of free radicals by oxidation of these amino acids was proposed as

269 the explanation (Clausen et al. 2009). As expected, the ABTS radical scavenging activity of the  
270 different milk remained constant during the 5 minutes of salivary incubation, whereas the ABTS  
271 radical scavenging activity rose as the digestion proceeded reaching the highest value at the end of  
272 the pancreatic phase of the digestion in all the analysed milks (**Figure 2**). This can be explained by  
273 an increased number of peptides and amino acids at higher hydrolysis available for interaction with  
274 the ABTS radical as already reported (De Gobba, Espejo-Carpio, Skibsted, & Otte, 2014; Kumar,  
275 Chatli, Singh, Mehta, & Kumar, 2016). On the other hand, previous studies reported an increase in  
276 radical scavenging activity of cow, goat and human milk after *in vitro* digestion (Tsopmo, et al.,  
277 2009; Nehir et al., 2015; Power Grant et al., 2016; Tagliacruzchi et al., 2016c). Comparison of the  
278 data at the end of the digestion showed that sheep and goat milk displayed the highest ABTS radical  
279 scavenging activity ( $P>0.05$ ) followed by cow ( $P<0.001$ ) and camel ( $P<0.001$ ) milk.

280 Hernández-Ledesma, Amigo, Recio and Bartolomé (2007) found that an equimolar free amino acids  
281 mixture had low antioxidant activity compared to those of the corresponding peptides. Accordingly,  
282 extensive hydrolysis, resulting in an increased amount of free amino acids, should bring about to a  
283 lower antioxidant activity. However, digested sheep and goat milks showed the highest ABTS  
284 radical scavenging activity but goat milk showed the highest digestibility whereas sheep milk the  
285 lowest. Therefore, the ABTS radical scavenging activity of digested milk seems more related to the  
286 specificity and amount of formed peptides than to the extent of hydrolysis.

287 To fully characterize the antioxidant properties of the digested milk and to evaluate the impact of  
288 the released peptides, peptidic fractions were further extracted from the post-pancreatic digested  
289 samples through ultrafiltration with a cut-off of 3 kDa and evaluated for their ABTS radical  
290 scavenging activity and for their ability to scavenge hydroxyl radical and to inhibit lipid  
291 peroxidation. The data regarding the antioxidant properties of the peptidic fractions of the post-  
292 pancreatic samples are reported in **Table 2**, together with the peptide content. The amount of  
293 released peptides after pancreatic digestion was not significantly different between cow, camel and  
294 goat milk whereas sheep milk digestion resulted in a release of significantly greater amount of

295 peptides. Normalizing the data for the peptide content, it was possible to compare the antioxidant  
296 capacity of the peptidic fractions of the different milks. All of the peptidic fractions exhibited a  
297 certain degree of ABTS and hydroxyl scavenging activity. ABTS radical scavenging activity of the  
298 peptidic fractions of sheep, goat and cow milk was not significantly different whereas camel milk  
299 peptidic fraction showed the lowest ABTS radical scavenging activity (**Table 2**). Peptidic fraction  
300 from cow milk was the most active against hydroxyl radical whereas fractions from goat and sheep  
301 milk showed the highest lipid peroxidation inhibitory activity (**Table 2**). The distinct antioxidant  
302 properties of the gastro-intestinal digested peptidic fractions should be mainly attributed to the  
303 specificity of the peptides released from the sequences of the protein present in the different milk.  
304

### 305 *3.3 ACE-inhibitory activity of the post-pancreatic peptidic fractions*

306 The ACE-inhibitory activity obtained for the peptidic fractions of the post-pancreatic samples were  
307 expressed as IC<sub>50</sub> (defined as the peptide concentration required to inhibit 50% of the ACE activity)  
308 and ranged from 625.4 ± 60.6 to 2396.5 ± 135.0 µg of peptides mL<sup>-1</sup> (**Table 2**). The hydrolysates  
309 produced by the action of digestive enzymes on sheep milk exhibited the highest ACE inhibitory  
310 activity whereas cow milk peptidic fraction showed the lowest inhibitory activity (**Table 2**).

