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

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Abstract

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

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

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

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

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

of SGF. The pH was adjusted to 2.0 with 6 mol L^{-1} of HCl and supplemented with porcine pepsin (1115 U mL^{-1} of simulated gastric fluid). After 2 h of incubation at 37°C, the final intestinal step was carried out by adding 36 mL of SIF (prepared by mixing 24 mL of pancreatic fluid and 12 mL of bile salts). Then, the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at 37°C for 2 h. All 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 preparation of the peptidic fractions from digested cow, camel, goat and sheep milks

Protein hydrolysis during the *in vitro* digestion was followed by measuring the amounts of released amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay and leucine as standard (Adler-Nissen, 1979). The obtained raw data were corrected by the contribution of the control digestion and normalised with respect to the initial content in proteins of the respective milk. Data are expressed as mmol leucine equivalent g^{-1} milk proteins and reported as a mean value and standard deviation from the three analytical replicates. Low molecular weight peptides were extracted by ultrafiltration (cut-off 3 kDa) from the post-pancreatic digested samples as described by Tagliazucchi et al. (2017). The peptide content in the peptidic fraction was determined by using the TNBS method as described above and expressing the results as mg of leucine equivalent mL^{-1}.

2.5. Biological activities analysis

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 µL of
123 appropriately diluted samples or standard (vitamin C) were mixed with 50 µL of TPTZ (2,4,6-tri(2-
124 pyridyl)-S-triazine) at a concentration of 3 mmol L^{-1}, 50 µL of 3 mmol L^{-1} $FeSO_4$, and 50 µL 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 µL of sample (at a peptide concentration of 1g
129 L^{-1}) were added to 200 µL of ethanol and 2.6 µL of linoleic acid, and the total volume was adjusted
130 to 500 µL with sodium phosphate buffer, 50 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 µmol of vitamin C g^{-1} milk proteins
136 or µmol vitamin C g^{-1} of peptides. Hydroxyl radical scavenging capacities was expressed as µmol
137 vitamin C 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_{50} 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_{50} was defined as the concentration of peptides required to inhibit 50% of the
148 enzymatic activity and expressed as μg of peptides mL^{-1}. The IC_{50} 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^{-1} Tris-HCl pH 8.0 buffer and sonicated in
156 a sonic bath (for 30 sec 4 times). After centrifugation at 10000*g* 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^{-1}) and the 0.1 mol L^{-1} 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^{-1}
165 Tris-HCl pH 8.0 buffer and 10 μL of enzyme solution (0.1 U mL^{-1}) were added. The reaction was
166 initiated by the addition of 5 μL of substrate solution (Gly-Pro-pNA 6.4 mmol L^{-1}). 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_{50}) was
170 determined by plotting the percentage of DPP-IV inhibition as a function of sample final
171 concentration (natural logarithm). IC_{50} values were expressed as mg of peptides mL^{-1}. 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 Tagliazucchi 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, ± 40 ppm; fragment mass tolerance, ± 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 ± 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 $P<0.05$.

3. Results and discussion

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

The chemical composition of skimmed cow, camel, goat and sheep milks is reported in **Table 1**. Sheep milk contained significant higher ($P<0.05$) amount of total proteins and caseins respect to the other milks. The content in total proteins and caseins was not significant different between cow, camel and goat milks. Indeed, no significant statistical differences were observed between the total whey proteins and lactose content as well as the pH value of the different milk.

The degradation of milk proteins by gastro-intestinal proteolytic enzymes was compared by measuring the amount of released free amino groups using TNBS assay (**Figure 1**). As expected, the amount of free amino groups before the digestion (corresponding to the time 0 of the salivary phase of digestion) was not significantly different between the different milk and remained constant during the 5 minutes of salivary incubation. An increase in the hydrolysis was observed for milk of different species during gastric digestion. After 30 minutes of gastric digestion the amount of free amino groups released from goat milk was significantly higher ($P<0.001$) than that released from cow, camel and sheep milk. No significant statistical differences were observed between the milk from sheep and camel, whereas cow milk showed significantly less amino groups that the other milks ($P>0.05$). The amount of released amino groups increased slightly but not significantly during the subsequent 90 minutes of peptic digestion in all the milks. The transition from gastric to pancreatic environment produced a significant increase in the amount of free amino groups in all the digested milks. Subsequently, the quantity of released amino groups showed a tendency to gradually increase during the entire pancreatic phase of the digestion. At the end of the digestion, goat milk showed a significant higher amount of released amino groups ($P<0.001$) compared to camel, cow and sheep milks. No significant differences were found between the amount of free amino groups released from cow, camel and sheep milk ($P>0.05$). These results showed that 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; Tagliazucchi 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_{50} (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^{-1} (**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_{50}
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_{50} value against DPP-IV
325 (6.9 ± 0.1 mg peptides mL^{-1}), 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 DPPIV-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, Paolella, & FitzGerald, 2017a).

