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Release of the anti-hypertensive tripeptides valine-proline-proline and isoleucine-proline-proline from bovine milk caseins during *in vitro* gastro-intestinal digestion

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1 **Abstract**

2 The aim of this study was to identify and quantify the release of antihypertensive tripeptides valine-
3 proline-proline (VPP) and isoleucine-proline-proline (IPP) during *in vitro* oro-gastro-intestinal
4 (OGI) digestion of bovine skimmed milk. The experimental approach combined the recently
5 developed harmonized static *in vitro* digestion (IVD) model and targeted mass spectrometry in
6 order to monitor peptide generation. We firstly demonstrated that VPP and IPP are released from
7 bovine milk proteins during *in vitro* OGI digestion at final concentrations of 354.3 ± 29.8 and 973.8
8 ± 155.7 $\mu\text{g/L}$, respectively. *In silico* analysis of cleavage sites and mass spectrometry revealed that
9 tetrapeptides VPPF, IPPL and IPPK are precursors of VPP and IPP. The release of other ACE-
10 inhibitory peptides, such as FVAP, VAP, AW and VY, was demonstrated and their fate and the
11 time course were investigated. This research underlines the suitability of IVD system to study the
12 release of short bioactive peptides during OGI transit.

13

14 **Keywords:** milk; IPP; VPP; mass spectrometry; *in vitro* human digestion.

15

16 **Introduction**

17 Bovine milk proteins contains encrypted in their sequences several bioactive peptides with
18 demonstrated biological functionalities such as anti-hypertensive, antioxidant, immunomodulatory
19 and anti-microbial activities.¹

20 Among the milk-derived bioactive peptides, the anti-hypertensive lacto-tripeptides valine-proline-
21 proline (VPP) and isoleucine-proline-proline (IPP) have attracted particular attention in the last
22 years.^{2,3} They have shown potentiality as anti-hypertensive agents due to their inhibitory effects on
23 angiotensin-converting enzyme (ACE) showing IC₅₀ values of 9 and 5 µmol/L, respectively.⁴

24 Furthermore, although several *in vivo* studies confirmed the antihypertensive effect of the two
25 lactotriptides in spontaneously hypertensive rats (SHR),^{5,6} the results from human clinical trials
26 are still controversial. In 2012 the European Food Safety Authority (EFSA) published an opinion of
27 the Panel on Dietetic Products, Nutrition and Allergies on the health claims related to IPP and VPP,
28 with special regards to the maintenance of normal blood pressure.⁷ The EFSA identified 25 human
29 intervention studies, 15 of which did not observe any effect of IPP and VPP on systolic or diastolic
30 blood pressure. However, three recent meta-analyses of human clinical trials demonstrated a small
31 but significant lowering effect on systolic and diastolic blood pressure, especially in pre-
32 hypertensive or mildly hypertensive patients.⁸⁻¹⁰ The exact mechanism of anti-hypertensive activity
33 of VPP and IPP is still not known and may involve, other than the ACE-inhibition, the production
34 of vasodilators such as nitric oxide^{11,12} or an effect on sympathetic nervous activity.¹³ A recent
35 study also demonstrated that VPP and IPP exhibit insulin-like activities on cultured adipocytes and
36 are able to suppress cytokine-mediated inflammatory responses in the same cell line, suggesting the
37 potential use of these lactotriptides in the management of metabolic syndrome and its
38 complications.¹⁴ Lactotriptides VPP and IPP are easily released from bovine caseins by starter
39 lactic acid bacterium *Lactobacillus helveticus* owing to its repertoire of cell wall bound proteinases
40 and cytoplasmic peptidases able to hydrolyze caseins to VPP and IPP.² Recently, strains of the non-
41 starter species *Lactobacillus casei* and *Lactobacillus rhamnosus* were also found to be able to

42 release VPP and IPP during milk fermentation.¹⁵ Thanks to the proteolytic activities of these
43 lactobacilli, VPP and IPP were frequently detected in several fermented food, such as hard and soft
44 cheeses.¹⁶ Therefore, fermentation processes driven by lactobacilli appear to be the primary way for
45 releasing VPP and IPP from bovine caseins. The milk-derived lacto-tripeptides VPP and IPP are the
46 active ingredients of the sour milk Calpis (Calpis Co., Tokyo, Japan) and Evolus (Valio, Helsinki,
47 Finland), produced by fermentation with *Lactobacillus helveticus*.²
48 These tripeptides have been shown to be highly resistant to the proteases secreted in the oro-gastro-
49 intestinal (OGI) tract and to the brush border peptidases.¹⁶ Moreover, IPP was found to be
50 bioavailable after oral consumption of a yogurt beverage enriched in antihypertensive peptides.¹⁷
51 Some evidences suggest the possible release of IPP from intact protein or large peptide sequences in
52 the intestinal tract. Foltz et al.¹⁷ found plasma IPP concentrations significantly higher than the
53 baseline concentrations after consumption of the placebo beverage instead the lacto-tripeptide
54 enriched beverage. The placebo beverage did not contain free IPP but only intact bovine milk
55 proteins, suggesting that IPP was generated in the intestinal tract of human subjects. Furthermore,
56 we recently showed that IPP was released from camel κ -casein during *in vitro* digestion.¹⁸
57 However, no demonstrations have been given so far about the release of VPP and IPP from bovine
58 caseins by mammalian OGI proteolytic enzymes.
59 Ohsawa et al.¹⁹ studied the *in vitro* release of bioactive peptides from bovine caseins using a
60 mammalian model of the gastro-intestinal tract. Despite they have found in the digested milk
61 several precursors of VPP and IPP, the presence of the lacto-tripeptides was not confirmed. The
62 study however suffered of some limitations. The gastro-intestinal model was far from the
63 physiological conditions and the software used for peptide identification were not able to detect
64 peptides of length less than four or five amino acids.^{20,21}
65 Recently, an *in vivo* study carried out by Boutrou and colleagues²² confirmed the presence of
66 numerous precursors of VPP and IPP in the jejunum of healthy subjects following caseins intake.

