

*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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## **Interpretative Summary**

### ***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**

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

**Running head: Milk protein digestion: bioactivity and peptidomics**

***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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## ABSTRACT

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

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

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

## INTRODUCTION

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

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

## MATERIALS AND METHODS

### *Samples and reagents*

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

### *In vitro digestion*

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

Briefly, 20 g of WPI, CP or CTR sample were mixed with 150 mL of distilled H<sub>2</sub>O and maintained on an orbital shaker at 150 rpm for 5 minutes. The digestion procedure involved three phases. For the oral phase 6.66 mg  $\alpha$ -amylase in 2.1 mL of 1 mM CaCl<sub>2</sub>, pH 7 was added to the samples and they were incubated for 30 min at 37 °C on a shaker. For the gastric phase, 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 added. The samples were then incubated for 120 min at 37 °C on a shaker. For the small intestinal phase, the pH was increased to 7 with 6 M NaOH and 0.2 mg pancreatin and 1.2 g bile in NaHCO<sub>3</sub> 0.5 M were added to the samples before the final incubation of 180 min at 37 °C on a shaker was performed.

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

#### ***Samples preparation***

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

#### ***Angiotensin-1-converting enzyme inhibitory activity***

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

#### ***Total antioxidant capacity-ABTS assay***



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

#### ***Proteomic and peptidomic profile***

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

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

## **FIGURE 1**

### ***Peptidomic and proteomic data analysis***

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

### ***Cell culture experiments***

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

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

### ***Metabolic activity***

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

Undifferentiated HT29-MTX-E12 cells were plated at a density of 1.5x10<sup>4</sup> cells/well in 96-well plates and cultured for 24 hours. Whey protein isolate, CP and CTR retentate were diluted in 0.05% FCS DMEM (Giromini et al., 2015) and added to the cell culture at different concentrations (from 42.62 to 0.16 mg/ml). For the control wells, 0.05% FCS DMEM was added.

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

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

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

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

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

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

The HT29-MTX-E12 cells were seeded at a density of  $5 \times 10^5$  cells/well in 12-well plates and maintained for up to 21 days in complete medium (Martinez-Maqueda et al., 2012a). Martinez-Maqueda et al. (2012a) reported that, after 21 days of culture, HT29-MTX-E12 cells express a stable amount of mucus. At day 21, the cells were washed with PBS, and the medium was replaced with 0.05% FCS DMEM medium for 24 h (cell starvation). The treatment medium (0.05% FCS) with WPI, CP or CTR protein retentate at different concentrations (0.33 up to 1.33 mg/g) was added to the cells, which were then incubated in a controlled atmosphere (37°C / 5% CO<sub>2</sub>) for 3 hours. The concentration range was chosen based on the MTT test results. Control cells (0mg/g) were not treated.

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

#### **TABLE 1**

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

#### ***Statistical analysis***

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

## RESULTS

### *In vitro digestion*

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

### *Angiotensin-1-Converting Enzyme Inhibitory activity*

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

## FIGURE 2

### *Total antioxidant capacity-ABTS assay*

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

### *Proteomic/peptidomic analysis of digesta*

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

## TABLE 2

### *Cell culture experiments*

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

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

#### ***MUC2 and MUC5AC gene expression***

#### **FIGURE 4**

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



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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## CONCLUSIONS

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

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

## REFERENCES

- Aletti, A., F., Maffioli, A., Negri, M.H., Santamaria, F.A., De Lano, B., Kistler, G.W., Schmid-Schönbein, G., Tedeschi. 2016. Peptidomic analysis of rat plasma: proteolysis in hemorrhagic shock. *SHOCK*. 45: 540-554. [doi: 10.1097/SHK.0000000000000532](https://doi.org/10.1097/SHK.0000000000000532).
- Baldi, A., I. Politis, C., Pecorini, E., Fusi, R., Chronopoulou and V., Dell'Orto. 2005. Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. *J Dairy Res.* 72:66-72. <https://doi.org/10.1017/S002202990500110X>.
- Calabrese, E.J. and L.A., Baldwin. 2002. Defining hormesis. *Hum. Exp. toxicol.* 21:91-97.
- Cheli, F., C., Giromini, and A., Baldi. 2015. Mycotoxin mechanisms of action and health impact: '*in vitro*' or '*in vivo*' tests, that is the question. *World Mycotoxin J.* 8: 573-589. <https://doi.org/10.3920/WMJ2014.1864>.
- Clausen, M.R., L.H., Skibsted, J., Stagsted, 2009. Characterization of major radical scavenger species in bovine milk through size exclusion chromatography and functional assays. *J Agric Food Chem.* 57:2912-2919. DOI: 10.1021/jf803449t.
- Cocchetti, P., F., Tripodi, G., Tedeschi, S., Nonnis, O., Marin, S., Fantinato, C., Cirulli, M., Vanoni, L., Alberghina. 2008. The CK2 phosphorylation of catalytic domain of Cdc34 modulates its activity at the G1 to S transition in *Saccharomyces cerevisiae*. *Cell cycle.* 7:1391-1401. <https://doi.org/10.4161/cc.7.10.5825>.
- Dell'Orco, M., P., Milani, L., Arrigoni, O., Pansarasa, V., Sardone, E., Maffioli, F., Polveraccio, M., Bordoni, L., Diamanti, M., Ceroni, F.A., Peverali. 2016. Hydrogen peroxide-mediated induction of SOD1 gene transcription is independent from Nrf2 in a cellular model of neurodegeneration. *Biochim. Biophys. Acta Gene Regul. Mech.* 1859: 315-323. <https://doi.org/10.1016/j.bbagrm.2015.11.009>.

