

In vitro digested milk proteins: Evaluation of angiotensin-1-converting enzyme inhibitory and antioxidant activities, peptidomic profile, and mucin gene expression in HT29-MTX cells

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Accepted Version

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To link to this article DOI: <http://dx.doi.org/10.3168/jds.2019-16833>

Publisher: Elsevier

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1 **Interpretative Summary**

2 ***In vitro* digested milk proteins: evaluation of Angiotensin-1-Converting Enzyme**

3 **Inhibitory and antioxidant activities, peptidomic profile and mucin gene expression in**

4 **HT29-MTX cells**

5 *by Giromini et al.* We studied the bioactive effect of *in vitro* digested whey protein isolate
6 (WPI), casein proteins (CP) and soy proteins (CTR) in term of Angiotensin-1-Converting
7 Enzyme Inhibitory activity (ACE1-I), antioxidant (AOX) and intestinal HT29-MTX-E12 cell
8 metabolic activity and mucus-production. Our results indicate that milk proteins possess higher
9 AOX and ACE1-I activity after *in vitro* digestion. Peptidomic analysis reveal that WPI and CP
10 generated potentially bioactive peptides mainly associated to ACE1-I bioactivity. We also
11 reported that specific concentrations of WPI, CP and CTR are able to promote HT29-MTX-E12
12 cells metabolic activity and, in the case of CP, the MUC5AC and MUC2 gene expression,
13 suggesting that the consumption of milk proteins can have a positive effect on intestinal
14 defenses.

15 **Running head: Milk protein digestion: bioactivity and peptidomics**
16 ***In vitro* digested milk proteins: evaluation of Angiotensin-1-Converting Enzyme**
17 **Inhibitory and antioxidant activities, peptidomic profile and mucin gene expression in**
18 **HT29-MTX cells**
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ABSTRACT

33 Over the past decades, a number of studies investigated the health-promoting functions of milk
34 peptides. However, to date many hurdles still exist regarding the widespread utilization of milk-
35 derived bioactive peptides as they may be degraded during gastrointestinal digestion. Thus, the
36 aim our study was to *in vitro* digest intact Whey Proteins Isolate (WPI) and Casein Proteins
37 (CP), mimicking *in vivo* digestion, to investigate their bioactive effects and to identify the
38 potential peptides involved. Whey protein isolate and CP were digested using a
39 pepsin/pancreatin protocol and ultra-filtered (3kDa cut-off membrane). A permeate (<3kDa)
40 and a retentate (>3kDa) were obtained. Soya protein was included as a control (CTR).
41 Angiotensin-1-Converting Enzyme Inhibitory (ACE1-I) and Antioxidant activity (AOX) were
42 assessed and compared with those observed in undigested proteins and CTR. Furthermore, the
43 permeate was characterized by LC-nano ESI MS/MS using a shotgun-peptidomic approach
44 while retentate was further digested with trypsin and analysed by mass spectrometry with a
45 shotgun-proteomic approach to identify potentially bioactive peptides. Further, the effect of
46 WPI, CP and CTR retentate on cell metabolic activity and on mucus production (MUC5AC and
47 MUC 2 gene expression) was assessed in intestinal goblet HT29-MTX-E12 cells. Results
48 showed that WPI permeate induced a significant ACE1-I inhibitory effect (49.2 ± 0.64 %)
49 compared with undigested WPI, CP permeate and retentate ($P < 0.001$) and with CTR permeate
50 (10.40 ± 1.07 %; $P < 0.001$). A significant increase in AOX (1.58 ± 0.04 and 1.61 ± 0.02 μmol
51 trolox AOX equivalents /mg protein, respectively) upon digestion was found in WPI.
52 Potentially bioactive peptides associated with ACE1-I and anti-hypertensive effects were
53 identified in WPI permeate and CP retentate. WPI, CP and CTR retentate, at specific
54 concentrations, were able to stimulate ($P < 0.05$) metabolic activity in HT29-MTX-E12 cells;
55 MUC5AC expression was increased ($P < 0.05$) by CP retentate and unaltered ($P > 0.1$) by WPI
56 retentate. MUC2 expression was significantly increased by 0.33 mg/g CP and reduced by 1.33

57 mg/g CP. Our results confirm that milk proteins may be rich sources of bioactive compounds
58 with a greatest beneficial potential of CP at intestinal goblet cell level.

59 **KEYWORDS:** milk protein; *in vitro* digestion; peptidomic; angiotensin-I-converting enzyme.

60

61

INTRODUCTION

62 Milk proteins are considered one of the most important source of bioactive peptides. Beyond
63 their well-known nutritional values, milk proteins exhibit an extensive range of physiological
64 effects that promote general health and the functions of specific organs and tissues (Meisel and
65 FitzGerald, 2003; Martínez-Maqueda et al., 2012b). The most relevant functional properties of
66 milk proteins are obtained following gastrointestinal digestion and milk peptides release (Baldi
67 et al., 2005; Vermeirssen et al., 2003). A recent study compared the peptidome of human jejunal
68 effluents after ingestion of casein and whey proteins with the peptidome obtained after their *in*
69 *vitro* gastrointestinal digestion. This study has allowed the identification of protein domains
70 that are resistant to gastrointestinal digestion (Sanchón et al., 2018). Some of these peptide
71 fragments correspond to previously described sequences that might exert their physiological
72 function at the intestinal epithelium level or after absorption where they can elicit unique effects
73 (Shimizu, 2004). It has been reported that peptides from the hydrolysis of milk proteins possess
74 functional properties such as antioxidant, angiotensin-I-converting enzyme inhibitory activity,
75 antimicrobial and cytomodulatory activities (Baldi et al., 2005; Martínez-Maqueda et al.,
76 2012b; Giromini et al., 2017). Petrat-Melin et al. (2015) found that beta-casein variants
77 exhibited antioxidant and angiotensin-I-converting enzyme inhibitory activity upon digestion.
78 Furthermore, larger peptides from milk protein digestion pool (i.e >3kDa) are unlikely to reach
79 the bloodstream and will therefore mediate their bioactive effect locally, at gastro intestinal
80 level (Miner-Williams et al., 2014). Beta-casomorphin 7, a casein-derived peptide, exhibited an
81 enhanced mucin gene expression mediated by opioid receptors in intestinal goblet cells (Zoghbi

82 et al., 2006). To date, however, most attention has been focused on the bioactive effect of single
83 peptides or hydrolysed proteins (Martínez-Maqueda et al., 2012a; Plaisaincié et al., 2015) with
84 limited data on the effect of the whole pool of peptides obtained after *in vitro* digestion of milk
85 proteins (Mukhopadhyaya et al., 2015; Volstatova et al., 2016), their potential mechanism of
86 action and the candidate peptides involved. Casein and whey proteins, however, are present in
87 milk and dairy products in their intact form and the total peptide pool produced from their
88 digestion may have diverse effects *in vivo*. Evidences from robust *in vitro* studies simulating
89 protein digestion, and the subsequent evaluation of the bioactive effect could offer invaluable
90 information on the physiological mechanisms by which these proteins may elicit their biological
91 effect *in vivo*. Consequently, we investigated the ACE1-Inhibitory (ACE1-I) and antioxidant
92 (AOX) activity of *in vitro* digested (permeate and retentate fractions) Whey Protein Isolate
93 (WPI) and Casein Proteins (CP) compared to undigested samples and to soya protein isolate
94 (CTR). A peptidomic/ proteomic analysis of WPI and CP was also performed to substantiate
95 the presence of bioactive peptides. Further, the effect of WPI, CP and CTR retentate was tested
96 on cell metabolic activity and on mucus production (MUC5AC and MUC2 gene expression) in
97 HT29-MTX-E12 cells as a human intestinal goblet cell model.

98 MATERIALS AND METHODS

99 *Samples and reagents*

100 The samples tested in the study were the following: Whey Protein Isolate sample (WPI) (Volac
101 Int Ltd, Cambridge) with a protein content of 93g/100g protein; Calcium caseinate (casein
102 protein, CP, Garret Ingredients) with a protein content of 90g/100g protein; and soya protein
103 isolate (CTR) (MyProtein, Northwich) with a protein content of 90g/100g.

104 *In vitro digestion*

105 Whey protein isolate, CP and CTR *in vitro* digestion was performed according to the method
106 of Minekus et al. (2014), and further adapted by our group (Giromini et al., 2017).
107

108 Briefly, 20 g of WPI, CP or CTR sample were mixed with 150 mL of distilled H₂O and
109 maintained on an orbital shaker at 150 rpm for 5 minutes. The digestion procedure involved
110 three phases. For the oral phase 6.66 mg α -amylase in 2.1 mL of 1 mM CaCl₂, pH 7 was added
111 to the samples and they were incubated for 30 min at 37 °C on a shaker. For the gastric phase,
112 the pH was decreased up to 2 with 6 M HCl and 0.9 g of pepsin in 8.3 mL of 0.1 M HCl was
113 added. The samples were then incubated for 120 min at 37 °C on a shaker. For the small
114 intestinal phase, the pH was increased to 7 with 6 M NaOH and 0.2 mg pancreatin and 1.2 g
115 bile in NaHCO₃ 0.5 M were added to the samples before the final incubation of 180 min at 37
116 °C on a shaker was performed.