333 The hydrolysates generated herein with cow, camel, goat and sheep milk proteins exhibited
334 higher DPP-IV IC_{50} 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, Paolella, Mudgil,
337 Maqsood, & FitzGerald, 2017b) hydrolysed with trypsin. Lower IC_{50} 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 (Tagliazucchi et al., 2016a; Tagliazucchi 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 β-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 β-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 β-casein, 8
366 from α_{S1}-casein, 4 from α_{S2}-casein and 4 from κ-casein. Only 3 bioactive peptides were released
367 from whey proteins (two from β-lactoglobulin and one from α-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_{50} 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 lactotripeptides VPP and IPP have been shown (at dosages between 5 and 100 mg day^{-1}) 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 α-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_{50} 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**

The present study integrated peptides identified by LC-MS/MS with *in vitro* bioactivities of milk from four different species (cow, camel, goat and sheep) after the application of the harmonized INFOGEST *in vitro* gastro-intestinal digestion protocol. Whereas goat milk showed the highest apparent digestibility, sheep milk appeared to be the best source of ACE-inhibitory peptides. Moreover, cow milk was found to be the best source of DPP-IV-inhibitory peptides and antioxidant peptides and amino acids. Peptidomic analysis showed that goat and sheep milk displayed the highest similarity in peptide sequences identified after *in vitro* digestion. Most of the released bioactive peptides were in common between two or more species and the peptides with the highest ACE-inhibitory activity had previously demonstrated to be bioavailable in humans.

Although this study lays the basis to distinguish milk from different species in the light of their bioactivities and bioactive peptides released during *in vitro* digestion, limitations have to be considered. The most important is that this research was conducted analysing one sample of milk from each species. Therefore, to expand the results, studies involving more milk samples are required. Finally, further investigations and *in vivo* trials are needed to establish which of the 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 μmol of vitamin C 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^{-1} of milk.

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

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.

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

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).
^{a}% of inhibition was determined using the < 3 kDa fractions of the post-pancreatic sample at a concentration of 1 g L^{-1} 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.

Sequence	*Activity*	*Sample*[a]	*Protein*[b]
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	α_{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	α_{S2}-casein
YLG	Peroxyl radical scavenging	Co, G, S	α_{S1}-casein
AWPQ	ABTS radical scavenging	G, S	α_{S2}-casein
VYPF	ABTS radical scavenging Inhibition of lipid peroxidation	G, S	β-casein
YIPI	ABTS radical scavenging	Co, G, S	κ-casein
YLPL	ABTS radical scavenging	G, S	α_{S1}-casein
YVEEL	Peroxyl radical scavenging	G, S	β-lactoglobulin
YQEPVLG	ABTS radical scavenging	G, S	β-casein

[a]Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)
[b]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.

Sequence	*IC_{50}*[a] *µmol L^{-1}*	*Sample*[b]	*Protein*[c]
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_{S1}-casein
LW	15	Co, G, S	α_{S1}-casein
TF	18	Ca	Various proteins
HLPLP	21	Co, Ca, G, S	β-casein
VIP	26	Co, Ca	α_{S2}-casein
IAV	27	G, S	α_{S1}-casein
LY	44	Ca, G, S	Various proteins
IVP	50	Co	α_{S1}-casein
IL	55	Co, Ca, G, S	Various proteins
RPK	92	Co, Ca, G, S	α_{S1}-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	β-casein
FP	205	Co, G, S	Various proteins
PYP	220	Co, Ca, G, S	κ-casein
VAV	260	G, S	κ-casein
ILP	270	Ca	β-casein
VYP	288	Co, G, S	β-casein
LNVPGEIVE	300	Co	β-casein
TGPIPN	316	G, S	β-casein
VLP	320	Co, Ca	β-casein
SLPQ	330	Co, G, S	β-casein
PL	337	Co, Ca, G, S	Various proteins
AVP	340	Co	β-casein
LF	349	G, S	Various proteins
VP	420	Co, Ca, G, S	Various proteins
VYPFPGPI	500	Co	β-casein
NILP	560	G, S	β-casein
EMPFPK	565	Co, G, S	β-casein
YP	720	Co, Ca, G, S	Various proteins
LPLP	720	Co, Ca, G, S	β-casein
ALPM	928	Co, G, S	β-lactoglobulin
IF	930	G	α_{S2}-casein

