Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder

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ABSTRACT

This study aims to evaluate the impact of heat treatment on the hydrolysis kinetics of milk proteins and on the peptide release during *in vitro* dynamic gastric digestion. SDS-PAGE and ELISA techniques were employed to assess the hydrolysis of proteins over time of digestion. The evolution of the peptidome generated through dynamic digestion of heated and non-heated milk, was studied at different times, using MS-based techniques (ion trap and MALDI-TOF/TOF) coupled to liquid chromatography. The peptide homology value between both samples at the end of digestion (48%), confirmed the impact of heat treatment on the identity of peptides generated during digestion, despite their identical initial protein content and being the same matrix in both cases. Heat treatment produced an increased resistance to hydrolysis by pepsin in the casein fraction. However, β-Lg was found to be more susceptible to hydrolysis. Although differences on the pattern of peptide release were found between both samples, also some common traits after digestion were observed. The regions comprised between the residues 76-93 of β-casein, where several binding epitopes are included, as well as the β-casein domains 126-140 and 190-209 were found to be resistant to pepsin.

Keywords: dynamic digestion / Peptidomics/ mass spectrometry/ heat treatment

1.Introduction

The study of the digestion process has gained importance in the last years, since the released products may elicit subsequent health effects. The evaluation of the peptides generated during digestion may be relevant to understand the behavior of proteins upon this process, and also nutritional, pharmacological and toxicological concerns. The formation of bioactive peptides derived from milk proteins digestion has been recently reviewed (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014a), pointing out the MS-based techniques as indisputable tools to perform targeted or untargeted analyses of digests. Several *in vitro* static digestion models have been developed and widely employed over the years (Guerra et al., 2012; Hur, Lim, Decker, & McClemens, 2011; Kopf-Bolanz et al., 2012). These models intend to mimic physiological conditions, however, they lack of mechanisms to control the sequential secretion of enzymes, the removal of digestion products, the appropriate mixing and the continuous changes in pH. Therefore, dynamic digestion models, have been proposed as more realistic with respect to physiological parameters (Guerra et al., 2012; Ménard et al., 2014; Wickham, Faulks, & Mills, 2009). The gastric phase plays an important role in digestion, as the peptides produced during this step are delivered to small intestine, where they can either interact in situ with gastrointestinal receptors or be absorbed, mainly in duodenum and upper jejunum (Shimizu et al., 2010; Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011).

However, it is increasingly evident that food processing can influence the behavior of proteins upon digestion, and therefore it may affect the release of peptides. Milk products are often thermally treated in food industry in order to

lengthen their self-life, to reduce pathogen risk, or to modify their functional properties (Guo, Fox, Flynn, & Kindstedt, 1996). The modifications that proteins usually undergo upon these technological processes include unfolding, protein aggregation, oxidations, and Maillard reaction, which leads to a blockage of Lys. Heat treatment may also produce the formation of non-natural amino acids, i.e. isopeptide bonds in lysinoalanine or lantionine, which are no longer substrate of digestive proteases. Wada and Lönnerdal (2014) reported high content of lactulosyl-lysine after *in vivo* and *in vitro* digestion of milks subjected to heat treatments at high temperatures i.e. sterilized and ultra high temperature (UHT) milks. The formation of heat-induced aggregates of casein and whey protein can occur (Patel, Singh, Anema, Creamer, 2006; Jean, Renan, Famelart, Guyomarc'h, 2006). Likewise, modifications on β-Lactoglobulin (β-Lg) structure produced by heat treatment have been evaluated under different conditions, for instance different values of pH, ionic strength, or protein concentration (Loveday, Wang, Rao, Anema & Singh, 2012; Aymard, Durand & Nicolai, 1996; Iametti, Cairoli, Gragory & Bonomi, 1995). These changes may have distinct effect on the allergenic response (Shandilya, Kapila, Haq, Kapila & Kansal, 2013), or on the formation of bioactive peptides through digestion (Meisel et al., 1998). The heat-induced changes in food proteins influence their stability to *in vitro* static digestion. Heating of milk has significant effect on the level of digestion and affect in a different manner the digestion of caprine and bovine milk (Almaas et al., 2006). Casein becomes more resistant to digestion when heat treatment is applied, as revealed in infant formulas subjected to different heating processes i.e. 80°C/20 s, 85°C/180 s, 105°C/60 s, where the latter one had the strongest effect (Dupont, Boutrou, Ménard, Jardin, Tanguy, et al., 2010a). These