493 Di Pierro, G., M.B., O’Keeffe, A., Poyarkov, G., Lomolino, R.J., FitzGerald, 2014. Antioxidant  
 494 activity of bovine casein hydrolysates produced by *Ficus carica* L.-derived proteinase. Food  
 495 chem. 156: 305-311. <https://doi.org/10.1016/j.foodchem.2014.01.080>.

496 Fekete, Á. A., C., Giromini, Y., Chatzidiakou, D. I., Givens and J. A., Lovegrove. 2016a. Whey  
 497 protein lowers blood pressure and improves endothelial function and lipid biomarkers in  
 498 adults with prehypertension and mild hypertension: results from the chronic Whey2Go  
 499 randomized controlled trial. Am. J. Clin. Nutr. 104: 1534-1544.  
 500 <https://doi.org/10.3945/ajcn.116.137919>.

501 Fekete, Á. A., C., Giromini, Y., Chatzidiakou, D. I., Givens and J. A., Lovegrove. 2018. Whey  
 502 protein lowers systolic blood pressure and Ca-caseinate reduces serum TAG after a high-fat  
 503 meal in mildly hypertensive adults. Sci rep. 8: 5026. [https://doi.org/10.1038/s41598-018-](https://doi.org/10.1038/s41598-018-23333-2)  
 504 [23333-2. https://doi.org/10.1038/s41598-018-23333-2](https://doi.org/10.1038/s41598-018-23333-2).

505 Fekete, Á. A., D. I., Givens and J. A., Lovegrove. 2016b. Can milk proteins be a useful tool in  
 506 the management of cardiometabolic health? An updated review of human intervention trials.  
 507 Proc Nutr Soc. 75: 328-341. <https://doi.org/10.1017/S0029665116000264>.

508 Fernández-Tomé, S., J., Sanchón, I., Recio, B., Hernández-Ledesma. 2018. Transepithelial  
 509 transport of lunasin and derived peptides: Inhibitory effects on the gastrointestinal cancer  
 510 cells viability. J. Food Compos. Anal. 68: 101-110.  
 511 <https://doi.org/10.1016/j.jfca.2017.01.011>.

512 Fusi, E., C., Giromini, R., Rebucci, L., Pinotti, V., Caprarulo, F., Cheli, F., Vitari, C.,  
 513 Domeneghini, A., Baldi. 2018. Ochratoxin A cytotoxicity on Madin–Darby canine kidney  
 514 cells in the presence of alpha-tocopherol: Effects on cell viability and tight junctions. J Anim  
 515 Physiol An N. 102:350-355.

516 Giromini, C., Á. A., Fekete, D. I., Givens, A., Baldi and J. A., Lovegrove. 2017. Short-  
 517 Communication: A Comparison of the In Vitro Angiotensin-1-Converting Enzyme



518 Inhibitory Capacity of Dairy and Plant Protein Supplements. *Nutrients*. 9:1352.  
519 <https://doi.org/10.3390/nu9121352>.

520 Giromini, C., F., Cheli, R., Rebucci, A., Baldi. 2019. Invited review: Dairy proteins and  
521 bioactive peptides: Modeling digestion and the intestinal barrier. *J. Dairy Sci.* 102:929-942.

522 Giromini, C., A., Baldi, E., Fusi, R., Rebucci and S., Purup. 2015. Effect of growth factors,  
523 estradiol 17- $\beta$ , and short chain fatty acids on the intestinal HT29-MTX cells. *Cell Biol.*  
524 *Toxicol.* 31:199-209. <https://doi.org/10.1007/s10565-015-9304-y>.

525 Giromini, C., R., Rebucci, E., Fusi, L., Rossi, F., Saccone, A., Baldi. 2016. Cytotoxicity,  
526 apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines  
527 exposed to ochratoxin A. *Cell Biol. Toxicol.* 32: 249-258. [https://doi.org/10.1007/s10565-](https://doi.org/10.1007/s10565-016-9332-2)  
528 [016-9332-2](https://doi.org/10.1007/s10565-016-9332-2).

529 Hartmann, R., and H., Meisel. 2007. Food-derived peptides with biological activity: from  
530 research to food applications. *Curr. Opin. Biotechnol.* 18:163-169.  
531 <https://doi.org/10.1016/j.copbio.2007.01.013>.