67 Unfortunately, the lacto-tripeptides were not detectable under the condition used in the mass
68 spectrometry analysis by Boutrou et al.²²

69 Simulated gastro-intestinal digestion models are widely employed in many fields of food and
70 nutritional sciences and are useful tools to study, in a simplified manner, the digestion process in
71 the upper gastro-intestinal tract applying physiological-based conditions, i.e., chemical and
72 enzymatic compositions of digestive fluids, pH and residence periods typical for each compartment.

73 Recently, a basic static *in vitro* digestive (IVD) model simulating human digestion has been
74 developed within the COST action INFOGEST²⁰ with the aim to harmonize inter-laboratory results
75 and to set experimental conditions that are most close as possible to the physiological situation. The
76 harmonized IVD system has been successfully utilized to study the release of antihypertensive
77 peptides from camel milk¹⁸ and gluten-derived sequences from pasta²³, as well as the
78 bioaccessibility of β -carotene²⁴ and phenolic compounds²⁵.

79 The aim of this study was to investigate the release and fate of the anti-hypertensive tripeptides
80 VPP and IPP and some other ACE-inhibitory peptides during simulated OGI digestion of bovine
81 skimmed milk. Differently from the previous efforts to detect VPP and IPP in milk digestates^{19,22} in
82 the present work, we combined an *in vitro* OGI digestion system, which exploited the same
83 conditions of the harmonized IVD system developed within the COST action INFOGEST,²⁰ with
84 targeted mass spectrometry analysis.

85

86 **Materials and methods**

87 *Materials*

88 Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine α -amylase, pepsin from
89 porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP), mucin II and III, bovine serum
90 albumin, urea and trinitrobenzensulfonic acid (TNBS) were supplied by Sigma (Milan, Italy). The
91 tripeptides VPP and IPP (95% purity) were synthesized by DBA (Milan, Italy). Amicon Ultra-0.5
92 mL regenerated cellulose 3 kDa were supplied by Millipore (Milan, Italy). Ultra-high-temperature-
93 treated (UHT) skimmed bovine milk was purchased from a local market (Reggio Emilia). Mass
94 spectrometry solvents and all the other reagents were purchased from Carlo Erba (Milan, Italy). The
95 absorbance values were determined through a Jasco V-550 UV/Vis spectrophotometer (Orlando,
96 FL, U.S.A.).

97

98 *In vitro gastro-intestinal digestion of skimmed milk using harmonized protocol*

99 For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST
100 and further validated for milk, was used.^{20,26,27} Briefly, simulated salivary (SSF), simulated gastric
101 (SGF), and simulated intestinal (SIF) fluids were prepared according to Kopf-Bolan et al.²⁶
102 Intestinal fluid was prepared by mixing pancreatic (PF) and bile (BF) fluids to final ratio 2:1 (v/v).
103 Skimmed bovine milk (18 mL) was mixed with 24 mL of SSF containing 150 U/mL of porcine α -
104 amylase and incubated for 5 min (oral phase). Gastric phase was mimed by adding 48 mL of SGF to
105 the digestion mixture, adjusting the pH to 3.0 with 6.0 N HCl, and supplementing porcine pepsin to
106 achieve the final concentration of 1115 U/mL. The resulting gastric chyme was further incubated
107 for 120 min. For intestinal phase, 72 mL of SIF (48 mL of PF and 24 mL of BF) was added to the
108 gastric chyme, the pH was adjusted to 7.0 with NaOH 1N and pancreatin was supplemented at the
109 final concentration of 0.4 mg/mL (corresponding to a final trypsin activity of 120 U/mL as
110 suggested by Minekus et al.²⁰). The digestion mixture was further incubated for 180 min (pancreatic
111 phase). All incubations were performed at 37 °C on a rotating wheel (10 rpm). The digested

112 samples were sampled at 12 time points (after 0 and 5 min of salivary digestion, after 30, 60, 90 and
113 120 min of gastric digestion and after 20, 30, 60, 90, 120, 180 min of intestinal digestion,
114 respectively), cooled on ice and immediately frozen at –80 °C for further analyses. The digestions
115 were performed in triplicate.