117 A blank sample (enzymes of the digestion alone), a positive control and negative control were
118 included as reference samples and for stability tests in all digestions performed (n=3).

119 *Samples preparation*

120 At the end of the digestion, the total digesta obtained was transferred to 3kDa cut-off membrane
121 (VIVASPIN 20 Sartorius). Each filter was previously activated with 0.1% BSA solution.
122 Samples were centrifuged for 20 min at 3500 x g to obtain permeate (peptides and polypeptides
123 < 3kDa) and retentate (peptide and polypeptides > 3kDa) fractions. Aliquots from permeate
124 and retentate fractions were sampled and snap frozen in liquid nitrogen to stop enzyme activity
125 before storing at -80 °C for further experiments.

126 *Angiotensin-1-converting enzyme inhibitory activity*

127 The ACE1-I of WPI, CP and CTR undigested, permeate and retentate samples was quantified
128 using the ACE1-I assay with furanacrololyl-Phe-Glu-Glu (FAPGG) as the synthetic substrate
129 for the ACE1-I enzyme, as described by Giromini et al. (2017). A synthetic specific ACE
130 inhibitor captopril at the concentration of 20nM was included as control. Data are expressed as
131 percentage of ACE1-I (% ACE1-I).

132 *Total antioxidant capacity-ABTS assay*

133 Antioxidant capacity (AOX) was determined in WPI, CP and CTR undigested, permeate and
134 retentate samples using the method described by Re et al. (1999) with modifications. Trolox
135 stock solution (2.5 mM in distilled water) was used to produce the standard curve. A solution
136 of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid (ABTS) (7 mM) was prepared with
137 potassium persulfate (140 mM) in distilled water and left to react in the dark for 12–16 hours
138 to produce the ABTS•+solution. For the study of AOX capacity of WPI, CP and CTR permeate
139 and retentate, the ABTS•+solution was diluted with phosphate buffered saline, pH 7.4, (PBS)
140 to reach the absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30°C. A volume of 20 μ L
141 of sample or Trolox standard was mixed with 2mL of ABTS•+ working solution and incubated
142 in dark for 6 min at room temperature before measuring absorbance at 734 nm on the
143 spectrophotometer. Appropriate solvent blanks were included in each assay. The percentage
144 inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of
145 samples and of Trolox for the standard reference data. AOX results are expressed as μ mol
146 Trolox equivalent (TE)/ mg protein.

147 ***Proteomic and peptidomic profile***

148 Permeate and retentate of WPI and CP were analysed by LC-nano ESI tandem mass
149 spectroscopy using a shotgun-label free approach to identify peptides and proteins. The
150 permeate was analysed without any digestion before MS/MS to identify endogenous peptides
151 presents in the samples, the so called peptidomic strategy (Aletti et al. 2016) (Fig. 1) while the
152 retentate was further digested using sequence-grade trypsin with a protein:protease ratio 20:1
153 (Iametti et al., 2001) with a proteomic strategy (Fig. 1). The digestion with trypsin is a normal
154 procedure in the proteomic MS/MS analysis in order to identify the proteins based on the
155 sequence of the corresponding tryptic peptides. Trypsin generates experimentally observable
156 peptides that can be used to uniquely identify a protein.

157 Mass analysis was carried out using an LTQ OrbitrapVelos (Thermo Fisher Scientific, Bremen,
158 Germany) as described in Maffioli et al. (2017) for the peptidomic strategy and in Coccetti et
159 al., (2008) for the proteomic analysis.

160 **FIGURE 1**

161 *Peptidomic and proteomic data analysis*

162 MS spectra were searched against the mammalian NCBI sequence database (release
163 24.01.2017) (casein and whey samples) by the Sequest search engine contained in the Proteome
164 Discoverer 1.4.0 software (Thermo Fisher Scientific Inc., USA). The following parameters
165 were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, Met oxidation, N-terminal
166 acetylation and Gln /Asn deamidation as variable modifications. Carbamidomethylation of Cys
167 as fixed modification and trypsin (2 misses) as protease were parameters further added in the
168 proteomic analysis. Only peptides with False Discovery Rate 1% (against decoy) and Xcorr 1.5
169 were included for positive identification (Dell'Orco et al. 2016). Two replicates were carried
170 out for each sample in the MS analysis. All peptides were searched in SATPdb (Singh et al.
171 2016) and BIOPEP-UWM database (Minkiewicz et al 2008), two databases of structurally
172 annotated therapeutic peptides, to find potentially bioactive peptides. In order to consider
173 possible further proteolysis, the search was performed keeping a minimum sequence length of
174 6 amino acids and applying a "IF" nested function to a matrix which compared the sequence
175 of each peptide found with the ones of the database (Microsoft Excel 2016 (version 15.27)).
176 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
177 Consortium via the PRIDE partner repository (Vizcaino et al 2016) with the dataset identifier
178 PXD012588 for the permeate and PXD012625 for the retentate.

179 *Cell culture experiments*

180 In our study, the permeate and retentate fractions obtained after IVD reproduced the pool of
181 peptides generated during the physiological digestion in humans (Giromini et al., 2019). Small
182 peptides (<3KDa, permeate fraction) could be absorbed by epithelial cells along the small

183 intestine and enter the blood circulation, whereas polypeptides (>3KDa, retentate fraction) can
184 reach the colonic environment. Thus, in a second set of experiments we tested the effect of
185 WPI, CP and CTR retentate fractions on intestinal HT29-MTX-E12 cell metabolic activity and
186 mucus production modulation.

187 *Metabolic activity*

188 The HT29-MTX-E12 cells were kindly donated by Dr. Stig Purup, Aarhus University, Research
189 Centre Foulum, DK. This human colorectal adenocarcinoma cell line, which is a clone of HT29
190 cells, is able to differentiate into a mucus-producing goblet-like cell line. Cell maintenance was
191 performed in 25 cm² flasks using 15 mL Dulbecco's modified Eagle's medium (DMEM) with
192 glucose (4.5 g/L) and supplemented with 10% of FCS, 1% 1 M HEPES, 1% (v/v)
193 penicillin/streptomycin, 2% Glutamax and 1% MEM NEAA. The cells were cultivated at 37°C
194 in a humidified incubator with 5% CO₂. All experiments were performed using HT29-MTX-
195 E12 cells within six cell passages (passages 40 to 45) to ensure reproducibility.

196 Undifferentiated HT29-MTX-E12 cells were plated at a density of 1.5x10⁴ cells/well in 96-well
197 plates and cultured for 24 hours. Whey protein isolate, CP and CTR retentate were diluted in
198 0.05% FCS DMEM (Giromini et al., 2015) and added to the cell culture at different
199 concentrations (from 42.62 to 0.16 mg/ml). For the control wells, 0.05% FCS DMEM was
200 added.

201 The retentate stock solutions of WPI, CP and CTR contained between 86.23 and 85.24 mg
202 protein/ml. Stock solutions have been normalised to 85.24 mg/ml, which corresponds to the
203 lowest protein concentration observed among WPI, CP and CTR. Considering the high
204 cytotoxicity of stock solutions and the absence of DMEM medium to regulate the osmotic
205 pressure, we have tested diluted concentrations of retentate from 42.62 up to 0.16mg/ml.

206 After 3 and 24 hours of treatment, the metabolic activity of HT29-MTX-E12 cells was
207 evaluated by MTT test. MTT assay measures the production of the chromophore formazan from
208 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide (MTT). Formazan is produced

209 in viable cells by the mitochondrial enzyme succinate dehydrogenase. Three replicates per
210 treatment were included and the experiments were repeated three times (n=3).

211 Specifically, the cell metabolic activity percentage induced by the protein retentate
212 was calculated as follows:

213 % cell metabolic activity = (mean optical density of treated cells / mean optical density of
214 control cells) x 100.

215 *Mucus production- real-time quantitative PCR (qRT-PCR)*

216 The HT29-MTX-E12 cells were seeded at a density of 5×10^5 cells/well in 12-well plates and
217 maintained for up to 21 days in complete medium (Martinez-Maqueda et al., 2012a). Martinez-
218 Maqueda et al. (2012a) reported that, after 21 days of culture, HT29-MTX-E12 cells express a
219 stable amount of mucus. At day 21, the cells were washed with PBS, and the medium was
220 replaced with 0.05% FCS DMEM medium for 24 h (cell starvation). The treatment medium
221 (0.05% FCS) with WPI, CP or CTR protein retentate at different concentrations (0.33 up to
222 1.33 mg/g) was added to the cells, which were then incubated in a controlled atmosphere (37°C
223 / 5% CO₂) for 3 hours. The concentration range was chosen based on the MTT test results.
224 Control cells (0mg/g) were not treated.