532 Iametti, S., G., Tedeschi, E., Oungre, and F., Bonomi. 2001. Primary structure of kappa-casein  
533 isolated from mares' milk. *J. Dairy Res.* 68: 53-61.

534 Livak, K.J., and T.D., Schmittgen. 2001. Analysis of relative gene expression data using real-  
535 time quantitative PCR and the 2- $\Delta\Delta CT$  method. *Methods*. 25: 402-408.  
536 <https://doi.org/10.1006/meth.2001.1262>.

537 Maffioli E., S. Nonnis, R., Angioni, F., Santagata, B., Calì, L., Zanolli, A., Negri, A., Viola,  
538 and G., Tedeschi. 2017. Proteomic analysis of the secretome of human bone marrow-derived  
539 mesenchymal stem cells primed by pro-inflammatory cytokines. *J. Proteomics*. 166:115-  
540 126. <https://doi.org/10.1016/j.jprot.2017.07.012>.

541 Martínez-Maqueda, D., B., Miralles, S., De Pascual-Teresa, I., Reverón, R., Muñoz and I.,  
542 Recio. 2012a. Food-derived peptides stimulate mucin secretion and gene expression in  
543 intestinal cells. *J. Agric. Food Chem.* 60: 8600-8605. <http://dx.doi.org/10.1021/jf301279k>.

544 Martínez-Maqueda, D., B., Miralles, I., Recio and B., Hernández-Ledesma. 2012b.  
 545 Antihypertensive peptides from food proteins: a review. Food Funct. 3: 350-361.  
 546 <http://dx.doi.org/10.1039/C2FO10192K>.  
 547 Martínez-Maqueda, D., B., Miralles, E., Cruz-Huerta and I., Recio. 2013. Casein hydrolysate  
 548 and derived peptides stimulate mucin secretion and gene expression in human intestinal  
 549 cells. Int Dairy J. 32:13-19. <https://doi.org/10.1016/j.idairyj.2013.03.010>.  
 550 Meisel, H. and R. J., FitzGerald. 2003. Biofunctional peptides from milk proteins: mineral  
 551 binding and cytomodulatory effects. Curr. Pharm. Des. 9: 1289-1296.  
 552 <https://doi.org/10.2174/1381612033454847>.  
 553 Minekus, M., M., Alving, P., Alvito, S., Ballance, T., Bohn, C., Bourlieu, F., Carrière, R.,  
 554 Boutrou, M., Corredig, D., Dupont, C., Dufour, L., Egger, M., Golding, S., Karakaya, B.,  
 555 Kirkhus, S., Le Feunteun, U., Lesmes, A., Macierzanka, A., Mackie, S., Marze, D. J.,  
 556 McClements, O., Ménard, I., Recio, C.N., Santos, R.P., Singh, G.E., Vegarud, M.S.J.,  
 557 Wickham, W., Weitschies, and A., Brodkorb. 2014. A standardised static in vitro digestion  
 558 method suitable for food—an international consensus. Food Funct. 5: 1113-1124.  
 559 <https://doi.org/10.1039/C3FO60702J>.  
 560 Miner-Williams, W. M., B. R., Stevens and P. J., Moughan. 2014. Are intact peptides absorbed  
 561 from the healthy gut in the adult human? Nutr Res Rev. 27: 308-329.  
 562 <https://doi.org/10.1017/S0954422414000225>.  
 563 Minkiewicz P., J., Dziuba, A., Iwaniak, M., Dziuba, M., Darewicz. 2008. BIOPEP database and  
 564 other programs for processing bioactive peptide sequences. J AOAC Int. 91: 965-980.  
 565 Moon, J. K., and T., Shibamoto. 2009. Antioxidant assays for plant and food components.  
 566 J. Agric. Food Chem. 57:1655-1666. DOI: [10.1021/jf803537k](https://doi.org/10.1021/jf803537k).  
 567 Mukhopadhyay, A., N., Noronha, B., Bahar, M.T., Ryan, B.A., Murray, P.M., Kelly, I.B.,  
 568 O'Loughlin, J.V., O'Doherty and T., Sweeney. 2015. The anti-inflammatory potential of a

moderately hydrolysed casein and its 5 kDa fraction in *in vitro* and *ex vivo* models of the gastrointestinal tract. Food Funct. 6:612-621.

Nielsen, D. S. G., B. B., Jensen, P. K., Theil, T. S., Nielsen, K. E. B., Knudsen and S., Purup. 2018. Effect of butyrate and fermentation products on epithelial integrity in a mucus-secreting human colon cell line. J Funct Foods. 40: 9-17. <https://doi.org/10.1016/j.jff.2017.10.023>.

Pecorini, C., R., Rebucci, S., Truchet, and A., Baldi. 2009. In vitro effects of lactoferrin on intestinal and mammary epithelial cell lines. Ital. J. Anim. Sci. 8: 643-645. <https://doi.org/10.4081/ijas.2009.s2.643>.