116 A control sample, which consisted of the gastro-intestinal juices, enzymes and water in place of
117 milk, was included in the experimental trials to evaluate the possible impact of the digestive
118 enzymes on the subsequent analyses.

119

120 *Determination of protein hydrolysis during the digestion*

121 The determination of protein hydrolysis in the digested samples was carried out by measuring the
122 peptide concentration by the TNBS method using leucine as standard.²⁸

123 The hydrolysis degree (DH) was expressed in percentage and calculated as reported in equation (1):

$$124 \text{ DH(\%)} = (\mathbf{h}/\mathbf{h_{tot}}) \cdot 100 \quad (1)$$

125 where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of
126 α-amino groups formed at the different stages of the simulated digestion, and **h_{tot}** is the hydrolysis
127 equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the
128 individual amino acids in 1 g of protein and considering caseins as the only proteins in milk).

129 According to Adler-Nissen,²⁸ the **h_{tot}** value was fixed at 8, which is the value calculated for caseins.

130 DH data were subtracted with the data obtained in the control digestion.

131

132 *Nanoflow liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with* 133 *electrospray ionization (LC-ESI-QTOF-MS/MS) analysis*

134 Samples (0.5 mL) collected during the *in vitro* digestion were subjected to ultrafiltration with
135 Amicon Ultra-0.5 mL nominal cut-off 3 kDa, at 12000g for 120 min at 4 °C.

136 Nano LC/MS and tandem MS experiments were performed on a 1200 Series Liquid

137 Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS via a

138 Chip Cube Interface (Agilent Technologies, Santa Clara, CA, U.S.A.). Chromatographic separation
139 was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and a 43 mm ×
140 75 µm analytical column, both packed with a Zorbax 300SB 5 µm C18 phase (Agilent
141 Technologies). The mobile phase composition and the gradient were the same as reported by
142 Tagliazucchi et al.²⁹ The mass spectrometer was tuned, calibrated and set with the same parameters
143 as reported by Dei Più et al.³⁰
144 Monoisotopic precursor selection was applied to identify the lactotripeptides, some possible
145 precursors and additional ACE-inhibitory peptides. The assignment process was complemented and
146 validated by the manual inspection of MS/MS spectra. The sequences of the peptides studied are
147 listed in **Table 1** together with the selected precursor and the product ions.
148 VPP and IPP was quantified using the method reported in Solieri et al.¹⁵ and their amount expressed
149 as µg/L of hydrolysates. All of the other selected peptides were quantified by integrating the area
150 under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained
151 for each peptide.

152

153 *Statistical analysis*

154 The samples obtained from each digestion were analyzed in singular by TNBS assay and by mass
155 spectrometry. All data are presented as mean ± SD. Univariate analysis of variance (ANOVA) with
156 Tukey *post-hoc* test was applied using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA).
157 The differences were considered significant with $P < 0.05$.

158

159 **Results and Discussion**

160

161 *Assessment of protein hydrolysis during simulated digestion*

162 The hydrolysis of bovine milk proteins during the *in vitro* digestion was evaluated with the TNBS
163 assay. **Figure 1** reports the resulting DH values, relative to control condition, at various steps of the
164 simulated OGI transit. As expected, simulated salivary digestion did not enhance the DH. No
165 significant differences were found between the DH values before and after salivary digestion. After
166 30 min of gastric digestion, the DH slightly increased from $4.3 \pm 0.3\%$ to $10.2 \pm 2.4\%$ (**Figure 1**).
167 Major but not significant increase in DH was observed during the subsequent 90 min of gastric
168 digestion, reaching the value of $12.2 \pm 2.2\%$ at the end of gastric phase. The transition from gastric
169 to pancreatic treatment determined a significant enhance of DH values ($26.4 \pm 2.9\%$ after 20 min of
170 pancreatic digestion; $P < 0.0001$) (**Figure 1**). Subsequently, the DH showed a tendency to gradually
171 increase. Degradation of protein occurs mainly in the intestinal fluid due to the digestive action of
172 pancreatin that is a mix of different proteases. After 120 min of intestinal incubation, DH reached
173 the plateau ($57.4 \pm 12.7\%$) (**Figure 1**).
174 The DH values measured after 120 min of simulated gastro-pancreatic digestion are in agreement
175 with those previously determined on similar milk-derived protein substrates and with the same
176 harmonized digestion model.^{26,31} In particular, comparison between bovine and camel milks showed
177 that bovine milk proteins are more resistant to OGI digestion than camel milk proteins. Complete
178 digestion of camel milk proteins with the same harmonized protocol resulted in a DH value of 69.6
179 $\pm 2.1\%$ ¹⁸ owing to the higher susceptibility of camel milk proteins to peptic digestion compared to
180 bovine milk proteins.¹⁸ The DH value measured after peptic hydrolysis of bovine and camel milk
181 proteins, digested with the same harmonized digestion model, was 12.2% and 20.5%,
182 respectively.¹⁸