225 qRT-PCR assay was carried out with the real-time fluorescence method using a Strategene
226 Mx3000p. At the end of 3 hours incubation, total RNA was extracted from the HT29-MTX-
227 E12 cells using a nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to the
228 manufacturer's protocol. The RNA (50ng) was reverse transcribed using the iScript cDNA
229 synthesis kit (BioRad), and the resulting cDNA was used as a template for qRT-PCR. MUC5AC
230 and MUC2 genes were amplified by qRT-PCR with primer sequences that have previously been
231 published by Martínez-Maqueda et al.(2012a) and Nielsen et al.(2018) (Table 1), and
232 cyclophilin and β -actin were included as a reference genes. The primers have been checked for
233 specificity in BLAST before the Real-time PCR experiments.

234 **TABLE 1**

235 Each reaction tube contained 2X SYBR Green real-time PCR Master Mix, gene-specific
236 forward and reverse primers and the cDNA (1 μ l). The master mix included Maxima® Hot Start
237 Taq DNA polymerase, dNTPs in an optimized PCR buffer, and SYBR® Green I dye
238 supplemented with ROX passive reference dye. All reactions were analyzed under the same
239 conditions and normalized to the ROX reference dye to correct eventually fluctuations in the
240 readings due to evaporation phenomena. The samples were tested in triplicate and non-reverse
241 transcribed controls and no-template controls were included in the assays. The thermal profile
242 began with 4 minutes at 95°C followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 minute.
243 Relative quantification was performed, and the values were normalized to the internal reference
244 gene cyclophilin. Two internal controls, cyclophilin and β -actin genes were tested as
245 endogenous genes and cyclophilin was selected because its amplification was more efficient
246 and had no variation across treatments. This verified the efficiency of its use as the endogenous
247 control.

248 *Statistical analysis*

249 Statistical analysis were performed using GraphPad-Prism version 8 software package
250 (GraphPad Software, San Diego, USA). Data are presented as least square means \pm SEM.
251 ACE1-I and AOX data were analysed using a two-way ANOVA to identify the significant
252 differences between undigested, permeate and retentate in WPI, CP and CTR. The differences
253 between means were compared using Tukey's test and considered statistically significant at
254 $P < 0.05$. For gene expression analysis, the comparative CT method was used (Livak and
255 Schmittgen, 2001) to determine the fold changes in gene expression, which were calculated
256 with the threshold method ($2^{-\Delta\Delta CT}$). A two-way ANOVA was also performed on cell
257 metabolic activity and gene expression data and means were compared using Tukey's test ($P <$
258 0.05).

RESULTS

259

260 *In vitro digestion*

261 WPI, CP and CTR samples were *in vitro* digested and the total digesta was filtered with a 3kDa
262 membrane to obtain a permeate (<3kDa) and a retentate (>3kDa) fraction. The WPI, CP and
263 CTR permeate fractions were further tested for ACE1-I and AOX activities. The WPI, CP and
264 CTR retentate fractions were tested for ACE1-I, AOX and cytomodulatory effect. A
265 peptidomic/proteomic characterization was performed on WPI and CP permeate and retentate
266 to check the presence of bioactive peptides.

267 *Angiotensin-1-Converting Enzyme Inhibitory activity*

268 Whey protein isolate permeate exhibited the highest ACE1-I effect. In particular, WPI permeate
269 induced a significant ACE1-I inhibitory effect (49.2 ± 0.64 %) compared with WPI undigested
270 ($P < 0.001$), CP permeate ($P < 0.001$) and CP retentate ($P < 0.001$) and with CTR permeate (10.40
271 ± 1.07 %; $P < 0.001$). CP permeate induced a significant ACE1-I effect (23.91 ± 0.64 %)
272 compared with CTR permeate ($P < 0.001$), which showed the lowest ACE1-I effect.

273 **FIGURE 2**

274 *Total antioxidant capacity-ABTS assay*

275 Whey protein isolate and CP permeate showed a significant increase in AOX (1.58 ± 0.04 and
276 1.61 ± 0.02 $\mu\text{mol trolox AOX equivalents /mg protein (TrAOX equ/mg P)}$ respectively,
277 compared with undigested WPI and CP (< 0.6 $\mu\text{mol TrAOX equ/mg protein}$; $P = 0.02$), CTR
278 permeate (1.07 ± 0.04 $\mu\text{mol TrAOX equ/mg protein}$; $P = 0.04$) and WPI, CP and CTR retentate.
279 Low AOX was observed in CTR retentate (< 0.6 $\mu\text{mol TrAOX equ/mg protein}$).

280 *Proteomic/peptidomic analysis of digesta*

281 Based on the results obtained with ACE1-I and AOX activities, WPI and CP permeate and
282 retentate samples were analyzed for their peptide content. Permeate was characterized by LC-
283 nano ESI MS/MS using a shotgun-peptidomic approach while retentate was further digested
284 with trypsin and analysed by mass spectrometry with a shotgun-proteomic approach as shown
285 in Fig.1. The list of the peptides identified in the retentate and permeate of WPI and CP are
286 reported in the Supplementary Tables S1 (permeate) and S2 (retentate), respectively. All the
287 data sets from CP and WPI were searched in SATPdb and BIOPEP-UWM databases taking
288 into account possible further proteolysis to find potentially bioactive peptides. In accordance
289 with the results reported above, peptides with ace inhibitory and anti-hypertensive activity were
290 identified in the WPI permeate together with one peptide with anti-hypertensive properties in
291 the casein retentate (TPEVDDEALE) (Table 2).

292 **TABLE 2**

293 *Cell culture experiments*

294 In our study, the retentate fraction contained molecules with a molecular size larger than 3kDa,
295 these molecules can reach the colonic tissue and ideally can escape the small intestinal
296 absorption. There is little evidence that dietary bioactive peptides other than di and tri peptides,
297 can cross the gut wall and enter the blood circulation. Di-and tri- peptides can be absorbed by
298 selective transport systems (such as paracellular pathway via tight junctions, passive diffusion
299 via enterocytes, endocytosis or by carrier-mediated transport systems), larger peptides and
300 protein particulates may cross the gut wall though only in very small quantities (Miner-
301 Williams et al., 2014). Therefore, we selected the >3kDa retentate fraction for colon cell
302 experiments.

303

304 The results presented here indicated that all of the protein fractions, at specific concentrations,
305 were able to modulate the metabolic activity of the HT29-MTX-E12 cells and to exert trophic
306 effects on the intestinal epithelia. Treatment of HT29-MTX-E12 cell monolayer with WPI, CP
307 and CTR (soya protein isolate) retentate caused stimulation or reduction of cell metabolic
308 activity depending on the dosage, as measured by MTT test (mitochondrial activity). As shown
309 in Figure 3, in the lowest range of concentrations tested (0.16mg/g-1.33mg/g), the CTR
310 retentate was effective in significantly enhancing HT29-MTX-E12 cell metabolic activity
311 compared with untreated cells (0 mg/g). In particular after 24 hours, CTR retentate at the
312 concentrations from 0.16 to 0.66 mg/g significantly enhanced cell metabolic activity ($P<0.01$).
313 After 24 hours treatment, CP at the concentration of 0.33 and 0.66 mg/g increased cell metabolic
314 activity compared with untreated cells ($P<0.05$); whereas after 3 hours of CP and WPI and after
315 24 h WPI treatment cell metabolic activity remained unchanged compared with untreated cells
316 (0 mg/g). Considering the highest concentration range (2.66-21.31mg/g), we observed that,
317 WPI, CP and CTR retentate reduced the metabolic activity of the HT29-MTX-E12 cells in a
318 dose-dependent manner. In contrast, CTR retentate after 3 h treatment at the concentration from
319 2.66 up to 21.31 mg/g maintained cell metabolic activity compared with untreated cells
320 (0mg/g).

321 *MUC2 and MUC5AC gene expression*

322 **FIGURE 4**

323 We cultured HT29-MTX-E12 cells for 3 h in the presence of WPI, CP or CTR retentate.
324 Specific concentrations of CP retentate modulated the expression of mucins MUC5AC and
325 MUC2 in HT29-MTX-E12 cells. In particular, CP retentate promoted the expression of
326 MUC5AC mRNA at 0.33 mg/g ($P= 0.02$), 0.66 mg/g ($P=0.04$) and 1.33 mg/g ($P= 0.008$)
327 compared with untreated cells (0 mg/g) and with WPI and CTR. MUC5AC mRNA expression

328 remained unaltered ($P>0.1$) by WPI and CTR retentate treatment. CP retentate at the
329 concentration of 0.33mg/g also promoted the expression of MUC2 mRNA. The MUC2 mRNA
330 gene expression by WPI and CTR retentate remained unaltered.

331 **DISCUSSION**

332 In the present study we *in vitro* digested WPI, CP and soya (CTR) proteins to assess their ACE1-
333 I and AOX activities and to study the release of **the bioactive peptides**.