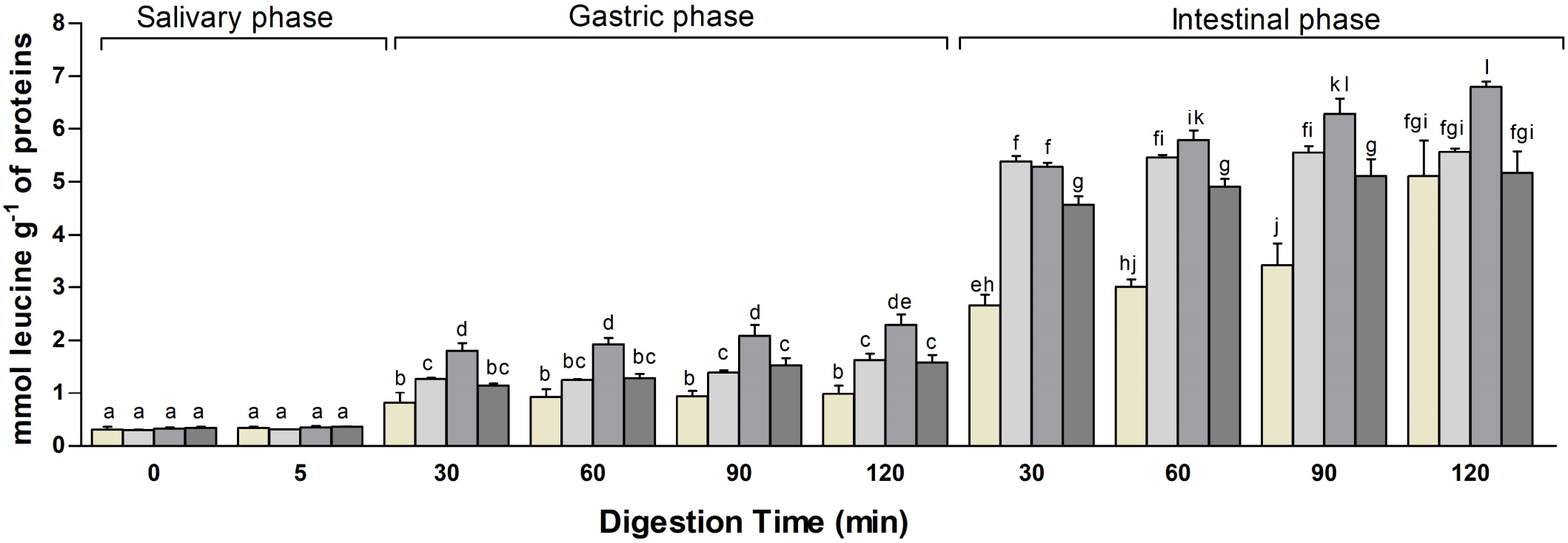
[a]IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity. The values are from BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).
[b]Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)
[c]Precursor protein