311 The different enzyme-to-substrate ratio in the case of sheep milk could have partially influenced the  
312 ACE-inhibitory activity of the peptidic fraction of digested sheep milk. Enzymatic hydrolysis can  
313 generate ACE-inhibitory peptides whereas further degradation of the peptides into much smaller  
314 fragments may result in a decrease in the ACE-inhibitory activity (Tagliazucchi et al., 2017).

315 Therefore, the lower digestibility of sheep milk could result in a lower amount of short peptides and  
316 a highest ACE-inhibitory activity. Previous reported data showed that the digestion of camel and  
317 goat milk, using the same harmonized *in vitro* model and the same ACE assay, resulted in an IC<sub>50</sub>  
318 value comparable with that found in this study (Tagliazucchi et al., 2016a; Tagliazucchi et al.,  
319 2017).

320

321 *3.4 DPP-IV-inhibitory activity of the post-pancreatic peptidic fractions*

322 Digests from cow, camel, goat and sheep milk showed DPP-IV inhibitory activity in the *in*  
323 *vitro* assay (**Table 2**). A dose dependent inhibition was observed for all digests but some differences  
324 were noted. Cow milk post-pancreatic peptidic fraction had the lowest IC<sub>50</sub> value against DPP-IV  
325 ( $6.9 \pm 0.1$  mg peptides mL<sup>-1</sup>), which means the highest inhibitory activity. The other digested milks  
326 showed a DPP-IV inhibitory power from 2.2 to 2.5 times lower than cow milk, with digested camel  
327 milk having a significant lower inhibitory activity than digested goat milk.

328 The different DPP-IV-inhibitory activity of the digested milks is probably related to differences in  
329 the amount and/or type of released peptides. However, at least in the case of sheep milk, the  
330 relatively low DPP-IV inhibitory potency may be partially linked with the lowest enzyme-to-  
331 substrate ratio, which resulted in a lower extent of hydrolysis respect to the other milks  
332 (Nongonierma, Mazzocchi, Paoletta, & FitzGerald, 2017a).

333 The hydrolysates generated herein with cow, camel, goat and sheep milk proteins exhibited  
334 higher DPP-IV IC<sub>50</sub> values than those reported in the literature with cow whey proteins and caseins  
335 (Nongonierma & FitzGerald, 2013; Power Grant, Fernández, Norris, Riera, & FitzGerald, 2014),  
336 caprine caseins (Zhang et al., 2016) or camel milk proteins (Nongonierma, Paoletta, Mudgil,  
337 Maqsood, & FitzGerald, 2017b) hydrolysed with trypsin. Lower IC<sub>50</sub> values were also obtained  
338 after *in vitro* digestion of cow milk protein concentrate and skimmed milk powder and camel milk  
339 (Lacroix & Li-Chan, 2012; Nongonierma, et al., 2017b).

340

341 *3.5. Peptidomic profile of in vitro digested cow, camel, goat and sheep milk peptidic fractions and*  
342 *identification of antioxidant, ACE-inhibitory and DPP-IV-inhibitory peptides*

343 The nano-LC-MS/MS system identified 522 peptides from the digested samples. In particular, 119,  
344 76, 164, and 163 peptides were identified in digested cow, camel, goat, and sheep milk, respectively  
345 (see online supplementary **Tables S1-S8**). This work reveals higher numbers of peptides released  
346 after *in vitro* digestion of camel and goat milk than previously reported using the same harmonized

347 protocol. Our previous research identified 65 and 50 peptides in digested camel and goat milk,  
348 respectively (Tagliacruzchi et al., 2016a; Tagliacruzchi et al., 2017). Concerning cow milk, several  
349 studies have already found higher amount of peptides (more than 119) than this study (Egger et al.,  
350 2016; Picariello et al., 2010). To the best of our knowledge, this is the first paper reporting a  
351 comprehensive peptidomic profile of digested sheep milk.