334 We reported that permeate and, to a lesser extent, retentate of WPI showed a significant ACE1-
335 I activity compared with undigested WPI, demonstrating that the *in vitro* digestion influenced
336 the liberation of ACE1-I peptides. Furthermore, also the permeate fraction of CP showed higher
337 ACE1-I activity after digestion, confirming the results reported in our previous study where the
338 ACE1-I activity was tested on a panel of dairy and plant protein supplements (Giromini et al.,
339 2017), and in the study of Petrat-Melin et al. (2015). In the CTR sample, the ACE1-I activity
340 was significantly reduced in permeate fraction, compared with CTR undigested. For peptides
341 to be effective ACE1-inhibitors, they must resist digestion and enter the circulation before
342 reaching the target organ. Therefore, the bioactivities observed *in vitro* may not directly
343 translate into significant effects *in vivo*, due to the differential bioavailability of peptides in
344 different individuals. The heterogeneity in individuals' responsiveness to proteins consumption
345 can prevent the identification of the association between dietary intakes and health, hinder the
346 identification of health benefits for specific population groups and limit our understanding of
347 the exact role of the different bioactive compounds. Moreover, also the absorption step in the
348 gastro-intestinal environment represents a key point in dairy peptide bioactivity. A bioactive
349 peptides to exert ACE1-I effect after oral administration must remain intact and active after
350 intestinal transport and brush border peptidases contact.

351 As reported by Miner-Williams et al (2014), only a few of the great number of milk peptides
352 with proven antihypertensive activity *in vitro* have so far proven to be clinically effective *in*

353 *vivo*. However, our recent studies (Fekete et al., 2016a; 2016b and 2018) demonstrated a
354 relationship between blood pressure reduction in humans as a result of chronic and acute
355 consumption of milk proteins-based supplements and their *in vitro* ACE1-I activity. Those
356 studies demonstrated a significant decrease in blood pressure in both whey and casein protein-
357 consumers groups, compared with the control group (no protein) in a double-blind cross-over
358 randomized controlled trial. The results reported in the present study further support the
359 aforementioned outcomes.

360 In the present study *in vitro* digestion significantly enhanced AOX capacity of WPI, CP and
361 CTR as evaluated by ABTS assay. In our experimental conditions, CP permeate and to a lesser
362 extent WPI permeate showed higher AOX properties, compared with CTR. Caseins have high
363 content of antioxidant amino acids as tyrosine and tryptophan, the oxidation of which may
364 quench free radicals inducing AOX effect (Clausen et al., 2009). Clausen et al. (2009) also
365 reported that caseins are the highest radical scavengers in milk. Di Pierro et al. (2014) reported
366 that the AOX capacity values for NaCN increased from 0.06 to 0.18 μmol trolox equivalents/mg
367 protein and for β -casein from 0.51 to 1.19 μmol trolox equivalents/mg protein, after enzymatic
368 hydrolysis. Petrat-Melin and co-authors (2015) assessed the AOX of *in vitro* digested β -casein
369 variants and they reported a significant increase in AOX capacity upon digestion. The AOX
370 capacity exhibited by the permeate of WPI, CP and CTR in this study enforces the claim that
371 food proteins are a natural source of AOX compounds. The distinct AOX properties of our
372 samples may be attributed to the specificity of the peptides or amino acids released from WPI,
373 CP and CTR primary sequences.

374 Based on ACE1-I and AOX activity results, we further analysed WPI and CP permeate and
375 retentate for their bioactive peptide content. In accordance with the results reported above,
376 peptides with ACE1-I and anti-hypertensive activity were identified in the WPI permeate
377 together with one peptide with anti-hypertensive property in the CP retentate. Only a few of
378 previous studies, such as those conducted by Petrat-Melin and co-authors (2015), assessed the

379 release of bioactive peptides after digestion. A lack of information about the comparison
380 between the protein bioactivity and the effective release of peptides associated to that
381 bioactivity exists. Therefore, the present study compared the biological activities of the digested
382 proteins with the identification of bioactive peptides. All together our data confirm WPI is a
383 rich source of ACE1-I peptides with tested bioactivity, produced upon digestion. Casein protein
384 permeate showed a significant ACE1-I activity upon digestion, although no bioactive peptides
385 associated to this activity have been found in permeate. With regard to antioxidant capacity,
386 although we observed a significant increase of AOX capacity after protein digestion, we did
387 not identify peptides associated to this bioactivity. This may be due to analytical limitations
388 associated with MS peptide identification as our method allowed to identify 6 or more amino
389 acids long peptides, shorter peptides or single amino acids may have not being identified.

390 A step forward in peptidomic analysis will be also to test ACE1-I peptides from WPI and CP
391 for their absorption capacity and for their ability of preserving their bioactivity after absorption
392 and cell internalization. This will massively help in the design of nutritional strategies to prevent
393 cardiovascular disease or to standardize the industrial production of ACE1-I peptides obtained
394 by enzymatic hydrolysis.

395 In our study, the retentate fraction contained molecules with a molecular size larger than 3kDa,
396 these molecules can ideally escape the small intestinal absorption and reach the colonic tissue.
397 There is little evidence that dietary bioactive peptides other than di and tri peptides, can cross
398 the gut wall and enter the blood circulation. Di- and tri- peptides can be absorbed by selective
399 transport systems (such as paracellular pathway via tight junctions, passive diffusion via
400 enterocytes, endocytosis or by carrier-mediated transport systems), larger peptides and protein
401 particulates may cross the gut wall though only in very small quantities (Miner-Williams et al.,
402 2014). Therefore, we selected the >3kDa retentate fraction for colon cell experiments.

403 Whey protein isolate ,CP and CTR retentate at the lower range of concentrations, maintained
404 or stimulated metabolic activity of undifferentiated HT29-MTX-E12 intestinal cells; whereas

405 the higher range of concentrations reduced cell
406 metabolic activity. Several food-derived peptides have been described to exert potential chemo-
407 preventative properties against the viability of malignant cells (Meisel and FitzGerald, 2003;
408 Fernández-Tomé et al., 2018). In this respect, the inhibitory role that we detected may be
409 interesting from the perspective that the HT29-MTX-E12 cells have a cancerous origin.
410 Moreover, we observed a hormetic effect induced by CTR retentate (3 and 24 hours treatment)
411 and, to a lesser extent, by CP retentate (24 hours treatment) in HT29-MTX-E12 cells. The
412 hormetic response is defined as a biological phenomenon whereby a stimulatory effect results
413 from exposure to low doses of a compounds that is otherwise inhibitory when given to a cell at
414 higher doses (Calabrese and Baldwin, 2002; Purup and Nielsen, 2012).
415 In general, *in vitro* cell-based models represent an effective tool to test nutritional ingredients
416 for food and feed evaluation (Cheli et al., 2015; Giromini et al., 2016; Fusi et al., 2018). Cell
417 culture studies have provided increasing evidence that milk-derived bioactive peptides
418 modulate metabolic activity, differentiation and/or apoptosis of different cell types (Hartmann
419 and Meisel, 2007). Pecorini and co-authors (2009) demonstrated that lactoferrin could be
420 involved in regulating the growth of both intestinal and mammary epithelial cells. Purup et al.
421 (2007) showed that whey fractions from bovine milk stimulated intestinal cells growth.
422 Furthermore, Meisel and FitzGerald (2003) hypothesized that milk peptides can cooperatively
423 stimulate the viability of intestinal cells, thus, the development of the digestive tract. In a recent
424 study, Purup and co-authors (2018) demonstrated that both casein and whey based supplements
425 exerted proliferative effects on intestinal cells, in agreement with our study. Tonolo et al. (2018)
426 reported the ability of milk peptides to increase cell proliferation. In general, cell proliferation is
427 an essential mechanism for the re-establishment of the surface epithelium after injury and food
428 proteins may have an important role in prevention and in the recovery of gut tissue integrity.
429 **However, whether the herein observed effect on cell metabolic activity is the result of individual**

430 peptides activity or of the synergistic activity of a peptide pool produced by *in vitro* digestion
431 is at present not known, and would need further investigations.

432 In our study, CP was able to significantly promote the expression of MUC5AC gene at all
433 concentrations tested in mucus-producing HT29-MTX-E12 cells (21 days of culture). Further,
434 we have found a relationship of the MUC2 expression effect of CP to dose: at the highest
435 concentrations tested, CP reduced MUC2 expression while at the lowest concentration tested
436 CP significantly improved MUC2 expression. Our results are in agreement with those of
437 previous studies that have demonstrated the role of milk peptides in the modulation of
438 gastrointestinal mucus production. Plaisancié et al. (2013) demonstrated that the total peptide
439 pool from yoghurt modulated cell proliferation and the secretion of mucins in HT29-MTX-E12
440 cells. Martínez-Maqueda et al. (2013) reported that casein hydrolysate stimulated HT29-MTX-
441 E12 cells and promotes the expression of MUC5AC. The same group demonstrated the mucin-
442 secreting role of whey protein hydrolysate in HT29-MTX-E12 cells: β -lactorphin increased the
443 synthesis of mucin proteins without eliciting differences in MUC5AC gene expression
444 (Martínez-Maqueda 2012a). Plaisancié et al. (2015) demonstrated an increase in mucin
445 secretion and MUC2 and MUC4 gene expression in HT29-MTX-E12. β -casomorphin-7
446 increases MUC5AC mRNA expression and the secretion of this mucin, as demonstrated by
447 Zoghbi et al. (2006). α -lactorphin, a whey-derived peptide, showed enhanced expression of the
448 MUC5AC gene in HT29-MTX-E12 cells (Martínez-Maqueda et al., 2012a).