Petrat-Melin, B., P., Andersen, J.T., Rasmussen, N.A., Poulsen, L.B., Larsen and J.F., Young. 2015. *In vitro* digestion of purified  $\beta$ -casein variants A 1, A 2, B, and I: Effects on antioxidant and angiotensin-converting enzyme inhibitory capacity. J Dairy Sci. 98: 15-26. <https://doi.org/10.3168/jds.2014-8330>.

Plaisancié, P., J., Claustre, M., Estienne, G., Henry, R., Boutrou, A., Paquet, J., Léonil. 2013. A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. J. Nutr. Biochem. 24: 213-221. <https://doi.org/10.1016/j.jnutbio.2012.05.004>.

Plaisancié, P., R., Boutrou, M., Estienne, G., Henry, J., Jardin, A., Paquet and J., Léonil. 2015.  $\beta$ -Casein (94-123)-derived peptides differently modulate production of mucins in intestinal goblet cells. J Dairy Res. 82: 36-46. <https://doi.org/10.1017/S0022029914000533>.

Purup, S. and T.S., Nielsen. 2012n. Cell-based models to test the effects of milk-derived bioactives. Animal. 6: 423-432. <https://doi.org/10.1017/S1751731111002540>.

Purup, S., S.D., Nielsen, T.T., Le, H., Bertelsen, J., Sørensen, and L.B., Larsen. 2018. Wound healing properties of commercial milk hydrolysates in intestinal cells. Int J Pept Res Ther. 1-9. <https://doi.org/10.1007/s10989-018-9692-y>.

594 Purup, S., M., Vestergaard, L. O., Pedersen, and K., Sejrsen. 2007. Biological activity of bovine  
 595 milk on proliferation of human intestinal cells. *J. Dairy Res.* 74:58-65.  
 596 <https://doi.org/10.1017/S0022029906002093>.  
 597 Re, R., N., Pellegrini, A., Proteggente, A., Pannala, M., Yang and C., Rice-Evans. 1999.  
 598 Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free*  
 599 *radicals in biology and medicine.* 26:1231-1237. <https://doi.org/10.1016/S0891->  
 600 [5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3).  
 601 Sanchón, J., S., Fernández-Tomé, B., Miralles, B., Hernández-Ledesma, D., Tomé, C.,  
 602 Gaudichon and I., Recio. 2018. Protein degradation and peptide release from milk proteins in  
 603 human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem.* 239:486-  
 604 494. <https://doi.org/10.1016/j.foodchem.2017.06.134>  
 605 Shimizu, M. 2004. Food-derived peptides and intestinal functions. *Biofactors.* 21:43-47.  
 606 Singh, S., K., Chaudhary, S.K., Dhanda, S., Bhalla, S.S., Usmani, A., Gautam, A., Tuknait, P.,  
 607 Agrawal, D. and Mathur Raghava, G.P. 2016. SATPdb: a database of structurally annotated  
 608 therapeutic peptides. *Nucleic Acids Res.* 44: D1119-26.  
 609 Thaipong, K., U., Boonprakob, K., Crosby, L., Cisneros-Zevallos and D. H., Byrne. 2006.  
 610 Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity  
 611 from guava fruit extracts. *J Food Compost Anal.* 19:669-675.  
 612 <https://doi.org/10.1016/j.jfca.2006.01.003>.  
 613 Tonolo, F., M., Sandre, S., Ferro, A., Folda, V., Scalcon, G., Scutari, E., Feller, O., Marin, A.,  
 614 Bindoli and M. P., Rigobello. 2018. Milk-derived bioactive peptides protect against  
 615 oxidative stress in a Caco-2 cell model. *Food Funct.* <https://doi.org/10.1039/C7FO01646H>.  
 616 Vermeirssen, V., J., Van Camp, K., Decroos, L., VanWijmelbeke, and W., Verstraete. 2003.  
 617 The impact of fermentation and *in vitro* digestion on the formation of angiotensin-I-converting  
 618 enzyme inhibitory activity from pea and whey protein. *J. Dairy Sci.* 86: 429–438.

619 Vizcaino, J. A., A., Csordas, N., del-Toro, J. A., Dianes, J., Griss Lavidas, I., G., Mayer, Y.,  
 620 Perez-Riverol, F., Reisinger, T., Ternent, Q.W., Xu, Rui Wang, R., and Hermjakob, H. 2016  
 621 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44: D447-D456. doi:  
 622 10.1093/nar/gkv1145.

623 Volstatova, T., J., Havlik, M., Potuckova, and M., Geigerova. 2016. Milk digesta and milk  
 624 protein fractions influence the adherence of *Lactobacillus gasseri* R and *Lactobacillus casei*  
 625 FMP to human cultured cells. *Food Funct.* 7:3531-3538.