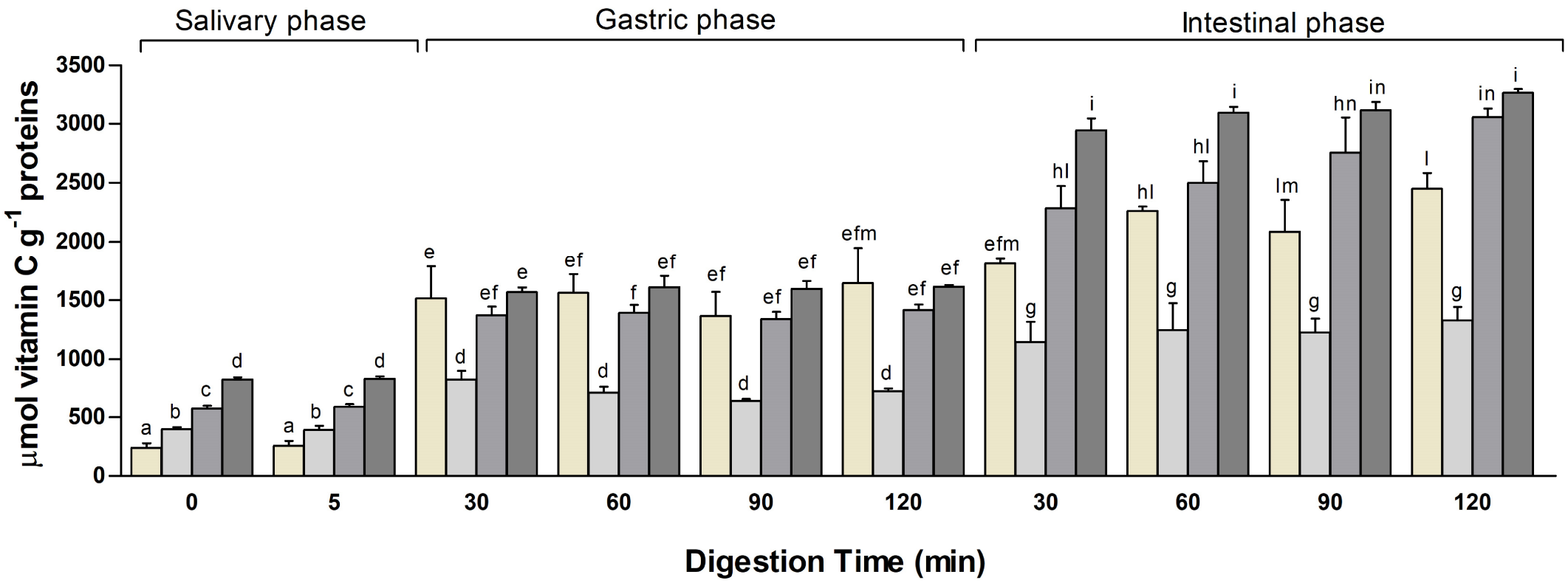
Table 5. Peptides with previously described dipeptidyl peptidase IV (DPP-IV)-inhibitory activity identified in the peptidic fractions (< 3 kDa) obtained from cow, camel, goat and sheep milk after *in vitro* gastro-intestinal digestion. Peptides are listed on the basis of their inhibitory potency.

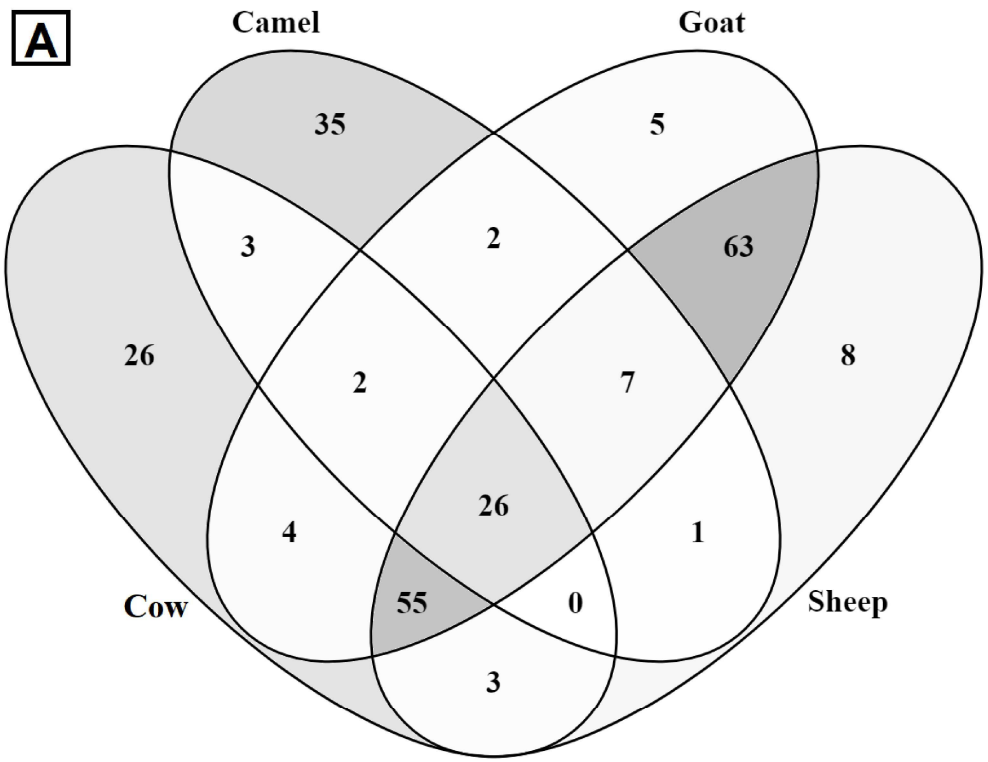
Sequence	*IC_{50}[a] µmol L^{-1}*	*Sample[b]*	*Protein[c]*
IPI	3.5	Co, G, S	κ-casein
VPL	16	Co	α_{S1}-casein
WL	43.6	Co	α-lactalbumin
LPVPQ	44	Co, Ca, G, S	β-casein
IPA	49	Co, S	Various proteins
VL	74	Co, Ca, G, S	Various proteins
LPQ	82	Co, Ca, G, S	Various proteins
YPVEPF	125	Co, G, S	β-casein
IP	150	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_{S1}-casein