449 The main limitation of our gene expression analysis is related to the lack of demonstrating
450 mucin secretion effect, which may precede gene expression stimulation (Martinez-Maqueda et
451 al., 2013) in the process of replenishing the intracellular mucin pool of goblet cells. This latter
452 point requires further analysis.

453 Overall, the proliferative and gene expression effects we observed in this study require further
454 investigations before the full influence of milk protein retentate can be determined at the
455 intestinal colon cells level.

456

CONCLUSIONS

457 Our study combines the identification of bioactive peptides by both a peptidomic and a
458 proteomic approach with *in vitro* bioactivities evaluated after simulated digestion. In summary,
459 our data show that whey proteins have the highest angiotensin-1-converting enzyme inhibitory
460 activity. Further, we found that whey proteins, after *in vitro* digestion, generate functional
461 peptides preserved, in particular those related to angiotensin-1-converting enzyme inhibitory
462 effect. This makes it possible to hypothesise that whey proteins can have similar effect *in vivo*.

463 Casein and soya proteins have stimulatory effects on cell metabolic activity in undifferentiated
464 HT29-MTX-E12 cells and, in particular, we found that casein proteins may promote mucus
465 related-gene expression in differentiated HT29-MTX-E12 cells. Furthermore, as whey, casein
466 and soya are effective in quite low concentrations, they may be useful as functional food
467 ingredients for the treatment of gut injury caused by inflammatory bowel diseases or diarrhoea
468 in new-borns mammals.

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630 **Figure legends:**

631 **Figure 1:**

632 Peptidomic and proteomic workflow on casein (CP), whey (WPI) and soy (CTR) proteins.

633 Retentates and permeates were analyzed by tandem mass spectroscopy using an LTQ

634 OrbitrapVelos and searched against the mammalia NCBI sequence database (casein and

635 whey samples) and UniProt glycine max (soy samples) by Sequest.

636

637 **Figure 2:**

638 ACE1- inhibitory effects of undigested (UND), permeate (P) and retentate (R) of WPI, CP and

639 soy (CTR). Data are normalized on the nitrogen content of each sample and are expressed

640 as percentage of ACE1-I. The synthetic ACE inhibitor captopril at the concentration of

641 20nM, included as control, inhibited 97% enzyme activity. Different superscript letters

642 denote significant differences ($P < 0.05$). ACEi, angiotensin-converting enzyme-1.

643

644 **Figure 3:**

645 Effects of WPI retentate (A), CP retentate (B) and CTR retentate (C) on HT29-MTX-E12 cell

646 metabolic activity (expressed as cell viability) after 3 and 24 h of treatment. Data are

647 expressed as means \pm SEM. Different superscript letters denote significant differences from

648 the metabolic activity obtained in the control cells (0 mg/ml) ($P < 0.05$).

649

650 **Figure 4:**

651 Effects of 3 hours treatment with WPI retentate, CP retentate and CTR retentate on MUC5AC

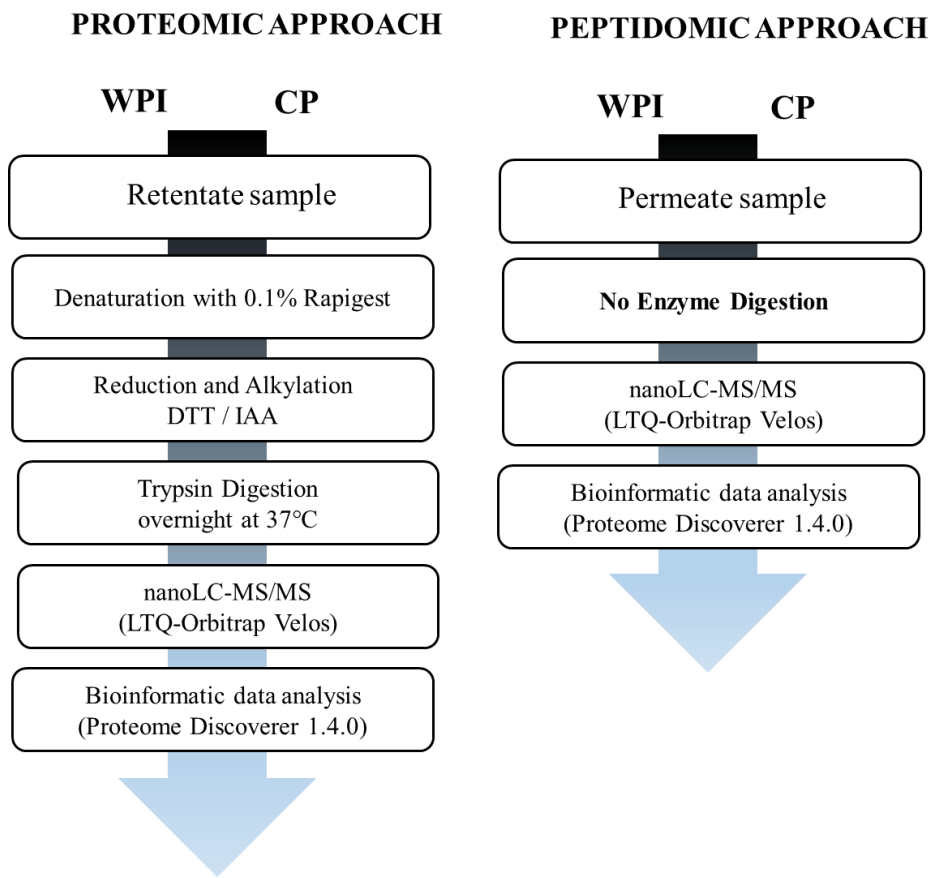
652 (A) and MUC (B) gene expression in the HT29-MTX-E12 cells (concentrations range 0.33-

653 1.33 mg/g) determined by qRT-PCR. The data are expressed as the relative gene expression

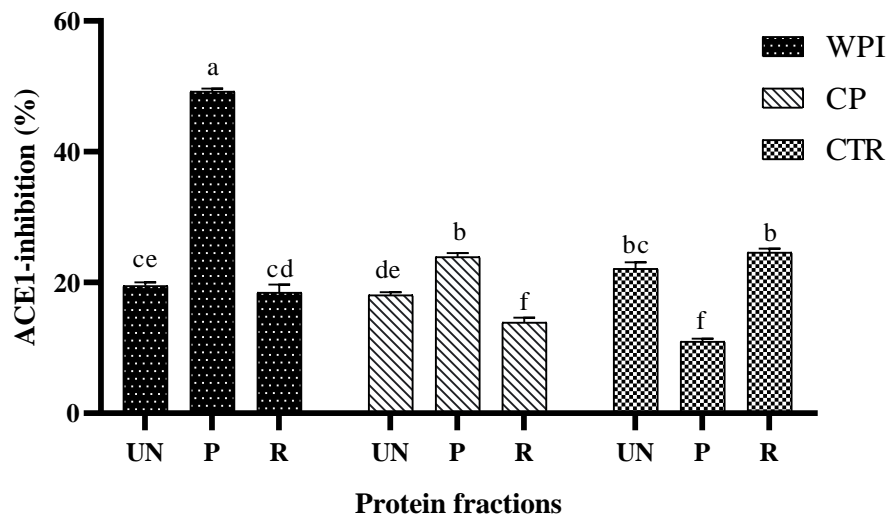
654 levels relative to the controls (untreated cells, 0 mg/g). Different superscript letters denote

655 significant differences ($P < 0.05$).