352 The majority of the peptides were from caseins (71.4, 73.7, 72.0 and 71.2% of the total identified  
353 peptides in digested cow, camel, goat and sheep milk, respectively) with  $\beta$ -casein which was the  
354 best source of peptides in all the digested milk (43.7, 51.3, 42.7 and 40.5% of the total identified  
355 peptides in digested cow, camel, goat and sheep milk, respectively). Whey proteins gave a lower  
356 amount of peptides respect to caseins, especially in camel milk that does not contain  $\beta$ -lactoglobulin  
357 (see online supplementary **Tables S1-S8**). In addition, 9 amino acids were also identified, 7 of them  
358 being essential amino acids (W, L, I, V, K, R and F).

359 The Venn diagram (**Figure 3A**) showed that 26, 35, 5, and 8 peptides were specific for *in vitro*  
360 digested cow, camel, goat, and sheep milk, respectively. Only 26 identified peptides were common  
361 for all the four digested milk, whereas goat and sheep milk showed the highest similarity in peptide  
362 sequences with 151 common peptides. Among them, 81 were in common also with cow milk and  
363 33 with camel milk, whereas 63 peptides were found only in goat and sheep digested milk.

364 **Tables 3-5** display the identified peptides with previously reported antioxidant, ACE-inhibitory and  
365 DPP-IV-inhibitory activities. In this study, 26 identified bioactive peptides are from  $\beta$ -casein, 8  
366 from  $\alpha_{S1}$ -casein, 4 from  $\alpha_{S2}$ -casein and 4 from  $\kappa$ -casein. Only 3 bioactive peptides were released  
367 from whey proteins (two from  $\beta$ -lactoglobulin and one from  $\alpha$ -lactalbumin). Finally, 19 peptides  
368 ranging from two to three amino acids arose from various milk proteins.

369 The Venn diagram (**Figure 3B**) showed that 19 identified bioactive peptides were common for all  
370 the four digested milks. The cow milk was the one that gave the highest number of unique bioactive  
371 peptides (8 specific peptides), whereas goat and sheep milk still showed the highest similarity in  
372 bioactive peptide sequences with 48 common peptides.

373 Three amino acids and 13 peptides with previously reported antioxidant properties were identified  
374 in the peptidic fraction of digested milk (**Table 3**). Some peptides such as VY and LK were found  
375 in the peptidic fractions of all the digested milk whereas others peptides were found only in specific  
376 fractions. In general, the majority of peptides with previously reported ABTS radical scavenging  
377 activity were found in digested goat and sheep milk, which showed the highest ABTS radical  
378 scavenging activity. On the contrary, camel milk peptidic fraction showed the lowest ABTS radical  
379 scavenging activity and contained the lowest number of ABTS radical scavenging peptides (**Tables**  
380 **2 and 3**). Three free amino acids (tryptophan, tyrosine and phenylalanine) with previously reported  
381 antioxidant properties were also identified in all the peptidic fractions of digested milk. These  
382 amino acids had been previously suggested as the major contributors to the antioxidant activity of  
383 digested human and cow milk (Tsopmo et al., 2009; Tagliazucchi et al., 2016c). In general, the  
384 presence of an antioxidant amino acid seems to be fundamental for the antioxidant properties of a  
385 peptide (Babini, Tagliazucchi, Martini, Dei Più, & Gianotti, 2017). As reported in the on line  
386 supplementary **Tables S1-S8**, several tyrosine- and tryptophan-containing peptides were found in  
387 the digested milk, which can contribute to the ABTS and hydroxyl radical scavenging activity of the  
388 peptidic fractions of milk.

389 In this study, 43 peptides identified presented ACE inhibition (**Table 4**). Some identified ACE-  
390 inhibitory peptides have very low IC<sub>50</sub> values and could be the primary contributors to the ACE-  
391 inhibitory activity of the digested milk (Matsufuji et al., 1994; Nakamura, Yamamoto, Sakai, &  
392 Takano, 1995; Kim, Byun, Park, & Shahidi, 2001; Quiros et al., 2007; Kaiser et al., 2016;  
393 Tagliazucchi et al., 2016a). Despite the differences in the ACE-inhibitory activity of the digested  
394 milk (**Table 2**) there is no clear species-specific release of ACE-inhibitory peptides. Probably, the  
395 diverse activity of the digested milk reflects differences in the amount of released ACE-inhibitory  
396 peptides. Three released peptides, namely VPP (identified in the digested cow, goat and sheep  
397 milk), IPP (identified in the digested milk of all the studied species) and WL (identified only in  
398 digested cow milk) have demonstrated anti-hypertensive activity in humans. In particular, the