183

184 *Release of valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) from bovine milk*
 185 *during in vitro digestion*

186 Even if different studies have suggested that bioactive peptides released from milk protein during
 187 digestion could exert *in vitro* ACE-inhibitory activity,²² no evidence has been given until now about
 188 the possible release of the anti-hypertensive peptides VPP and IPP from bovine milk protein during
 189 *in vitro* OGI digestion. The only study aimed at the identification of VPP and IPP after gastro-
 190 intestinal digestion failed to detect the lacto-tripeptides in the intestinal digesta.¹⁹

191 Bovine caseins have encrypted in their sequence both the lactotripeptides VPP and IPP. This last
 192 tripeptide was located both in β - and κ -caseins (fragments 74-76 and 108-110, respectively),
 193 whereas VPP was found only in β -casein (fragment 84-86). The producibility of these tripeptides by
 194 sequential digestion with pepsin and pancreatic enzymes (trypsin, chymotrypsin, elastase,
 195 carboxypeptidase A and B) was evaluated by analyzing the hydrolyzates collected at different times
 196 of simulated OGI digestion with nanoflow-LC-ESI-QTOF-MS/MS. VPP and IPP were not detected
 197 at the end of the gastric phase, whereas the addition of the pancreatic enzymes determined the
 198 release of both the antihypertensive lactotripeptides. As reported in **Figure 2**, their amounts tended
 199 to increase during the intestinal phase. In particular, IPP amount reached $414.6 \pm 11.3 \mu\text{g/L}$ of
 200 hydrolysates after the first 20 minutes of intestinal phase, and remained stable for the successive 70
 201 minutes of digestion. After this time, the concentration of IPP sharply increased until 180 minutes
 202 of digestion without reaching a plateau. In contrast, the VPP concentration exhibited a linear trend
 203 of increase during the first 60 minutes of digestion, did not change significantly during the
 204 pancreatic digestion, and raised during the late intestinal digestion. At the end of simulated
 205 digestion, IPP and VPP amounts were 973.8 ± 155.7 and $354.3 \pm 29.8 \mu\text{g/L}$ of hydrolysates,
 206 respectively (**Figure 2**). Nineteen randomized clinical intervention trials showed that daily doses
 207 (2–10 mg) of milk casein-derived lactotripeptides reduce the systolic (4.0 mmHg) and diastolic (1.9
 208 mmHg) blood pressure in hypertensive patients.¹⁰ Based on our data, a daily consumption of 300
 209 mL of skimmed milk would be sufficient to obtain an intake of VPP and IPP of about 4 mg.

210 Simulated OGI digestion demonstrated that IPP is released from milk caseins at higher amounts
 211 than VPP. The κ - and β -caseins in bovine milk represents the 9.3% and 33.7% of the total milk
 212 proteins, respectively.³² As the protein concentration in the digestive system was 3.9 g/L (35 g/L in
 213 milk, 9-fold diluted with digestive fluids), the κ - and β -casein concentrations were estimated about
 214 363 and 1314 mg/L, respectively (corresponding to 19.2 and 54.8 $\mu\text{mol/L}$, respectively).
 215 Considering that 2.99 $\mu\text{mol/L}$ of IPP was found at the end of the digestion and that IPP is present in
 216 both κ - and β -caseins, the recovery yield of IPP was approximately 4.0%. Similarly, considering
 217 that VPP is present only in β -casein, the recovery yield was approximately 2.1%, given the final
 218 VPP concentration of 1.14 $\mu\text{mol/L}$. Furthermore, the amount of IPP released from bovine milk was
 219 about 4 times more than that released from camel milk after 120 min of pancreatic digestion.¹⁸
 220 However, taken into consideration that IPP is present only in position 100-102 of camel milk κ -
 221 casein, the recovery yield of IPP from camel milk is about 4-fold higher than that obtained from
 222 bovine milk.¹⁸
 223
 224 *Reconstruction of possible release pathway of VPP and IPP during intestinal digestion*
 225 As detailed in **Figure 3**, *in silico* analysis of theoretical cleavage sites in the VPP or IPP-containing
 226 sequences of bovine milk caseins suggested that several digestive proteolytic enzymes might be
 227 involved in releasing these tripeptides. In particular, pepsin was found to cleave preferentially
 228 bonds with aromatic residues, such as F, Y and W, or L in position P1 and P1'.³³ Therefore,
 229 possible cleavage sites in the long sequence 70-92 of β -casein are L₇₀—P₇₁, L₇₇—T₇₈, F₈₇—L₈₈ and
 230 L₈₈—Q₈₉. Schmelzer et al.³⁴ found additional cleavage sites for pepsin in β -casein, such as I₇₄—P₇₅,
 231 T₈₀—P₈₁, V₈₃—V₈₄ and V₈₄—P₈₅. The peptide bonds N₇₂—I₇₃, L₇₇—T₇₈ and F₈₇—L₈₈ can be also
 232 hydrolyzed by chymotrypsin which preferentially cleaves at W, Y and F in position P1 and to a
 233 lesser extent at L, M and N in position P1'.³³ Elastase shows specificity towards A, V, S and L in
 234 position P1,³⁵ and could be involved in the hydrolysis of peptidic bonds L₇₇—T₇₈, V₈₃—V₈₄ and