97 modifications induced by heat may also affect the kinetics of protein digestion *in*
98 *vivo,* in a mini-pig model (Barbé et al., 2013). Although it has been reported that
99 β-Lg is resistant to pepsin digestion (Miranda, & Pelissier, 1983), there is also
100 evidence that under certain conditions it can be susceptible to hydrolysis by this
101 enzyme. Heating temperatures above 90ºC, and especially 100ºC, induce
102 conformational changes in this protein, which provoke the exposure of the
103 hydrophobic regions, increasing its susceptibility to hydrolysis by pepsin
104 (Guo, Fox, & Flynn, 1995; Peram, Loveday Ye, & Singh, 2013). In this regard, the
105 assessment of the peptidomic profile generated through dynamic digestion of
106 milk products subjected to different heat treatments can help to understand the
107 protein behavior during gastric digestion. Using a dynamic model represents a
108 step forward in terms of mimicking physiological conditions. The pH is an
109 important parameter that will affect the optimum activity of the enzyme. The
110 model used in this work followed a pH curve previously used in TIM-1 by
111 Minekus, Marteau, Havenaar, and Huis in Veld (1995), based on human trials
112 conducted by Marteau et al. (1991), and that showed good correlation. The units
113 of pepsin used were chosen from a pig model (Chiang, Croom, Chuang, Chiou, &
114 Yu, 2008), and the gastric emptying rates were selected from a trial conducted in
115 mini-pig fed the same milk samples employed in the present work (Barbé et al.,
116 2013). The pig is a suitable model to predict differences among dietary protein
117 digestibility in men, showing good inter-species correlation in terms of true N and
118 amino acid digestibility (Deglaire, Bos, Tomé, & Moughan, 2009). Therefore, the
119 aim of this work is to evaluate the impact of heat treatment on the hydrolysis
120 kinetics of milk proteins and on the evolution of the peptidome generated at

different times of gastric dynamic digestion under these conditions, using two mass spectrometry analyzers (Ion trap and MALDI TOF/TOF).

2.Materials and methods

2.1 Samples

Two milk samples (unheated and heat treated skim milk) were used for the experiments. They were prepared using a skimmed milk powder as described by Barbé et al. (2013). Briefly, the powder was ultra low heat cow skim milk containing 4% of humidity, 34% of protein, 54% of lactose and 8% of ash. The powder was reconstituted in distilled water to reach a final concentration of 50 g/L of protein, using a stirrer for 15 min to achieve full solubilization. Heat treatment (90ºC, 10 min) was applied to the reconstituted liquid to obtain the heat-treated milk sample. The unheated milk did not undergo any further treatment after reconstitution of the powder. For digestion process, 200 mL of each sample were used.

2.2 Dynamic digestion

A dynamic digester available at STLO (INRA Rennes, France) was used to perform digestions on the two samples (unheated and heated milk) in triplicate. The digester instrument and the software were previously described by Ménard et al. (2014). The half–time of gastric emptying used in the present study was set at 191 and 283 min for unheated and heated milk, respectively, as estimated by Barbé et al. (2013) in mini-pigs fed these milk samples. The beta (β) parameter for the Elashoff equation that controlled the gastric emptying was set at 0.8. The pH curve followed during digestions was controlled by the software (Ménard et al., 2014), using the pH data previously reported (Minekus et al., 995). Porcine

pepsin (P-6887, Sigma) was employed at 1000 Units/mL (Chiang et al., 2008) of simulated gastric fluid (SGF) (NaCl 150mM). The continuous flow of SGF into the gastric compartment was fixed at 0.5 mL/min as previously described (Minekus et al., 1995), and controlled by the software. The sampling was done at 4, 10, 20, 50, 105, 165, 225, 315 and 405 min of digestion time.

2.3 SDS-PAGE:

SDS-PAGE analyses were performed using 4-12% Bis-Tris polyacrilamide precast gels (1.5 mm x 15 wells; NuPAGE Novex, Invitrogen). Triplicates of digestion at different sampling times, above mentioned, were included in electrophoretic analyses. In addition, each milk sample, previous to digestion, was also analyzed in each SDS-PAGE run. These analyses were carried out as described by Bouzerzour et al. (2012). The molecular marker used for the experiments was Mark 12 Unstained Standard NuPAGE 4-12% (Invitrogen). The image analyses of the gels were performed using Image scanner III (GE Health-care Europe GbmH, Velizy-Villacoublay, France). Densitometry analyses of the gel images were carried out. The relative quantification of the β-lactoglobulin (β-Lg) over time of digestion, was performed by measuring the colored area volume of the band on the SDS-PAGE gel image. Both milk samples, previous to digestion, present in each run of SDS-PAGE experiments, were used to establish the initial amount of protein. The volume of their colored area was measured and considered as 100%. The hydrolysis of this protein through digestion was estimated by referring the colored areas at different digestion times to that of the initial amount.