626 Zoghbi, S., A., Trompette, J., Claustre, M. E., Homsí, J., Garzón, G., Jourdan, J. Y., Scoazec  
 627 and P. Plaisancié, 2006.  $\beta$ -Casomorphin-7 regulates the secretion and expression of  
 628 gastrointestinal mucins through a  $\mu$ -opioid pathway. *Am. J. Physiol. Gastrointest. Liver*  
 629 *Physiol.* 290: G1105-G1113. <https://doi.org/10.1152/ajpgi.00455.2005>.

**Figure legends:**

**Figure 1:**

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

Retentates and permeates were analyzed by tandem mass spectroscopy using an LTQ OrbitrapVelos and searched against the mammalia NCBI sequence database (casein and whey samples) and UniProt glycine max (soy samples) by Sequest.

**Figure 2:**

ACE1- inhibitory effects of undigested (UND), permeate (P) and retentate (R) of WPI, CP and soy (CTR). Data are normalized on the nitrogen content of each sample and are expressed as percentage of ACE1-I. The synthetic ACE inhibitor captopril at the concentration of 20nM, included as control, inhibited 97% enzyme activity. Different superscript letters denote significant differences ( $P < 0.05$ ). ACEi, angiotensin-converting enzyme-1.

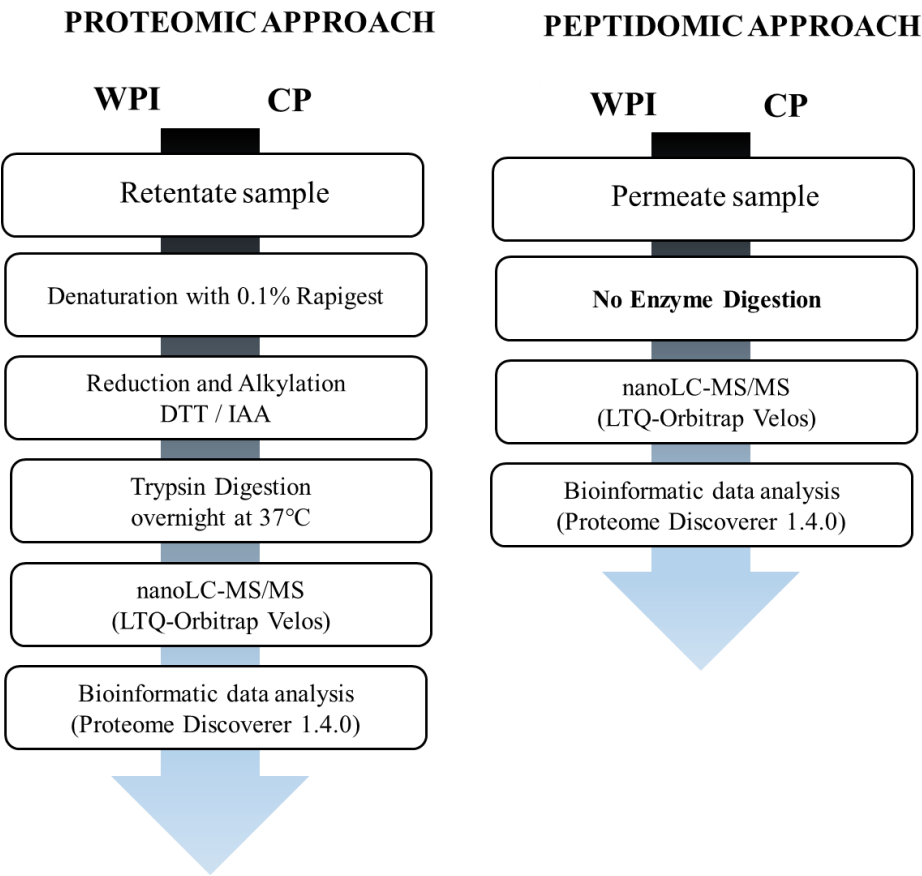
**Figure 3:**

Effects of WPI retentate (A), CP retentate (B) and CTR retentate (C) on HT29-MTX-E12 cell metabolic activity (expressed as cell viability) after 3 and 24 h of treatment. Data are expressed as means  $\pm$  SEM. Different superscript letters denote significant differences from the metabolic activity obtained in the control cells (0 mg/ml) ( $P < 0.05$ ).