399 lactotriptides VPP and IPP have been shown (at dosages between 5 and 100 mg day<sup>-1</sup>) to  
400 decrease the systolic (4.0 mmHg) and diastolic (1.9 mmHg) blood pressure in hypertensive patients  
401 and to positively modulate pulse wave velocity in mildly hypertensive subjects (Cicero, Fogacci, &  
402 Colletti, 2017). Two recent studies showed that VPP and IPP could be released from cow and goat  
403 milk during *in vitro* digestion at doses, which can elicit physiological effects (Rutella et al., 2016;  
404 Tagliazucchi et al., 2017). The  $\alpha$ -lactalbumin derived dipeptide WL was found to be bioavailable in  
405 human subjects, reducing *in vivo* ACE activity (Kaiser et al., 2016). One additional peptide  
406 (LHLPLP) was found to be able to decrease systolic and diastolic blood pressure in spontaneously  
407 hypertensive rats (Quiros et al., 2007). Some other peptides with very low IC<sub>50</sub> values (IY, VF and  
408 LPP) have been found in plasma of human volunteers after consumption of dairy products (van  
409 Platerink, Janssen, Horsten, & Haverkamp, 2006; Foltz et al., 2007). The peptide VY seems to be  
410 particularly interesting, behaving as a multifunctional bioactive peptide with high ACE-inhibitory  
411 and antioxidant activities (Cheng, Che, Xiong, 2010; Tagliazucchi et al., 2016a). The release of VY  
412 was common in milk from the different species studied. VY has been also found in human plasma  
413 after consumption of a milk beverage, indicating that this peptide is also released *in vivo* from cow  
414 milk caseins and is bioavailable in humans (Foltz et al., 2007).

415 Finally, 20 peptides with previously demonstrated DPP-IV-inhibitory activity were identified in the  
416 peptidic fractions of digested milk (**Table 5**). Cow milk was the best source of DPP-IV-inhibitory  
417 peptides (17 out of 20) and the sample with the highest DPP-IV-inhibitory activity (**Table 2**). Two  
418 well-known DPP-IV inhibitors, namely IPI (also known as Diprotin A) and VLP (also known as  
419 Diprotin B), were released from cow milk after digestion and could be the primary contributor to the  
420 DPP-IV-inhibitory activity of the digested cow milk (**Table 5**). Diprotin A but not Diprotin B was  
421 also found in the peptidic fractions of digested goat and sheep milk.

422

#### 423 **4. Conclusion**

424 The present study integrated peptides identified by LC-MS/MS with *in vitro* bioactivities of milk  
425 from four different species (cow, camel, goat and sheep) after the application of the harmonized  
426 INFOGEST *in vitro* gastro-intestinal digestion protocol. Whereas goat milk showed the highest  
427 apparent digestibility, sheep milk appeared to be the best source of ACE-inhibitory peptides.  
428 Moreover, cow milk was found to be the best source of DPP-IV-inhibitory peptides and antioxidant  
429 peptides and amino acids. Peptidomic analysis showed that goat and sheep milk displayed the  
430 highest similarity in peptide sequences identified after *in vitro* digestion. Most of the released  
431 bioactive peptides were in common between two or more species and the peptides with the highest  
432 ACE-inhibitory activity had previously demonstrated to be bioavailable in humans.  
433 Although this study lays the basis to distinguish milk from different species in the light of their  
434 bioactivities and bioactive peptides released during *in vitro* digestion, limitations have to be  
435 considered. The most important is that this research was conducted analysing one sample of milk  
436 from each species. Therefore, to expand the results, studies involving more milk samples are  
437 required. Finally, further investigations and *in vivo* trials are needed to establish which of the  
438 observed bioactive peptides have physiological significance.

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## Figure captions

**Figure 1. Comparison between the *in vitro* digestibility of skimmed cow, camel, goat and sheep milk.** Release of free amino groups during *in vitro* gastric and pancreatic digestion of skimmed cow (□), camel (▤), goat (▥) and sheep (▧) milk. Data were corrected by the contribution of the control digestion and normalised with respect to the initial protein content of the different milks studied and expressed as mmol of leucine equivalent per g of protein. Values are means of three independent digestions ± standard deviation (SD). Different letters indicate significantly different values ( $P<0.05$ ).