2.4 ELISA

Inhibition ELISA was performed on the triplicates of digestion from each sample (unheated and heat treated milk) at different times of digestion (0, 4, 10, 20, 50, 105, 165, 205, 315 and 405 min). ELISA was carried out as previously described (Dupont, Mandalari, Molle, Jardin, Role-Répécaud, et al., 2010b) using caseins-specific polyclonal antibodies to estimate the residual immunoreactivity of this protein during digestion process. Samples were first homogenized with a thurrax (Ultra Thurrax T8 IKA, Fischer Scientific, 20,000 tr/min-5 min). Each digestion time, mentioned above, was analyzed in triplicate.

2.5 LC-MS/MS-based analysis by ion trap and MALDI TOF/TOF

The triplicates of digestion from unheated and heated milk were individually analyzed by the two MS-based techniques: RP-HPLC-ion trap and nanoLC-MALDI-TOF/TOF at three digestion times (4, 50 and 405 min).

The RP-HPLC-MS/MS analyses of samples were carried out on a HPLC-ion trap described by Sánchez-Rivera Recio, Ramos, and Gómez-Ruiz (2013). The samples were eluted at 0.2 mL/min. A linear gradient was used from 0 to 45% of solvent B (trifluoroacetic acid-TFA 0.027% in acetonitrile) and 55% of solvent A (TFA 0.037% in water) in 120 min.

The samples were also analyzed by a nano-LC (Easy-nLC II; Bruker Daltonik, GmbH Bremen, Germany) coupled to a fraction collector (Proteineer fc II; Bruker Daltonik) and finally by MALDI-TOF/TOF (Autoflex speed; Bruker Daltonik). The column used was a pepMap 100 column, C_{18} 3µm of particle size, 75 µm x 15 cm (Dionex Acclaim pepMap 100; Thermo Scientific). The elution of peptides was

carried out with a linear gradient from 0 to 40% of solvent A (water/formic acid 0.1%) and 60% of solvent B (acetonitrile/formic acid 0.1%) in 90 min. The flow was set at 300 nL/min and the injection volume was 18 µL. The fraction collector gathered the sample from the nano-LC and deposited a drop every 15 sec on a prespotted Anchorchip α-Cyano-4-hydroxycinnamic acid (HCCA) plate (set for proteomics II; PAC-II 384 well plate; Bruker Daltonics). Later on, the plate was washed with 10 mM monohydrated ammonium phosphate in aqueous solution at 0.1% of TFA, to perform the MS/MS analyses off-line using MALDI-TOF-TOF. The mass range was set from 500 to 3500 m/z using an exclusion list containing the peaks from the matrix to avoid interferences.

Data processing was performed by using Data AnalysisTM (version 4.0; Bruker Daltoniks) and the peptide sequencing was done by MASCOT, as previously described by (Sánchez-Rivera, Diezhandino, Gómez-Ruiz, Fresno, Miralles, et al., 2014b).

2.6 Statistical analyses

Two-way analysis of variance (ANOVA) was applied to ELISA and densitometry data using Graphpad Prism 5.0 software. The effect of digestion time and the heat treatment on the hydrolysis of proteins were evaluated. The mean values are presented with the standard error of mean (SEM), and the effects were found significant at $p \leq 0.001$. Data were expressed as the percentage (%) of remaining protein over time of digestion with regard to the initial value (previous to digestion).

3.Results and discussion

220 3.1 *Hydrolysis of milk proteins by the action of pepsin*

221 Figure 1 shows the electrophoretic profile of unheated and heat treated milk
222 at different time points of digestion. In the case of the unheated milk (Figure 1A),
223 it is observed that the casein fraction (ca. 31 kDa) is rapidly hydrolyzed compared
224 to the heated one (Figure 1B). At 4 min, the electrophoretic bands that
225 corresponded to casein showed a drastic decrease in the non-heated sample,
226 remaining barely visible. This rapid degradation of casein by the action of pepsin
227 is also in accordance with other results reported after static gastric digestion of
228 caseins (Picariello et al., 2010), or β-casein using adult and infant *in vitro*
229 digestion models (Dupont, Mandalari, Molle, Jardin, Léonil, et al., 2010c).
230 However, in heated milk, the bands corresponding to the casein fraction
231 remained visible up to 50 min. These results point out that food processing, such
232 as heat treatment, affects the kinetics of protein hydrolysis during dynamic gastric
233 digestion, as previously observed in a static model (Almaas et al., 2006).