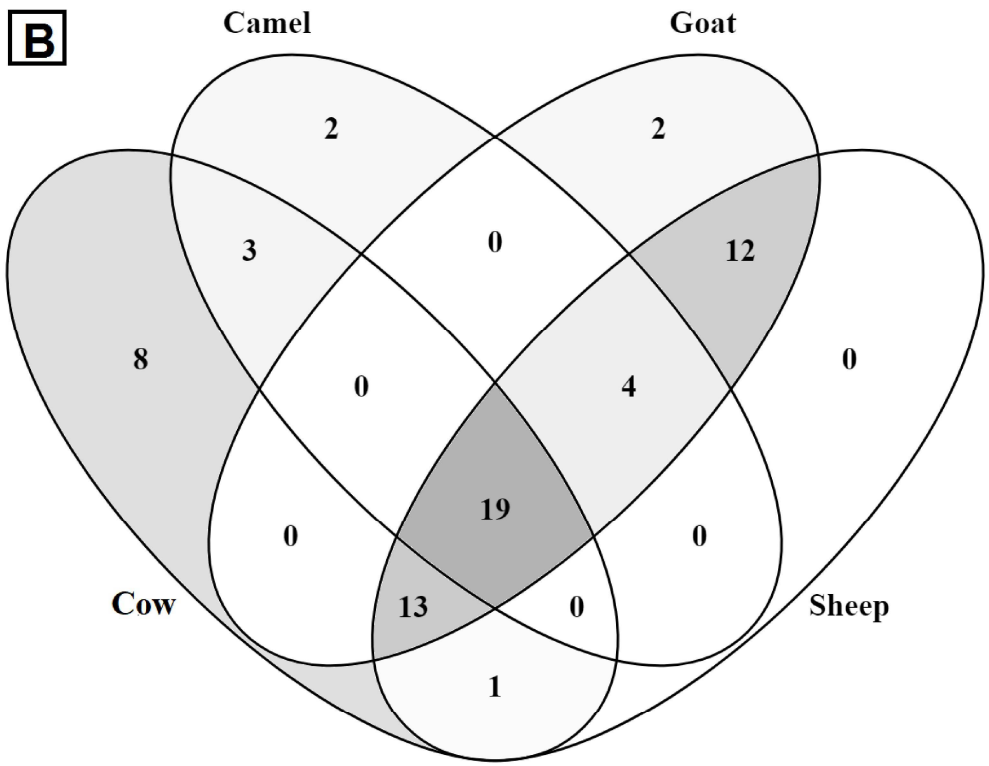
[a]IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity. The values are from BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

[b]Sample in which the peptide was identified (Co: digested cow milk; Ca: digested camel milk; G: digested goat milk; S: digested sheep milk)
[c]Precursor protein


Salivary phase
Gastric phase
Intestinal phase
mmol leucine g-1 of proteins
0
1
2
3
4
5
6
7
8
a a a a
a a a a
b c d bc
b bc d bc
b c d c
b c de c
eh f f g
hj fi ik g
j fi kl g
fgi fgi l fgi
0
5
30
60
90
120
30
60
90
120
Digestion Time (min)


Salivary phase
Gastric phase
Intestinal phase
μmol vitamin C g-1 proteins
3500
3000
2500
2000
1500
1000
500
0
0
5
30
60
90
120
30
60
90
120
Digestion Time (min)


A
Camel
Goat
Cow
Sheep
35
5
3
2
63
26
2
7
8
4
26
1
55
0
3


B
Camel
Goat
Cow
Sheep
2
2
3
0
12
8
0
4
0
19
0
0
13
0
1