657 **Figure 1**



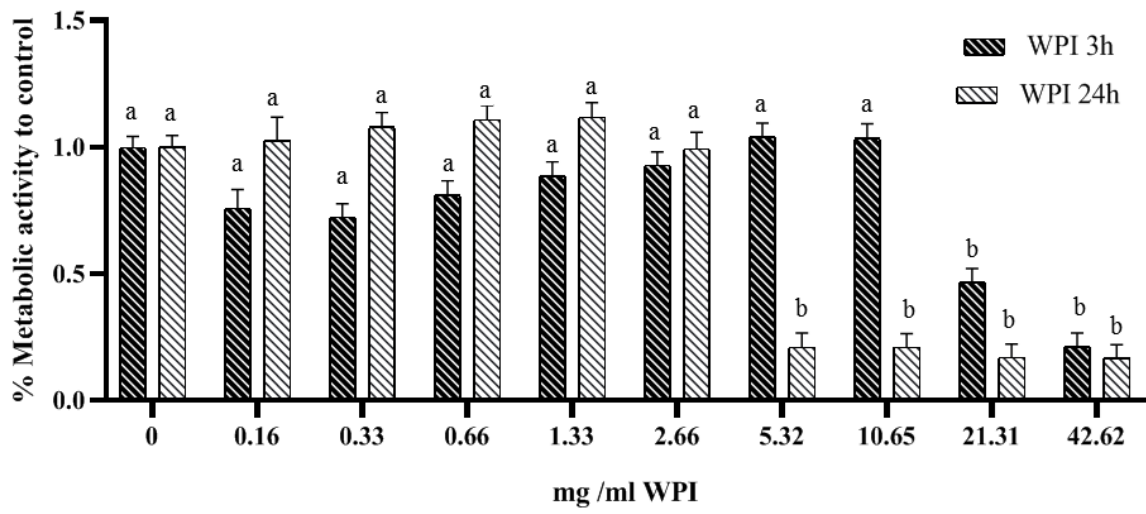
660 **Figure 2**



661

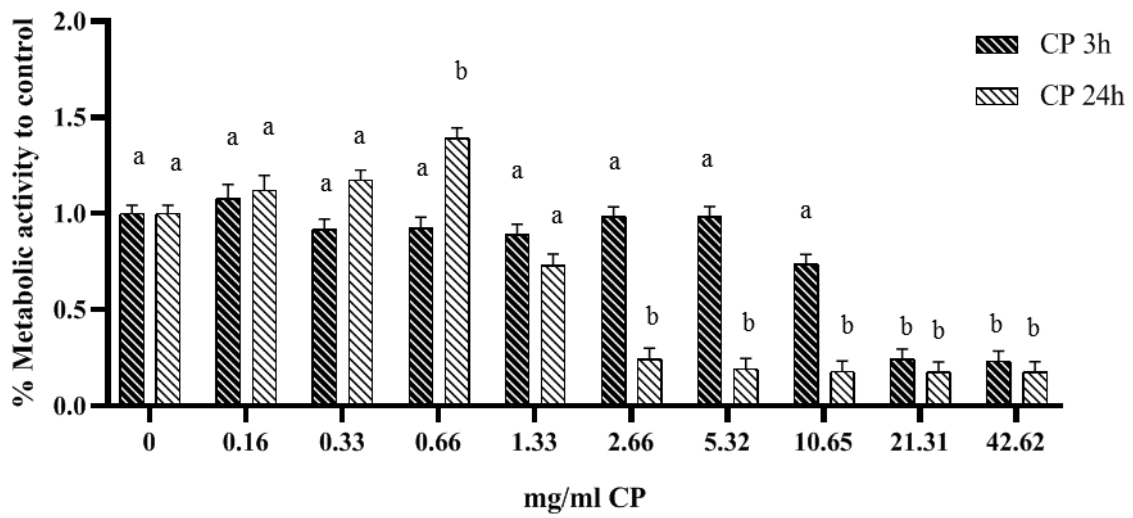
663 **Figure 3**

A



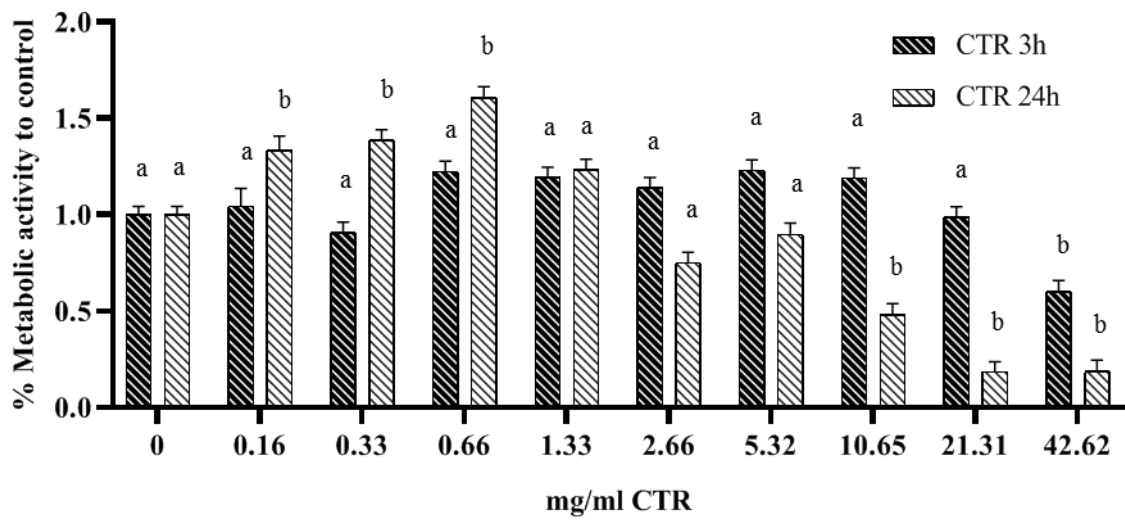
664

B



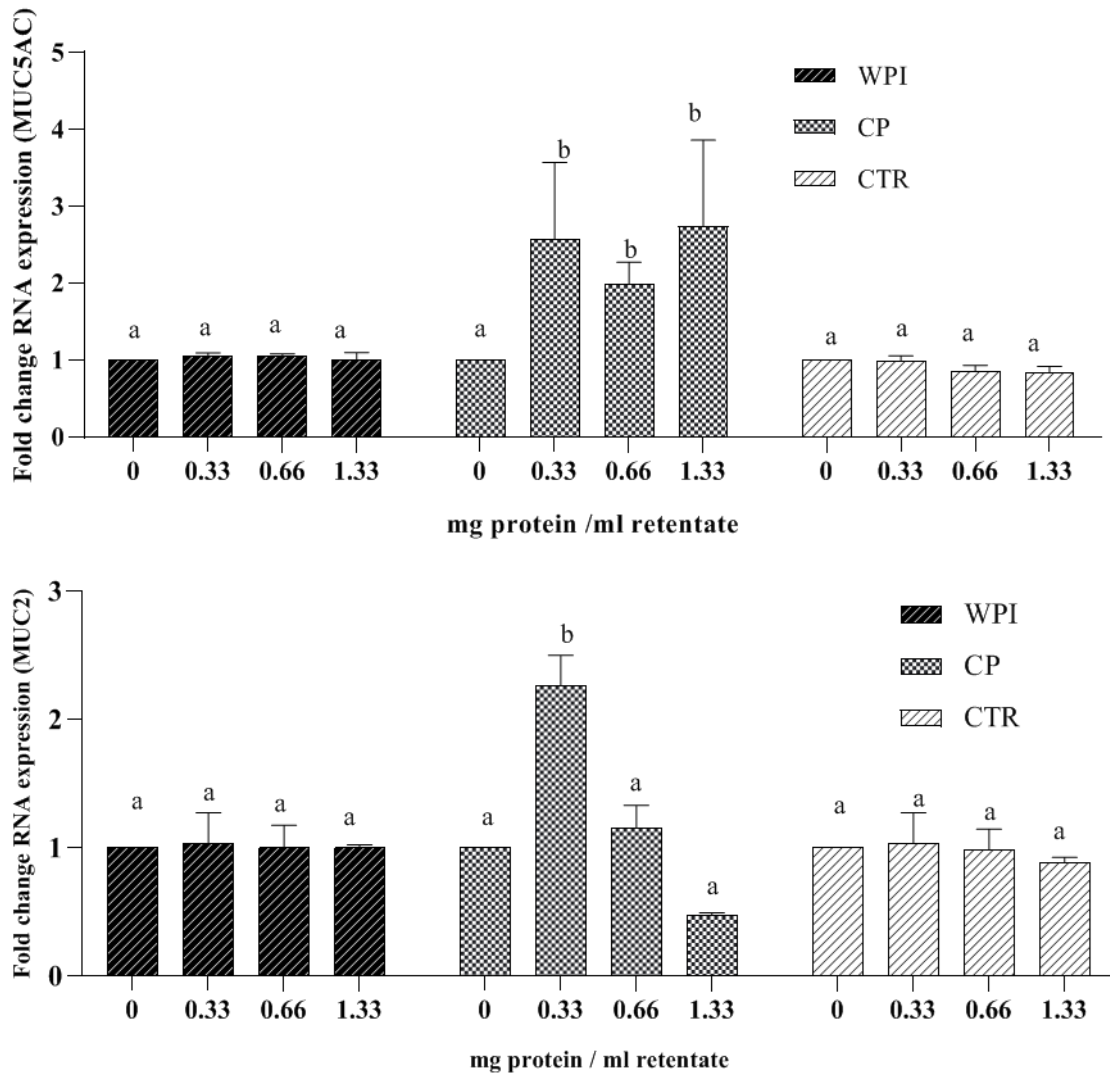
665

C



666

668 **Figure 4**



671 Giromini et al.

672 **Table 1**

Gene (&bp)	Primers	References
Mucin-5AC (MUC5AC) 240bp	5'- CGACCTGTGCTGTGTACCAT-3' 3'-CCACCTCGGTGTAGCTGAA-5'	Martínez-Maqueda et al., 2012a
Mucin-2 (MUC 2)	5'- ACCCCAAGCCCTTCTACGAG-3' 3'-GAGTGGATGCCGTTGATGGT-5'	Nielsen et al., 2018
β -actin (197bp)	5'-CTTCCTGGGCATGGAGTC-3' 3'-GCAATGATCTTGATCTTCATTGTG-5'	Martínez-Maqueda et al., 2012a
Cyclophillin (160bp)	5'-CTTCCTGGGCATGGAGTC-3' 3'-GCAATGATCTTGATCTTCATTGTG-5'	Martínez-Maqueda et al., 2012a