**Figure 2. Evolution of ABTS radical scavenging activity during the *in vitro* digestion of skimmed cow (□), camel (▤), goat (▥) and sheep (▧) milk.** ABTS radical scavenging activity was expressed as  $\mu\text{mol}$  of vitamin C  $\text{g}^{-1}$  of milk protein. Data were corrected by the contribution of the control digestion and normalised with respect to the initial protein content of the different milks studied. Values are means of three independent digestions ± standard deviation (SD). Different letters indicate significantly different values ( $P<0.05$ ).

**Figure 3. Venn diagrams of peptides obtained from skimmed cow, camel, goat and sheep milk.** (A) Venn diagram created with all the identified peptides released after *in vitro* gastro-

intestinal digestion (see on line supplementary material **Tables S1-S8** for the peptide sequences).

(B) Venn diagram created with only the bioactive peptides released and identified after *in vitro* gastro-intestinal digestion (see **Tables 3-5** for the peptide sequences and bioactivity).

**Table 1.** Chemical composition of skimmed cow, camel, goat and sheep milks. Data are expressed as g 100 g<sup>-1</sup> of milk.

	<i>Cow milk</i>	<i>Camel milk</i>	<i>Goat milk</i>	<i>Sheep milk</i>
<i>Total proteins</i>	3.60 ± 0.11 <sup>a</sup>	3.48 ± 0.14 <sup>a</sup>	3.78 ± 0.12 <sup>a</sup>	5.68 ± 0.21 <sup>b</sup>
<i>Caseins</i>	2.88 ± 0.07 <sup>a</sup>	2.68 ± 0.10 <sup>a</sup>	2.92 ± 0.09 <sup>a</sup>	4.76 ± 0.18 <sup>b</sup>
<i>Whey proteins</i>	0.72 ± 0.04 <sup>a</sup>	0.80 ± 0.08 <sup>a</sup>	0.86 ± 0.10 <sup>a</sup>	0.92 ± 0.08 <sup>a</sup>
<i>Lactose</i>	4.80 ± 0.18 <sup>a</sup>	4.87 ± 0.15 <sup>a</sup>	4.55 ± 0.14 <sup>a</sup>	4.74 ± 0.12 <sup>a</sup>
<i>Fat</i>	< 0.05	< 0.05	< 0.05	< 0.05
<i>pH</i>	6.65 ± 0.03 <sup>a</sup>	6.61 ± 0.05 <sup>a</sup>	6.64 ± 0.05 <sup>a</sup>	6.67 ± 0.04 <sup>a</sup>

Values represent means ± standard deviation of triplicate determination; different superscript letters within the same row indicate that the values are significantly different ( $P < 0.05$ ).

**Table 2.** Radical scavenging properties, lipid peroxidation inhibitory activity, and angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPPIV) inhibitory activities of peptidic fractions (< 3 kDa) obtained from cow, camel, goat and sheep milks after *in vitro* gastro-intestinal digestion.