235 V₈₄—P₈₅. None of these endoproteases should be able to hydrolyze the bonds P₇₆—L₇₇ and P₈₆—
 236 F₈₇.
 237 Based on these considerations, the tripeptides IPP and VPP can be released from β-casein in the
 238 form of the tetrapeptides IPPL and VPPF (**Figure 3**). The C-terminal residues L₇₇ and F₈₇ can be
 239 subsequently removed by pancreatic carboxypeptidase A (C-terminal exopeptidase) releasing the
 240 tripeptides IPP and VPP. Bovine κ-casein contains IPP in position 108-110. The peptide bond
 241 A₁₀₇—I₁₀₈ can be easily hydrolyzed by pepsin or elastase, whereas the peptide bond K₁₁₀—K₁₁₁ is a
 242 cleavage site for trypsin. The action of these enzymes should result in the release of the tetrapeptide
 243 IPPK (**Figure 3**). The residue K₁₁₀ can be removed by the action of the pancreatic carboxypeptidase
 244 B (C-terminal exopeptidase) which cleaves specifically C-terminal K and R residues.
 245 To confirm the pathway of IPP and VPP release reconstructed by *in silico* analysis, three putative
 246 precursors (IPPL, IPPK and VPPF) and three possible alternative by-products of hydrolysis (PPL,
 247 PPF and PPK) were tentatively identified and quantified by nanoflow-LC-ESI-QTOF-MS/MS
 248 during the intestinal digestion (**Figure 4**). Among the three selected precursors, only VPPF was
 249 found at the end of the gastric digestion, confirming that the bonds V₈₃—V₈₄ and F₈₇—L₈₈ are
 250 cleavage sites for pepsin. The amount of VPPF gradually increased in the hydrolysates during the
 251 pancreatic digestion thanks to the action of elastase and chymotrypsin. VPPF concentration reached
 252 a peak after 120 minutes of pancreatic digestion and then decreased in the remaining digestion time.
 253 The decrease in VPPF amount after 120 of minutes coincides with the observed increase in VPP
 254 concentration (**Figure 2**). Both IPPL and IPPK were released only during intestinal phase of
 255 digestion following different trends (**Figure 4**). The amount of IPPL gradually increased during the
 256 entire time of digestion without reaching a plateau, whereas IPPK reached the maximum amount
 257 after 90 minutes of digestion. Overall, these data confirmed that the tetrapeptides VPPF, IPPL and
 258 IPPK are precursors of IPP and VPP, probably due to the cleavage by pancreatic carboxypeptidases.
 259 Regarding the alternative by-products of the hydrolysis, PPL, PPF and PPK can be released instead
 260 of IPP and VPP and represent un-desired peptides, which reduce the recovery yields of the anti-

hypertensive tripeptides. As shown in **Figure 4**, only PPF was found in the hydrolysates. Trace amount of PPF were found at the end of the gastric digestion, suggesting that pepsin is able to cleave the bond V₈₄—P₈₅ with low efficacy. Subsequently, the amount of PPF greatly increased during pancreatic digestion because of the action of elastase. No evidence for the formation of PPL and PPK was found either during gastric or pancreatic digestions, suggesting that the bond I₇₄—P₇₅ is resistant to gastro-pancreatic proteases. This result could account for the higher recovery yield observed for IPP compared to VPP.

268

Time course and fate of additional ACE-inhibitory peptides

The fate of additional four selected peptides with known ACE-inhibitory activity was monitored during the pancreatic phase of digestion. FFVAP (f23-27) and VAP (f25-27) are α_{S1} -casein derived peptides which display high ACE-inhibitory activity and low IC₅₀ values (IC₅₀ values of 6 and 2 μ mol/L, respectively)^{36,37}. The pentapeptide FFVAP was firstly isolated from casein sequentially hydrolyzed with trypsin and prolyl-endopeptidase,³⁶ whereas the tripeptide VAP was chemically synthesized to reproduce the C-terminal portion of a fragment-peptide derived from an enzymatic hydrolysate of casein³⁷. However, until now, these ACE-inhibitory peptides were never found in *in vitro* digestive hydrolysates of milk proteins. **Figure 5** shows that combination of the harmonized IVD model with targeted mass spectrometry enables the detection of both peptides already at the end of the gastric digestion. The amount of FFVAP increased until 120 minutes of pancreatic digestion, after that it was further degraded to the shorter tripeptide VAP.