**Figure 4:**

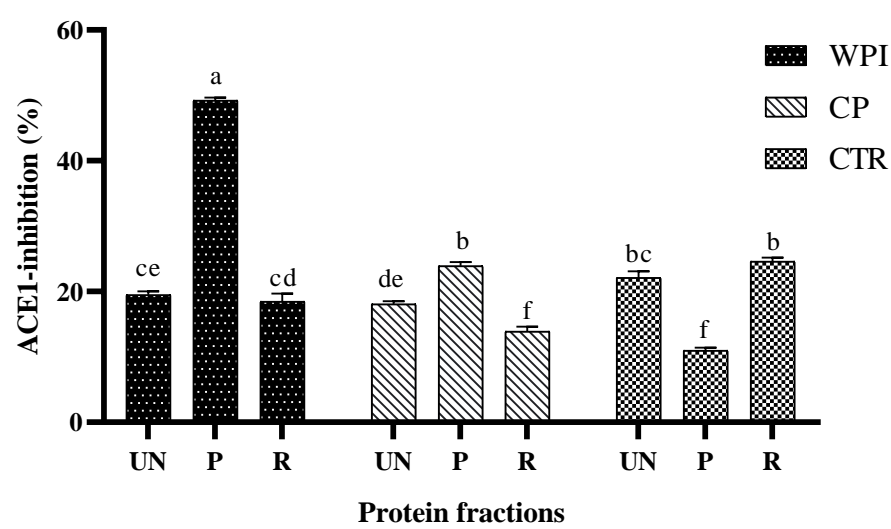
Effects of 3 hours treatment with WPI retentate, CP retentate and CTR retentate on MUC5AC (A) and MUC (B) gene expression in the HT29-MTX-E12 cells (concentrations range 0.33-1.33 mg/g) determined by qRT-PCR. The data are expressed as the relative gene expression levels relative to the controls (untreated cells, 0 mg/g). Different superscript letters denote significant differences ( $P < 0.05$ ).

657 **Figure 1**



658

660 **Figure 2**

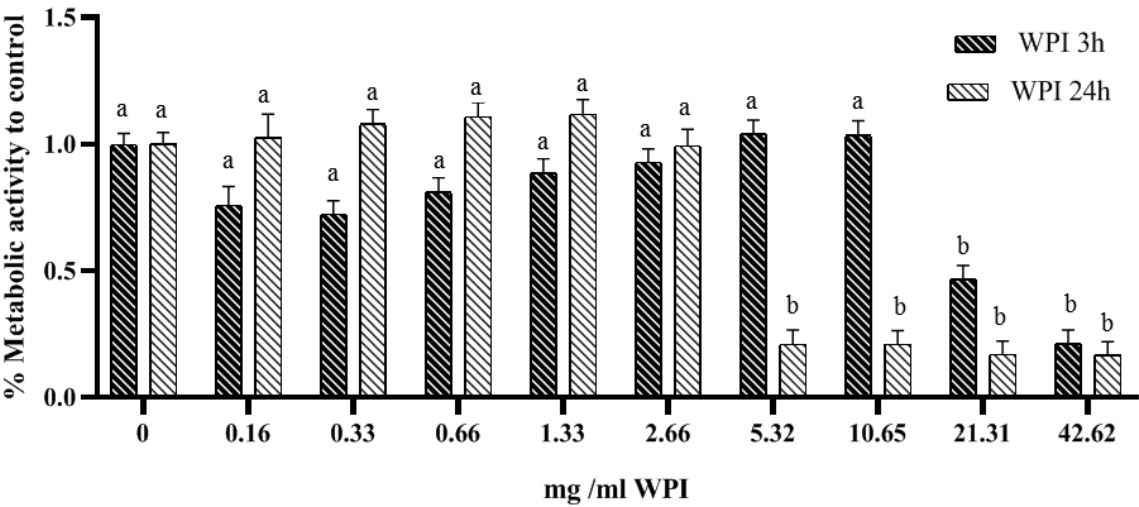


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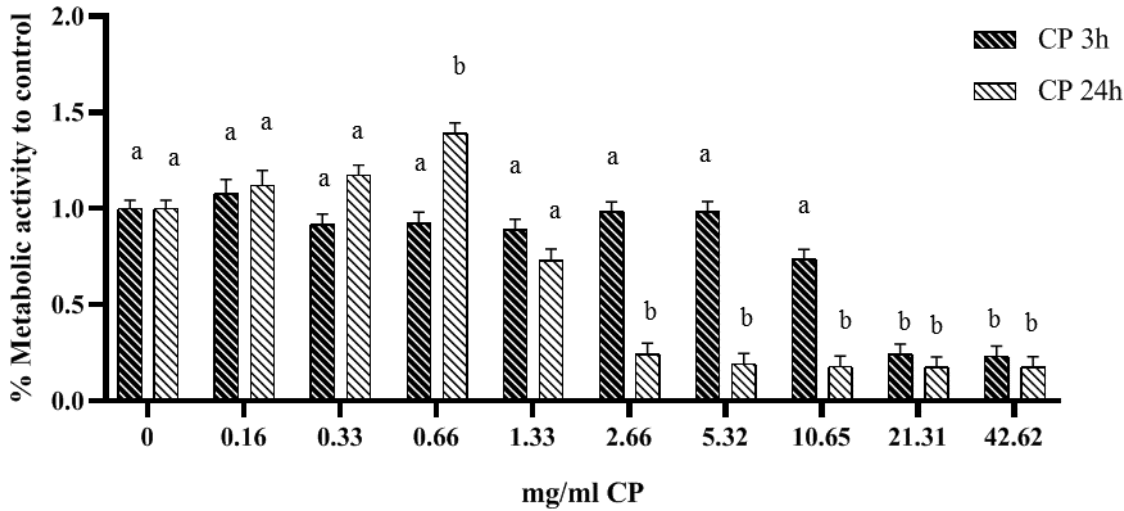
663 **Figure 3**