<i>Peptidic fractions (&lt; 3 kDa)</i>	<i>Peptide content (mg mL<sup>-1</sup>)</i>	<i>ABTS radical scavenging (μmol vitamin C g<sup>-1</sup> of peptides)</i>	<i>Hydroxyl radical scavenging (μmol vitamin C g<sup>-1</sup> of peptides)</i>	<i>Inhibition of lipid peroxidation (% inhibition<sup>a</sup>)</i>	<i>ACE-inhibition IC<sub>50</sub> (μg peptides mL<sup>-1</sup>)</i>	<i>DPPIV-inhibition IC<sub>50</sub> (mg peptides mL<sup>-1</sup>)</i>
<i>Cow milk</i>	23.2 ± 1.3 <sup>a</sup>	2016.7 ± 441.6 <sup>a,b</sup>	25.5 ± 1.6 <sup>a</sup>	80.2 ± 6.0 <sup>a</sup>	2396.5 ± 135.0 <sup>a</sup>	6.9 ± 0.1 <sup>a</sup>
<i>Camel milk</i>	21.7 ± 0.5 <sup>a</sup>	1513.0 ± 98.0 <sup>b</sup>	11.9 ± 1.8 <sup>b</sup>	78.2 ± 2.5 <sup>a</sup>	1748.2 ± 13.1 <sup>b</sup>	17.2 ± 0.8 <sup>b</sup>
<i>Goat milk</i>	22.8 ± 1.4 <sup>a</sup>	2243.7 ± 450.7 <sup>a,b</sup>	11.6 ± 2.2 <sup>b</sup>	92.6 ± 2.9 <sup>b</sup>	1156.3 ± 10.5 <sup>c</sup>	15.3 ± 0.8 <sup>c</sup>
<i>Sheep milk</i>	34.3 ± 1.7 <sup>b</sup>	2592.6 ± 291.6 <sup>a</sup>	11.2 ± 0.6 <sup>b</sup>	93.1 ± 0.8 <sup>b</sup>	625.4 ± 60.6 <sup>d</sup>	16.3 ± 1.2 <sup>b,c</sup>

Values represent means ± standard deviation of triplicate determination; different superscript letters within the same column indicate that the values are significantly different ( $P < 0.05$ ).

<sup>a</sup>% of inhibition was determined using the < 3 kDa fractions of the post-pancreatic sample at a concentration of 1 g L<sup>-1</sup> of peptides

**Table 3.** Peptides and amino acids with previously described antioxidant properties identified in the peptidic fractions (< 3 kDa) obtained from cow, camel, goat and sheep milk after *in vitro* gastro-intestinal digestion.

<i>Sequence</i>	<i>Activity</i>	<i>Sample<sup>a</sup></i>	<i>Protein<sup>b</sup></i>
F	Hydroxyl radical scavenging Inhibition of lipid peroxidation	Co, Ca, G, S	Various proteins
Y	ABTS radical scavenging Hydroxyl radical scavenging Inhibition of lipid peroxidation	Co, Ca, G, S	Various proteins
W	ABTS radical scavenging Hydroxyl radical scavenging Inhibition of lipid peroxidation	Co, Ca, G, S	Various proteins
IY	ABTS radical scavenging	Ca, G, S	Various proteins
LK	Peroxyl radical scavenging	Co, Ca, G, S	Various proteins
LW	Hydroxyl radical scavenging	Co, G, S	$\alpha_{S1}$ -casein
LY	ABTS radical scavenging	Ca, G, S	Various proteins
VY	ABTS radical scavenging Inhibition of lipid peroxidation	Co, Ca, G, S	Various proteins
FPQ	Inhibition of lipid peroxidation	Co, G, S	$\alpha_{S2}$ -casein
YLG	Peroxyl radical scavenging	Co, G, S	$\alpha_{S1}$ -casein
AWPQ	ABTS radical scavenging	G, S	$\alpha_{S2}$ -casein
VYPF	ABTS radical scavenging Inhibition of lipid peroxidation	G, S	$\beta$ -casein
YIPI	ABTS radical scavenging	Co, G, S	$\kappa$ -casein
YLPL	ABTS radical scavenging	G, S	$\alpha_{S1}$ -casein
YVEEL	Peroxyl radical scavenging	G, S	$\beta$ -lactoglobulin
YQEPVLG	ABTS radical scavenging	G, S	$\beta$ -casein

<sup>a</sup>Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)

<sup>b</sup>Precursor protein

**Table 4.** Peptides with previously described angiotensin-converting enzyme (ACE)-inhibitory activity identified in the peptidic fractions (< 3 kDa) obtained from cow, camel, goat and sheep milk after *in vitro* gastro-intestinal digestion. Peptides are listed on the basis of their inhibitory potency.