The ACE-inhibitory dipeptides AW and VY have been proved to exhibit low IC₅₀ values (5 μ mol/L for both the peptides) and to be absorbed in human plasma.³⁸ AW was found only in α_{S1} -casein (f163-164), whereas VY was found in β -casein (f59-60), α_{S2} -casein (f198-199) and in β -lactoglobulin (f41-42). As reported in **Figure 5**, neither AW nor VY were detected at the end of gastric digestion, but they were released from milk proteins by intestinal proteases. We recovered

286 the maximum amount of AW after 20 minutes of digestion, after that, the amount of AW dropped
287 and did not significantly change during the remaining time of digestion (**Figure 5**). The trend of VY
288 production was different. **Figure 5** shows a biphasic release consisting of a first maximum value
289 after 20 minutes of intestinal digestion, followed by a decrease during the subsequent 60 minutes of
290 digestion. During the successive 30 minutes of digestion, there was a threefold increase of VY
291 amount, followed by a further decrease in the last phase of digestion. The biphasic release of VY is
292 probably due to the presence of this dipeptide in the sequences of different milk proteins, which can
293 release VY thanks to the action of different pancreatic enzymes and at different digestion times.
294 Previous studies based on simplified and in-house digestive models failed to detect short bioactive
295 peptides in the digested milk. Here, we demonstrated that the harmonized gastro-intestinal IVD
296 model in combination with targeted mass spectrometry enables the identification of short ACE-
297 inhibitory and anti-hypertensive peptides released during digestion of milk proteins. Therefore, the
298 harmonized digestive system is confirmed to be an effective model to study the fate, kinetics of the
299 release and concentrations of short bioactive peptides during milk digestion in a reproducible
300 manner.

301 As a major result, our study provides the first evidence that VPP and IPP are released during the
302 OGI digestion of bovine milk caseins. The recovery yield of the two lactotripeptides was low for
303 both VPP and IPP at the first stages of OGI transit, but, as the digestion proceeds, they are released
304 to a greater extent from precursors present in the jejunum. Furthermore, to the best of our
305 knowledge we firstly demonstrate that other short ACE-inhibitory peptides, such as AW, VY, VAP
306 and FFVAP are present in the gastro-intestinal digested milk. Even if further investigation and *in*
307 *vivo* trials are required to corroborate our results, the present work opens the way to study the fate
308 of VPP, IPP and other bioactive peptides under *in vitro* digestive conditions most close as possible
309 to human physiology.

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Figure captions

Figure 1. Changes in hydrolysis degree (DH%) of bovine skimmed milk proteins during *in vitro* gastric and pancreatic digestion. Values are means of three independent digestion \pm standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 2. Release of the antihypertensive lactotripeptides valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) during intestinal digestion. Time zero represents the sample collected at the end of the gastric digestion. Values are means of three independent digestion \pm standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 3. Theoretical cleavage sites of pepsin (\blacktriangle), chymotrypsin (\bullet), elastase (\blacklozenge), and trypsin (\blacksquare) in the VPP and IPP-containing sequences of bovine β - and κ -caseins.

Figure 4. Fate and time course of the VPP and IPP precursors (VPPF, IPPL and IPPK) and of the un-desired product of hydrolysis PPF during intestinal digestion. Amounts are expressed as area under the peak (AUP). The end of the gastric digestion is considered as the starting point of sampling. Values are means of three independent digestion \pm standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 5. Fate and time course of short ACE-inhibitory peptides during intestinal digestion. Amounts are expressed as area under the peak (AUP). The end of the gastric digestion is considered as the starting point of sampling. Values represent means \pm SD of triplicate digestions. Different letters indicate significantly different values ($P < 0.05$).

Table 1. Sequences, Precursor Ions Selected for Fragmentation and Monitored Product Ions of the Peptides Identified and Quantified Using Mass Spectrometry.

Peptides	Fragments	Selected precursor ions ^a	Monitored product ions ^a
<i>β-casein</i>			
VY	f(59-60)	281.15	182.08
IPP	f(74-76)	326.21	213.12; 211.14
IPPL	f(74-77)	439.29	326.21; 308.20; 229.15
PPL	f(75-77)	326.21	229.15; 195.11
VPP	f(84-86)	312.19	213.12; 197.13
VPPF	f(84-87)	459.26	360.19; 294.18; 263.14
PPF	f(85-87)	360.19	263.14; 195.11
<i>α_{S1}-casein</i>			
FFVAP	f(23-27)	580.31	433.24; 286.18; 187.11
VAP	f(25-27)	286.18	187.11; 171.11
AW	f(163-164)	276.13	205.10
<i>α_{S2}-casein</i>			
VY	f(198-199)	281.15	182.08
<i>κ-casein</i>			
IPP	f(108-110)	326.21	213.12; 211.14
IPPK	f(108-111)	454.30	341.22; 308.20; 244.17
PPK	f(109-111)	341.22	244.17; 195.11
<i>β-lactoglobulin</i>			
VY	f(41-42)	281.15	182.08