A



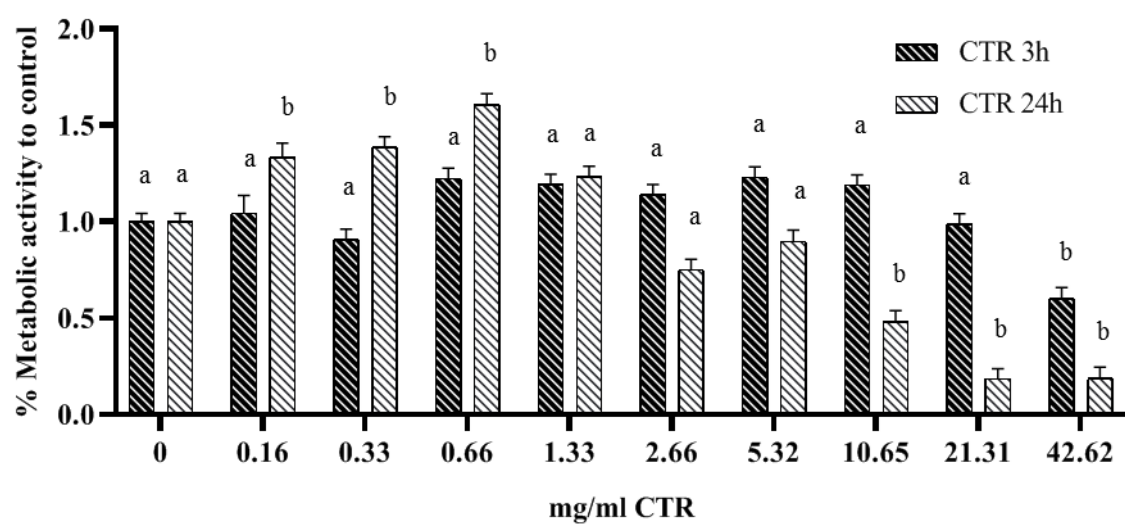
664

B



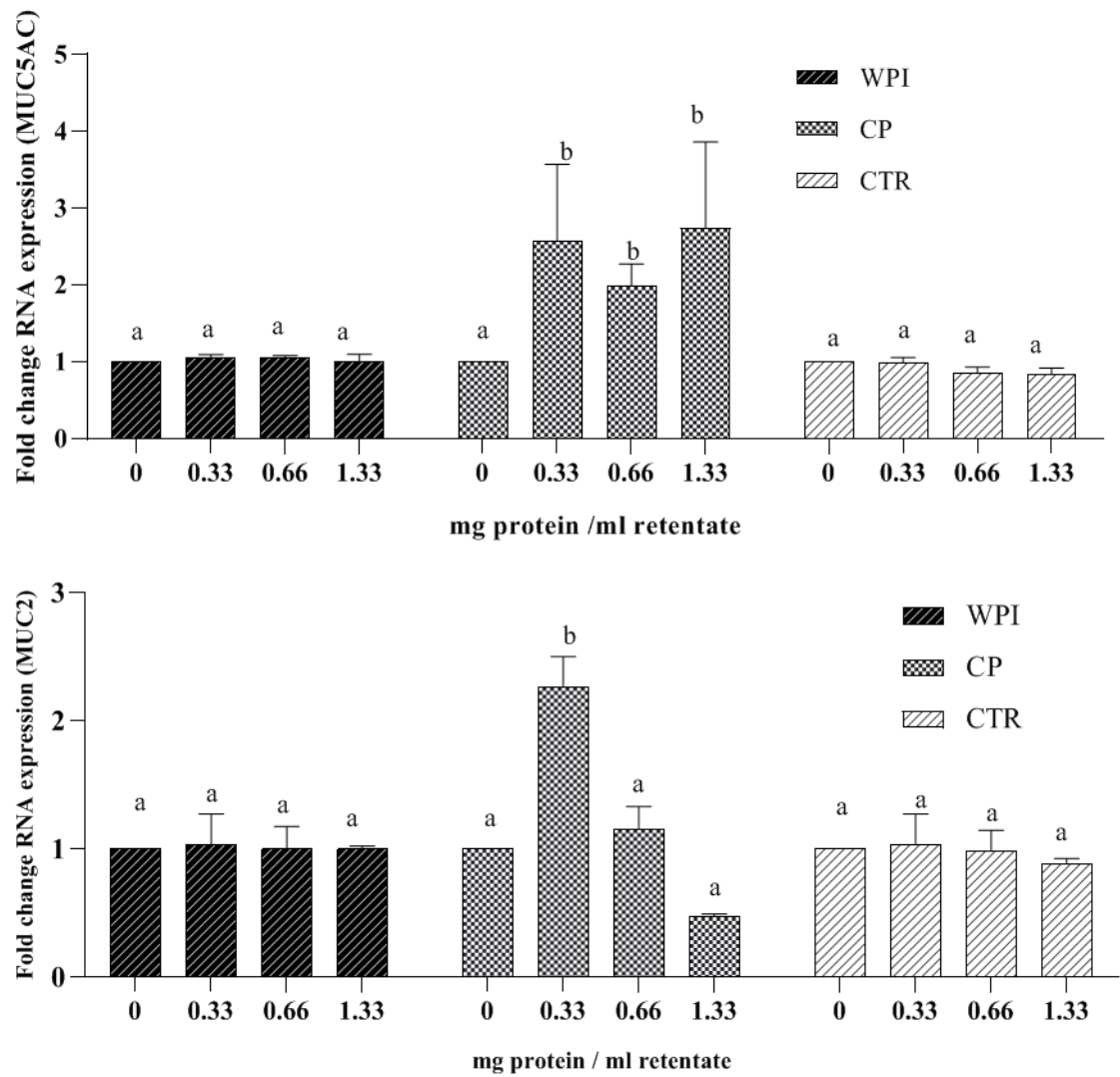
665

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
<b>Casein protein (CP)</b>			
<b>Anti hypertensive</b>	TPEVDDEALE		<u>TPEVDDEALEK</u>
	TPEVDDEALE		<u>TPEVDDEALEKFDK</u>
<b>Whey protein isolate (WPI)</b>			
<b>Anti hypertensive</b>	IDALNENK	<u>KIDALNENK</u> VLVLDTDYK	
	VLDTDYK	KIDALNENKVL <u>VLDTDYK</u>	
	LKPTPEGN	VEEL <u>LKPTPEGNLE</u>	
	VEELKP	<u>VEELKP</u> TPEGNLE	
	LKPTPEGN	VYVEEL <u>LKPTPEGNLE</u>	
	VEELKP	VY <u>VEELKP</u> TPEGNLE	
	VYVEELKPTPE	<u>VYVEELKPTPE</u> GNLE	
<b>Ace inhibitory</b>	VLDTDYK	KIDALNENKVL <u>VLDTDYK</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.



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704 **Table S1**

PERMEATE					
WP1					
Accession	Description	Coverage	Unique Peptides		
5EED_B	Chain B, Ovine	19,14	6		
	Lactoglobulin				
	Complex With				
	Decanol				
	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	NAC-alpha domain-	1,28	1		
4470.1	containing protein 1				
	isoform X3				
	(predicted)				
	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	insulin receptor	3,60	1		
0262.1	substrate 1				
	(predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	gAAELAAHSsLLG	N-Term(Acetyl);	5,74	4593,16811	1
	GPqGPGGMsAFTR	S10(Phospho);			
	VNLSPNRNQSAKV	Q16(Deamidated)			
	IRADS	; S22(Phospho)			
Accession	Description	Coverage	Unique Peptides		
XP_01951	keratin, type II	8,17	1		
5287.1	cytoskeletal 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-	2,19	1		
8010.1	containing RING finger protein 4 isoform X1 (predicted)				

Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
ISEsGKLsDQEQsSS	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		
	EGIHAAQQKEPMIG		4,20515	1907,922533	1
	VNQE		1558		
	YTDAPSFSDIPNPI		4,19215	2268,011278	1
	GSENSEK		5838		
	EGIHAAQQKEPMIG		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		