234 The bands corresponding to β-Lg (18 kDa, Figure 1A) were visible until the
235 end point of gastric digestion in unheated milk. The resistance of this protein to
236 gastric digestion has been previously reported during *in vitro* static digestion of
237 whey proteins (Picariello et al., 2010) or β-Lg (Dupont et al., 2010b), and of
238 unheated milk in rats (Miranda & Pelissier, 1983). However, in heat treated milk,
239 at 50 min, the action of pepsin caused a pronounced decrease in its band (Figure
240 1B), being completely degraded during gastric digestion. Indeed, certain
241 processes, such as the application of heat either at low pH or under high
242 pressure, induces conformational changes in this protein, which provokes the
243 exposure of the hydrophobic regions, increasing its susceptibility to hydrolysis by
244 pepsin (Peram et al., 2013; Bateman et al., 2010; Zeece et al., 2008). It has to be

taken into account, that under our digestion conditions, faster gastric emptying rate was established for unheated versus heated milk. In this regard, the protein in the non-heated sample was exposed shorter time to the action of the enzyme, as non-heated milk was emptied from the gastric compartment faster than the heated sample. The remaining amount of β-Lg was estimated by using densitometry (figure 2A). At 105 min, 70 % β-Lg remained in unheated milk while in heat treated milk only 35% of β-Lg remained intact.

Hydrolysis of milk proteins was also followed by ELISA, using casein-specific polyclonal antibodies (figure 2B). These results also confirmed the difference on casein hydrolysis kinetics between heated and non-heated milk. These differences were more evident at short times of digestion. After 4 min, a decrease of 80% of the signal for casein was detected in unheated milk while for heat treated milk was only 8%. The formation of aggregates of caseins and whey proteins during heat treatment of milk has been described, which may promote the casein resistance (Patel et al., 2006; Jean et al., 2006; Guyomarc'h, Law, & Dalgleish, 2003).

3.2 *Evolution of peptidomic profile of digests*

Overall, sequence coverage was found to increase over time of digestion, since more peptides and gradually smaller ones were identified, and thus, fitting better the selected target mass during the analyses. The sequence coverage at the end point of digestion reached 80% and 76%, for β- and α_{S1}-casein, respectively, in the case of heated milk; and 83% for both, β- and α_{S1}-casein, in the case of unheated milk. No peptides from the N-terminal phosphorylated

region of the protein were identified, since in complex samples detection of phosphorylated peptides is rather impaired by the presence of non-phosphorylated fragments, whose ionization is predominant with respect to the first ones (Picariello et al., 2010). This fact explains why the sequence coverage obtained did not reach higher values. The results obtained using two analyzers, ion trap and MALDI TOF/TOF, were complementary. Indeed, the latter instrument provided 18% of additional peptides, not detected by ion trap, which were broadly larger fragments than those identified by the ion trap instrument. In general, a higher number of peptides was identified from the casein fraction in unheated milk compared to the heat treated one. Better sequence coverage was also obtained in the first case, suggesting that the heat treatment could influence the release and/or peptide identification under the same analysis conditions. Partly, this could be due to different phenomena undergone by proteins upon heat treatment, i.e., lactosylation, glycosylation, as well as protein-protein interaction, that represent an additional difficulty for peptide sequencing by MS.

A study of peptide homology (considering as homologous identical sequences) was carried out to assess the similarities between heated and non-heated milk. The total peptide homologies in samples at 4, 50 and 405 min were found to be 34%, 37% and 48%, respectively, showing the difference in the peptide release in both samples. At the end of the gastric digestion only reached 48%, in spite of being the same matrices, with the same initial protein content and composition, suggesting an impact of heat treatment on peptide release.