^a: mono-charged ions

Figure 1

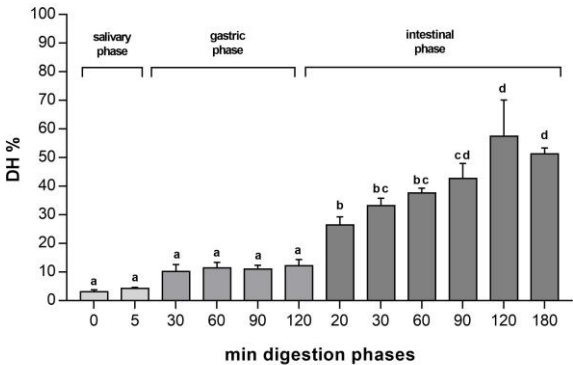


Figure 2

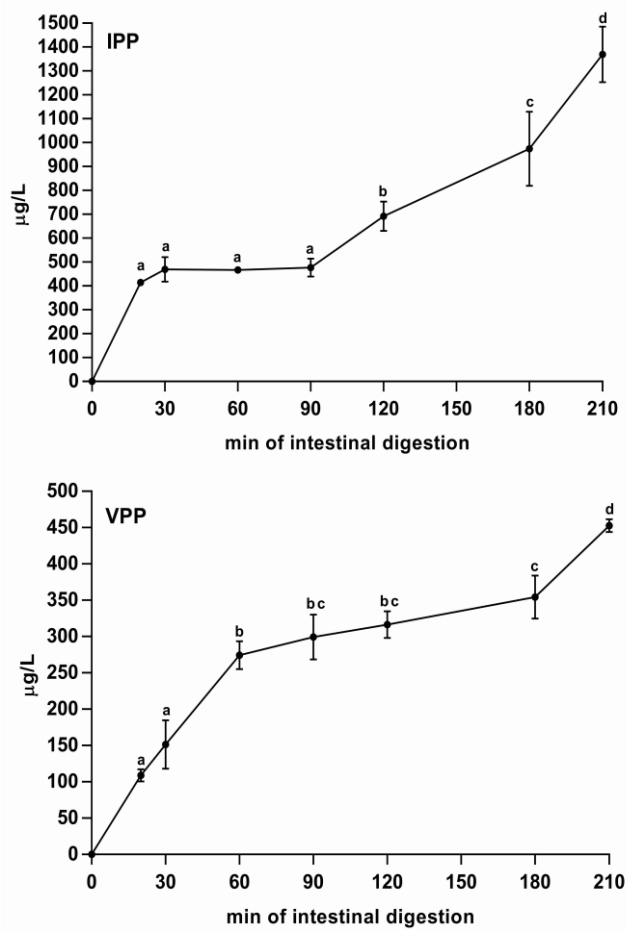


Figure 3

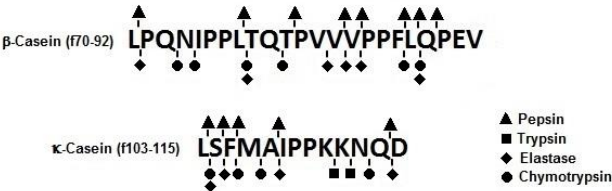