	EGIHAAQQKEPMIG	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		

	FQSEEQQTEDEL		4,69068	1853,763597	1
	QD		0027		
	FQSEEQQTEDEL		4,51901	1981,858677	2
	QDK		9127		
	FQSEEQQTEDEL		4,16888	1610,674364	1
			5708		
	FQSEEQQTEDEL		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
	VEELKPTPEGDL		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
	gYVVHNHKKCVLqI	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
	IPPGCELVVGGEp	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		
<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
P81265.1	Polymeric	1,59	1		
	immunoglobulin				
	receptor (predicted)				
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
NLDTVTKEDEGW			3,73467	1406,641771	1
			6361		
<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
XP_01981	alpha-S2-casein	5,86	1		
8430.1	(predicted)				
Peptide Sequence		Modifications	XCorr	MH+ [Da]	# PSMs
LTEEEKNRLNFLK			3,72728	1633,886034	1
			014		
<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
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**

<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
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
<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
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	
<b>Accession</b>	<b>Description</b>	<b>Coverage</b>	<b>Unique Peptides</b>		
XP_01984	39S ribosomal protein L43,	8,18	1		
4211.1	mitochondrial (predicted)				
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	EVQNPAPTQRPAQ	Q3(Deamidated)	3,381425	1437,7003	1
		;	858	65	
		N4(Deamidated)			

## 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	8,64	2		
	Lactoglobulin Complex				
	With Decanol				
	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	8,64	1		
	Lactoglobulin Complex				
	With Decanol				
	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

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