673

675 **Table 2**

	DataBase	Permeate	Retentate
Casein protein (CP)			
Anti hypertensive	TPEVDDEALE		<u>TPEVDDEALEK</u>
	TPEVDDEALE		<u>TPEVDDEALEKFDK</u>
Whey protein isolate (WPI)			
Anti hypertensive	IDALNENK	<u>KIDALNENKVLVLDTDYK</u>	
	VLDTDYK	<u>KIDALNENKVLVLDTDYK</u>	
	LKPTPEGN	<u>VEELKPTPEGNLE</u>	
	VEELKP	<u>VEELKPTPEGNLE</u>	
	LKPTPEGN	<u>VYVEELKPTPEGNLE</u>	
	VEELKP	<u>VYVEELKPTPEGNLE</u>	
	VYVEELKPTPE	<u>VYVEELKPTPEGNLE</u>	
Ace inhibitory	VLDTDYK	<u>KIDALNENKVLVLDTDYK</u>	

677 **Table legends**

678 **TABLE 1.** Primer sequences used for quantitative real time polymerase chain reaction.

679 Quantification by SYBR Green was used for all genes.

680 **TABLE 2.** List of Anti-hypertensive and ACE-Inhibitory peptides found in WPI and CP

681 permeate and retentate samples.

682 **TABLE S1: List of the peptides identified in the permeate of Whey protein isolate (WPI**

683 **and casein protein (CP).**

684 Permeate was analysed by LC-nano ESI tandem mass spectroscopy using a shotgun-label free

685 approach to identify ~~and quantify~~ peptides without any digestion before MS/MS with a

686 peptidomic approach. MS spectra were searched against the mammalia NCBI sequence

687 database (release 24.01.2017) (casein and whey samples) and UniProt glycine max (release

688 21.02.2018) (soy samples) by Sequest. Only peptides with False Discovery Rate 1% (against

689 decoy) and $Xcorr \geq 1.5$ were included for positive identification . Two replicates were carried

690 out for each sample in the MS analysis. PSMs displays the total number of identified peptide

691 sequences (peptide spectrum matches) for the corresponding protein, including those

692 redundantly identified.

693 **TABLE S2: List of the peptides identified in the retentate of Whey protein isolate (WPI**

694 **and casein protein (CP).**

695 Retentate was analysed by LC-nano ESI tandem mass spectroscopy using a shotgun-label free

696 approach to identify ~~and quantify~~ proteins and peptides with a proteomic approach. MS spectra

697 were searched against the mammalia NCBI sequence database (release 24.01.2017) (casein and

698 whey samples) and UniProt glycine max (release 21.02.2018) (soy samples) by Sequest. Only

699 peptides with False Discovery Rate 1% (against decoy) and $Xcorr \geq 1.5$ were included for

700 positive identification . Two replicates were carried out for each sample in the MS analysis.

701 PSMs displays the total number of identified peptide sequences (peptide spectrum matches) for
702 the corresponding protein, including those redundantly identified.

704 **Table S1**

PERMEATE					
WP1					
Accession	Description	Coverage	Unique Peptides		
5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	19,14	6		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	YVEELKPTPEGnLE	N12(Deamidated)	4,86	1618,77910	2
	TPEVDnEALEKFD	N6(Deamidated)	4,68	1635,76689	1
	K				
	VRTPEVDnEALEK	N8(Deamidated)	4,55	1890,93682	5
	FDK				
	TPEVDnEALEKFD	N6(Deamidated)	4,47	1507,67900	3
	TPEVDnEALEKFD	N6(Deamidated)	4,18	1706,80022	2
	KA				
	VEELKPTPEGnLE	N11(Deamidated)	4,08	1455,71477	8
	VEELKPTPEGNLE		4,07	1454,73503	1
Accession	Description	Coverage	Unique Peptides		
XP_01061	sodium channel	2,07	1		
5055.1	protein type 4 subunit alpha (predicted)				

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
GVNLFAGKFYYCI	T17(Phospho);	6,03	4535,88361	1
NTTtSERFDISEVN	M36(Oxidation);			
NKSECDSLmYt	T38(Phospho)			

Accession	Description	Coverage	Unique Peptides
XP_01981 4470.1	NAC-alpha domain- containing protein 1 isoform X3 (predicted)	1,28	1

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
sPPLQDSDLPLVqG	N-Term(Acetyl);	5,97	3720,59292	1
SVsEAsPEPQSEED	Q13(Deamidated)			
LTASPP	; S17(Phospho); S20(Phospho)			

Accession	Description	Coverage	Unique Peptides
XP_01061 0262.1	insulin receptor substrate 1 (predicted)	3,60	1

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
gAAELAHSsLLG	N-Term(Acetyl);	5,74	4593,16811	1
GPqGPGGMsAFTR	S10(Phospho);			
VNLSPNRNQSAKV	Q16(Deamidated)			
IRADS	; S22(Phospho)			

Accession	Description	Coverage	Unique Peptides
XP_01951 5287.1	keratin, type II cytoskeletal 1	8,17	1

isoform X2					
(predicted)					
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	GSKSISISVAGGGR	S16(Phospho);	5,70	4644,95571	1
	RsGFGGGYGGsGF	S25(Phospho)			
	GGGGFGGSGFGG				
	GFGSGGFGGGFGS				
	G				
Accession	Description	Coverage	Unique Peptides		
XP_01978	histone-lysine N-	11,55	1		
2783.1	methyltransferase				
	EHMT2 (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	GRILmGHATKSFP	M5(Oxidation);	5,67	4535,15249	1
	SSPSKGGACPSRA	S29(Phospho);			
	KMsMTGAGKsPPS	S36(Phospho);			
	VqSLA	Q41(Deamidated)			
Accession	Description	Coverage	Unique Peptides		
XP_01906	helicase POLQ-like	6,56	1		
2163.1	isoform X1				
	(predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	SVsEKFNLPRGsIQn	S3(Phospho);	5,63	4515,94692	1
	LLTGAAAsFSSCVLH	S12(Phospho);			
	FCEELEEFW	N15(Deamidated)			
		; S22(Phospho)			

Accession	Description	Coverage	Unique Peptides		
XP_01981	osteopontin	5,04	1		
8048.1	(predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	RIshELDsASSEVN	S3(Phospho); S8(Phospho)	4,63	1703,67278	1
Accession	Description	Coverage	Unique Peptides		
P02670.2	Kappa-casein;	7,29	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	AIVNTVDNPEAsSE	S12(Phospho)	4,39	1525,63445	1
Accession	Description	Coverage	Unique Peptides		
D3ZGB1.1	T-cell transcription factor NFAT5	1,16	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VSGNETSTTtPqVA tPG	T10(Phospho); T11(Phospho); Q13(Deamidated) ; T16(Phospho)	4,25	1988,70659	1
Accession	Description	Coverage	Unique Peptides		
XP_01930	PDZ domain- containing RING finger protein 4 isoform X1 (predicted)	2,19	1		

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
ISEsGKLDQEQsSS	S4(Phospho);	4,08	2087,73418	1
EH	S8(Phospho); S13(Phospho)			

CP

Accession	Description	Coverage	Unique Peptides		
P02662.2	Alpha-S1-casein	35,51	17		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	SDIPNPIGSEnSEKT	N11(Deamidated)	4,40062	1820,81267	4
	TM		3322		
	LHSMKEGIHAQQK		4,35386	2133,082079	1
	EPMIGV		1332		
	SDIPNPIGSENSEK		4,34833	1819,83452	1
	TTM		3359		
	EGIHAQQKEPMIG		4,20515	1907,922533	1
	VNQE		1558		
	YTDAPSFSDIPNPI		4,19215	2268,011278	1
	GSENSEK		5838		
	EGIHAQQKEPMIG		4,09921	2092,045702	4
	VNQELA		7415		
	YVPLGTQYTDAPS		4,09641	1411,665941	1
			5997		
	aPSFSDIPnPIGSEN	N-Term(Acetyl);	3,91413	1931,870409	1
	SEK	N9(Deamidated)	9986		
	EGIHAQQKEPMIG	Q16(Deamidated)	3,89798	2021,982225	4
	VNqEL		3789		