Figure 4

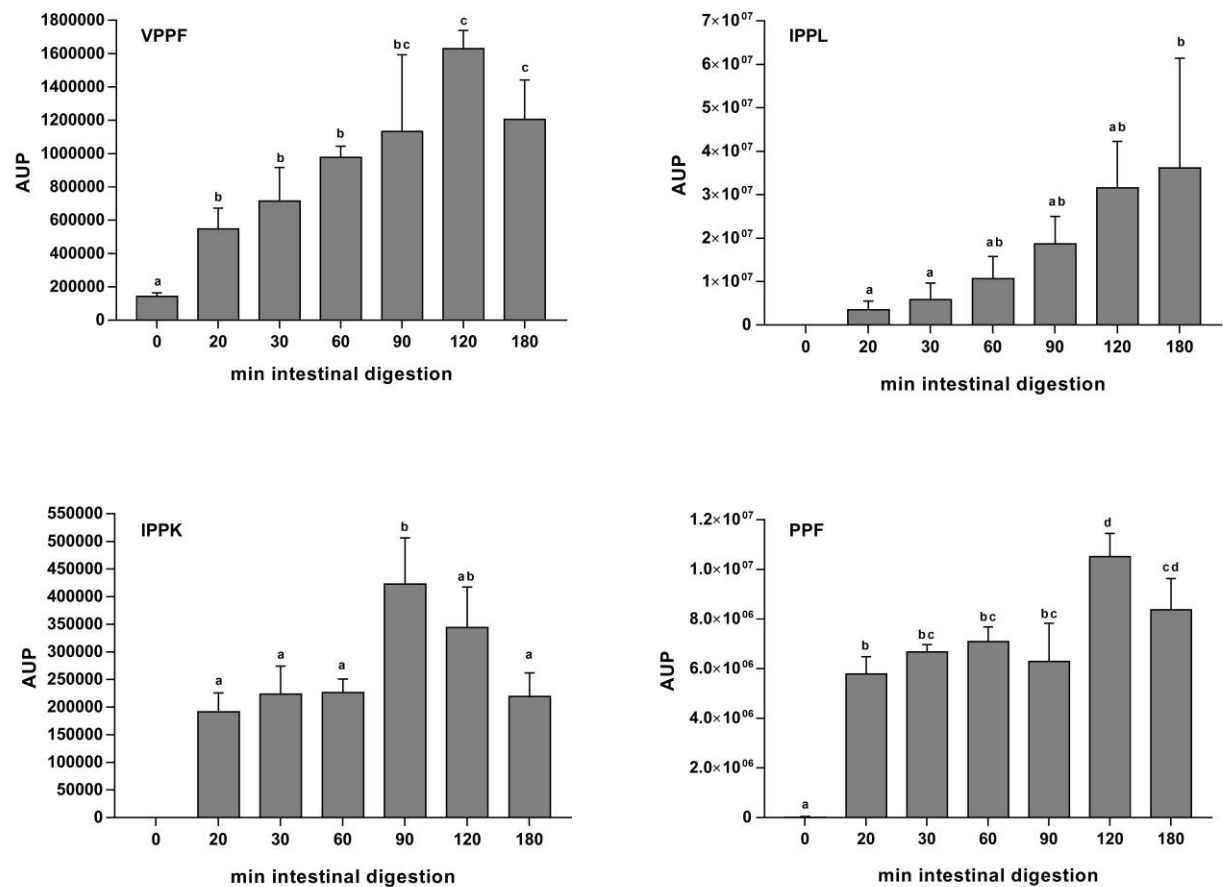


Figure 5

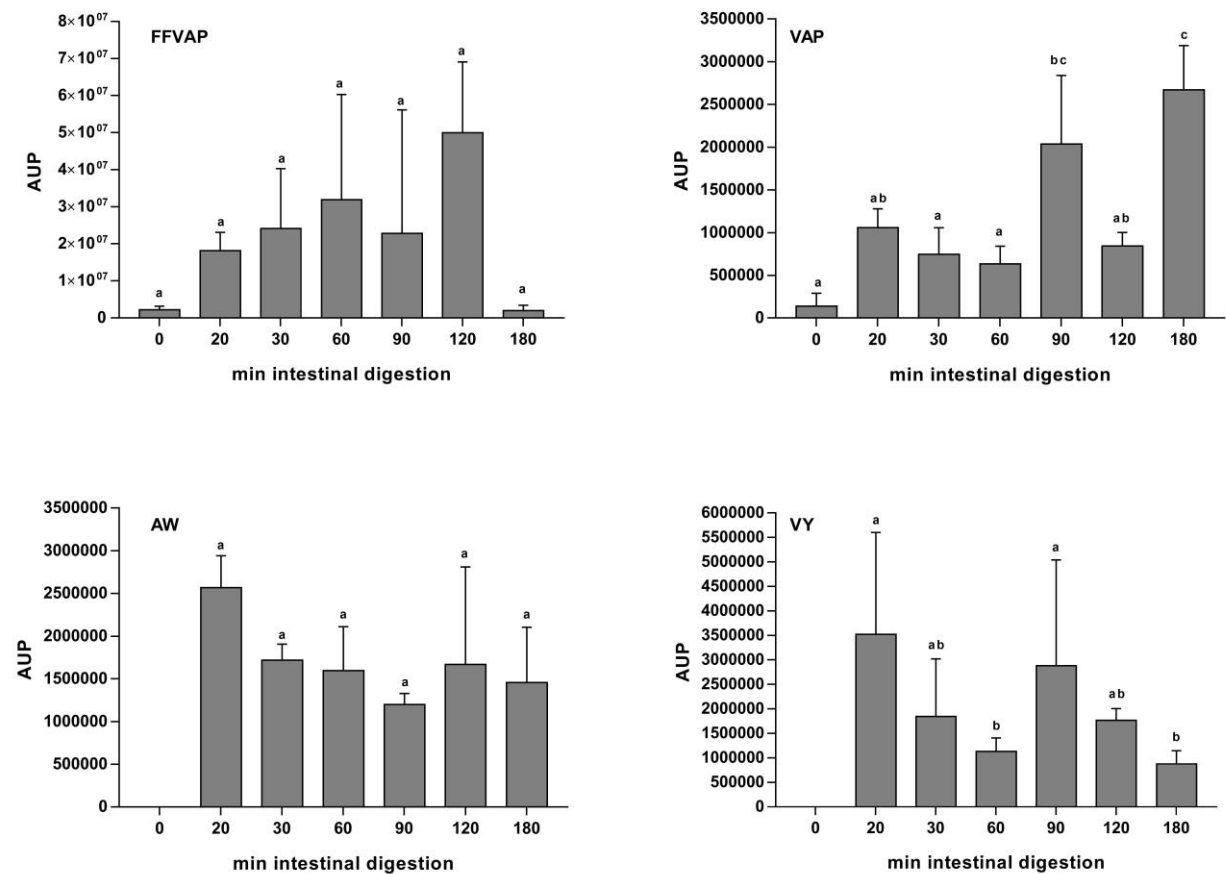


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