<i>Sequence</i>	<i>IC<sub>50</sub><sup>a</sup></i> <i>μmol L<sup>-1</sup></i>	<i>Sample<sup>b</sup></i>	<i>Protein<sup>c</sup></i>
IY	2.1	Ca, G, S	Various proteins
GPV	4.7	Co, G, S	β-casein
IPP	5.0	Co, Ca, G, S	β-casein
LHLPLP	5.8	Co, Ca, G, S	β-casein
VY	7.1	Co, Ca, G, S	Various proteins
VPP	9.0	Co, G, S	β-casein
VF	9.2	Co, Ca, G, S	Various proteins
LPP	9.6	Co, Ca	β-casein
WL	10	Co	α-lactalbumin
FVAP	10	Co	α <sub>S1</sub> -casein
LW	15	Co, G, S	α <sub>S1</sub> -casein
TF	18	Ca	Various proteins
HLPLP	21	Co, Ca, G, S	β-casein
VIP	26	Co, Ca	α <sub>S2</sub> -casein
IAV	27	G, S	α <sub>S1</sub> -casein
LY	44	Ca, G, S	Various proteins
IVP	50	Co	α <sub>S1</sub> -casein
IL	55	Co, Ca, G, S	Various proteins
RPK	92	Co, Ca, G, S	α <sub>S1</sub> -casein
YL	122	Ca, G, S	Various proteins
IP	130	Co, Ca, G, S	Various proteins

IPA	141	Co, S	Various proteins
LVYP	170	G, S	$\beta$ -casein
FP	205	Co, G, S	Various proteins
PYP	220	Co, Ca, G, S	$\kappa$ -casein
VAV	260	G, S	$\kappa$ -casein
ILP	270	Ca	$\beta$ -casein
VYP	288	Co, G, S	$\beta$ -casein
LNVPGEIVE	300	Co	$\beta$ -casein
TGPIP	316	G, S	$\beta$ -casein
VLP	320	Co, Ca	$\beta$ -casein
SLPQ	330	Co, G, S	$\beta$ -casein
PL	337	Co, Ca, G, S	Various proteins
AVP	340	Co	$\beta$ -casein
LF	349	G, S	Various proteins
VP	420	Co, Ca, G, S	Various proteins
VYFPFGPI	500	Co	$\beta$ -casein
NILP	560	G, S	$\beta$ -casein
EMPFPK	565	Co, G, S	$\beta$ -casein
YP	720	Co, Ca, G, S	Various proteins
LPLP	720	Co, Ca, G, S	$\beta$ -casein
ALPM	928	Co, G, S	$\beta$ -lactoglobulin
IF	930	G	$\alpha_{S2}$ -casein

<sup>a</sup>IC<sub>50</sub> is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity. The values are from BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

<sup>b</sup>Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)

<sup>c</sup>Precursor protein

**Table 5.** Peptides with previously described dipeptidyl peptidase IV (DPP-IV)-inhibitory activity identified in the peptidic fractions (< 3 kDa) obtained from cow, camel, goat and sheep milk after *in vitro* gastro-intestinal digestion. Peptides are listed on the basis of their inhibitory potency.

<i>Sequence</i>	<i>IC<sub>50</sub><sup>a</sup></i> <i>μmol L<sup>-1</sup></i>	<i>Sample<sup>b</sup></i>	<i>Protein<sup>c</sup></i>
IPI	3.5	Co, G, S	κ-casein
VPL	16	Co	α <sub>S1</sub> -casein
WL	43.6	Co	α-lactalbumin
LPVPQ	44	Co, Ca, G, S	β-casein
IPA	49	Co, S	Various proteins
VL	74	Co, Ca, G, S	Various proteins
LPQ	82	Co, Ca, G, S	Various proteins
YPVEPF	125	Co, G, S	β-casein
IP	150	Co, Ca, G, S	Various proteins
LPL	241	Co, Ca, G, S	β-casein
LPLPL	325	Ca, G, S	β-casein
MHQPPQPL	350	G	β-casein
FP	363	Co, G, S	Various proteins
FL	400	G, S	Various proteins
PQNIPPL	500	Co	β-casein
YP	658	Co, Ca, G, S	Various proteins
LP	712	Co, Ca, G, S	Various proteins
GPV	795	Co, G, S	β-casein
VP	880	Co, Ca, G, S	Various proteins
LW	993	Co, G, S	α <sub>S1</sub> -casein

<sup>a</sup>IC<sub>50</sub> is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity. The values are from BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

<sup>b</sup>Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)

<sup>c</sup>Precursor protein