	DAPSFSDIPNPIGSE		3,87243	2003,899828	2
	NSEK		3901		
	PSFSDIPNPIGSENS		3,83802	1817,84038	1
	EK		9146		
	LHSMKEGIHAQQK		3,78493	2504,234911	2
	EPMIGVNQE		0229		
	SDIPNPIGSEnSEK	N11(Deamidated)	3,74387	1487,68059	1
			8603		
	SFSDIPNPIGSENSE		3,71012	1720,789598	1
	K		64		
	DIGSESTEDQAME		3,69360	1767,755785	1
	DIK		3516		
	AMEDIKqMEAE	Q7(Deamidated)	3,62951	1295,541063	1
			3741		
	EGIHAQQKEPMIG	N15(Deamidated)	3,58607	2093,042528	2
	VnQELA		0538		
	TDAPSFSDIPNPIGS		3,57744	2104,950243	1
	ENSEK		9322		
	SDIPNPIGSEnSEKT	N11(Deamidated)	3,51720	1689,766771	1
	T		19		
Accession	Description	Coverage	Unique	Peptides	
XP_01981	beta-casein	27,23	10		
8429.1	(predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	IEKFQSEEQQQTE		5,00189	2352,073582	1
	DELQDK		5905		
	KIEKFQSEEQQQT		4,80392	2480,175449	1
	EDELQDK		9806		

	FQSEEQQTDEL		4,69068	1853,763597	1
	QD		0027		
	FQSEEQQTDEL		4,51901	1981,858677	2
	QDK		9127		
	FQSEEQQTDEL		4,16888	1610,674364	1
			5708		
	FQSEEQQTDEL		3,85110	1738,735643	1
	Q		4736		
	IEKFQSEEQQTE		3,73675	1980,895067	1
	DEL		4656		
	YQEPVLGPVRGPF		3,64272	1881,063524	1
	PIIV		213		
	TEDELQDKIHPFA		3,58944	1899,897386	1
	QTQ		8452		
	YPVEPFTESqSLTL	Q10(Deamidated)	3,55397	1827,859667	1
	TD		4628		
Accession	Description	Coverage	Unique	Peptides	
5EEE_A	Chain A, Bovine Lactoglobulin Complex With Decanol	24,07	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VEELKPTPEGDLE		4,15981	1455,715868	1
			0066		
	YVEELKPTPEGDL		4,15354	1618,773851	2
	E		4426		
	TPEVDDEALEKFD		4,07667	1635,772997	1
	K		0647		

	VEELKPTPEGDL		4,00140	1326,669481	1
			1901		
	SLLDAQSAPLR		3,64488	1170,645433	1
			7447		
Accession	Description	Coverage	Unique Peptides		
5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	24,07	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VEELKPTPEGnLE	N11(Deamidated)	4,15981	1455,715868	1
			0066		
	YVEELKPTPEGnLE	N12(Deamidated)	4,15354	1618,773851	2
			4426		
	TPEVDnEALEKFD	N6(Deamidated)	4,07667	1635,772997	1
	K		0647		
	VEELKPTPEGnL	N11(Deamidated)	4,00140	1326,669481	1
			1901		
	SLLDAQSAPLR		3,64488	1170,645433	1
			7447		
Accession	Description	Coverage	Unique Peptides		
P02668.1	Casoxin-C;	10	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	ASGEPTSTPTTEAV		4,26080	1763,809496	2
	ESTV		5607		
	IASGEPTSTPTTEA		3,57952	1777,827806	1
	VEST		6186		

Accession	Description	Coverage	Unique Peptides		
O88799.1	Zonadhesin;	0,8	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	gYVVHNNHKCVLqI	N-Term(Acetyl);	5,28981	4604,144722	1
	HCGCKDAQGGFV	Q12(Deamidated)	3519		
	PAGKTWISRGCTQ				
	SCACV				
Accession	Description	Coverage	Unique Peptides		
XP_01978	zinc finger protein	5,81	1		
0943.1	211-like (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	WEEWCLLDEVqIH	Q11(Deamidated)	4,71433	3828,778463	1
	LYLDVmVENFAL	; M19(Oxidation)	0673		
	VCMLGNS				
Accession	Description	Coverage	Unique Peptides		
XP_01887	ras and Rab interactor	4,6	1		
2274.1	2 (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sGGRPGAGPELEL	N-Term(Acetyl)	4,62434	3567,642233	1
	GTAGSPGGAPPEA		2918		
	APGDCTRAPPPSS				
Accession	Description	Coverage	Unique Peptides		
XP_01982	serine/threonine-	1,75	1		
2162.1	protein kinase WNK2 isoform X12 (predicted)				

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
qAGAGPTAASDPC	Q1(Deamidated);	4,29783	3291,473484	1
GKAVqTqQPCSVR	Q18(Deamidated)	4873		
ASLSADIC	;			
	Q20(Deamidated)			

Accession	Description	Coverage	Unique Peptides
XP_01888	Golgi integral	2,99	1
0271.1	membrane protein 4 isoform X4 (predicted)		

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
EENLPDENEEQKq	Q13(Deamidated)	4,24904	2432,032444	2
SNqKQEN	;	9187		
	Q16(Deamidated)			

Accession	Description	Coverage	Unique Peptides
NP_00103	aspartate beta-	25,69	1
4734.1	hydroxylase domain- containing protein 1 isoform 2 (predicted)		

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
IPPGCELVVGGEPq	Q14(Deamidated)	4,23613	2971,359531	1
CWAEGHCLLVDD		2145		
SF				

Accession	Description	Coverage	Unique Peptides
XP_01984	pancreatic secretory	3	1
3937.1	granule membrane		

major glycoprotein					
GP2 (predicted)					
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
DSTISVEENGVSAE			3,79421	1679,768846	1
SR			5202		
Accession	Description	Coverage	Unique Peptides		
P81265.1	Polymeric immunoglobulin receptor (predicted)	1,59	1		
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
NLDTVTKEDEGW			3,73467	1406,641771	1
			6361		
Accession	Description	Coverage	Unique Peptides		
XP_01981	alpha-S2-casein (predicted)	5,86	1		
8430.1					
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
LTEEEKNRLNFLK			3,72728	1633,886034	1
			014		
Accession	Description	Coverage	Unique Peptides		
XP_01967	zinc finger protein	0,89	1		
1843.1	462 isoform X2 (predicted)				
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
dFAQDIDINPGAVY		N-Term(Acetyl)	3,70081	2206,984911	1
KCRHC			7823		

Accession	Description	Coverage	Unique Peptides		
XP_01978	gem-associated	1,19	1		
0354.1	protein 5 isoform X4 (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sDqETEEEAREPEL	N-Term(Acetyl);	3,53098	2060,866991	1
	PCGV	Q3(Deamidated)	2018		
Accession	Description	Coverage	Unique Peptides		
XP_01889	proline-rich protein	6,1	1		
5067.1	20A-like (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sVEADGPAqPAqP	N-Term(Acetyl);	3,52627	1310,577684	1
		Q9(Deamidated);	7065		
		Q12(Deamidated)			

705

707 **Table S2****RETENTATE**

WPI					
Accession	Description	Coverage	Unique Peptides		
Q01523.1	Defensin-5	12,77	1		
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	ESLSGVCEISGR	C7(Carbamido methyl)	3,107130	1293,6067	
			766	37	1
Accession	Description	Coverage	Unique Peptides		
2OXS_A	Chain A, Crystal Structure Of The Trypsin Complex With Benzamidine At High Temperature (35 C)	8,97	1		
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	LGEDNINVVEGNEQFISA		3,179255	2163,0579	1
	SK		962	09	
Accession	Description	Coverage	Unique Peptides		
XP_01984	39S ribosomal protein L43, mitochondrial (predicted)	8,18	1		
4211.1					
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	EVQNPAPTQRPAQ	Q3(Deamidated)	3,381425	1437,7003	1
		; N4(Deamidated)	858	65	

CP

Accession	Description	Coverage	Unique Peptides		
O02772.3	Fatty acid-binding protein 3	9,77	1		
	Peptide Sequence	Modifications	Xcorr A2	MH+ [Da]	# PSMs
	LGVEFDETTADDR		3,4	1467,6462	
				9	1
Accession	Description	Coverage	Unique Peptides		
5EEE_A	Chain A, Bovine Lactoglobulin Complex With Decanol	8,64	2		
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	TPEVDDEALEK		3,684055	1245,5748	
			805	77	1
	TPEVDDEALEKFDK		4,296661	1635,7678	
			377	7	5
Accession	Description	Coverage	Unique Peptides		
5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	8,64	1		
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	TPEVDNEALEK	N6(Deamidated)	3,684055	1245,5748	
			805	77	1
	TPEVDNEALEKFDK	N6(Deamidated)	4,296661	1635,7678	
			377	7	5
Accession	Description	Coverage	Unique Peptides		
P11839.3	Beta-casein	7,21	1		

Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
FQSEEQQTEDELQDK		4,379714	1981,8427	
		012	47	1
Accession	Description	Coverage	Unique Peptides	
Q4GZT4.2	ATP-binding cassette sub-family G member 2	2,29	1	
Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
EDIGDEANETEEPSK		3,325044	1662,6938	
		632	95	1

708