Figure 3 shows the peptides from β-casein identified in non-heated (3A) and heated (3B) milk at 4, 50 and 405 min of digestion. At 4 min, in both samples, most of the peptides belonged to the C-terminal region comprised between

295 residues 190-209, indicating that the hydrolysis starts at this part of the protein.
296 Peptides from this region were more numerous in unheated milk (14 peptides)
297 compared to the heated sample (seven peptides), although some of these were
298 common in both i.e. f193-209. Furthermore, the regions 125-142 and 141-163 led
299 to different peptide profiles and in heated milk a lower number of peptides and
300 larger peptides were found compared to the unheated sample. At 50 min,
301 peptides from the C-terminal part of the protein still dominated the peptidome, as
302 also observed by Picariello et al. (2010) after static gastrointestinal digestion of
303 casein. Moreover, regions containing residues 1-14, 45-58 and 75-107 and 128-
304 140 gave rise to new peptides in unheated milk digests. Peptides from the latter
305 domain were especially abundant. In regard to these domains, similar patterns
306 were observed in heated milk, although it is noteworthy the presence of only one
307 sequence from region comprised between residues 1-14, i.e. 1-5, suggesting a
308 resistance of this area of the protein in heated samples. On the other hand,
309 peptides from region 58-80 were present in heat-treated milk as early as 50 min,
310 yet absent from unheated samples until 405 min. This delayed release may
311 indicate a slight resistance of this area in non-heated milk. At the end of digestion
312 (405 min) the C-terminal part of the protein was not the main source of peptides.
313 Nonetheless, numerous peptides from this part of the protein were still detected
314 in both, non-heated (16 peptides) and heated milk (17 peptides), among them
315 f193-209, which was identified at all digestion times. Peptides from this domain
316 were found to be abundant in effluents collected from mini-pigs after ingestion of
317 heated and unheated milk (Barbé et al., 2014). The N-terminal part of the protein,
318 gave rise to some new peptides in heated milk, which had not been identified in
319 previous sampling times (f1-5, f1-6, f1-11, f6-11). On the contrary, in unheated

320 milk, this part of the protein had been previously hydrolyzed into smaller peptides
321 i.e. f1-5 (RELEE) and f6-11 (LNVPGE). This suggests that the N-terminal end of
322 the protein may be hydrolyzed faster in non-heated *vs* heated milk. In addition to
323 the C-terminal end of the protein, other regions seemed to be resistant in
324 unheated and heated samples i.e. 76-93. Interestingly, this region was also
325 reported as resistant after static gastrointestinal digestion of blue cheese
326 (Sánchez-Rivera et al., 2014b), and of raw, pasteurized, sterilized milks and
327 yogurt using infant digestion model (Dupont et al., 2010b). Likewise, many
328 peptides from this domain were identified in piglets fed infant formula
329 (Bouzerzour et al., 2012). Similarly, peptide fragments from region 128-140 were
330 highly abundant in both unheated and heated milk, and all of them displayed
331 Leu^{140} at C-terminal position, which is a residue often cleaved when pepsin is
332 involved (Savoie, Agudelo, Gauthier, Marin & Pouliot, 2005). The resistance to
333 pepsinolysis of these regions (76-93 and 128-140) was determined by the high
334 content of Pro residues in both cases and their hydrophobicity at pH 3 (Vanhoof,
335 Goossens, De Meester, Hendriks, & Scharpe, 1995; Dupont et al., 2010c). These
336 domains of the protein, 67-90, 128-140, and 190-209 have been also found to be
337 resistant cores during adult human digestion regardless of the matrix ingested,
338 such as milk or casein, or the heat treatment applied (Svedberg, J., Haas, J, D.,
339 Leimenstoll, G., Paul, F., & Teschemacher, H., 1985; Boutrou et al., 2013).

340 Figure 4 shows the identified peptides from α_{S1}-casein in unheated (4A) and
341 heat treated milk (4B) at different times of digestion (4, 50 and 405 min). The
342 hydrolysis of this protein started from the N-terminal end (region 1-39), and
343 therefore determined the peptide release at short digestion times. Large
344 fragments from this part of the protein were abundant at 4 min in both samples.

However, the sequences present in heated milk (i.e f1-14/16/18/20/21/23, f3-20, f12/17/19-23) were larger than those found in the unheated sample (i.e f1-9/12/14/16/18/20, 15/16/17/19-23). Several sequences containing N-terminal Phe_{24} (five peptides) were found in both digests. In both milk samples, the release of N-terminal peptides during digestion occurred from the domains 1-23 and 24-39. This confirms the high susceptibility of the bond Phe^{23}-Phe^{24}. However, differences in the peptide bond cleavage were detected between heated and unheated milk within the first domain. For instance, the cleavage Gln_9-Gly_{10}, Pro_{12}-Gln_{13} and Val_{15}-Leu_{16} were found distinct of unheated milk. On the contrary, in heated samples the enzyme rather cleaved at Pro_2-Lys_3, Leu_{11}-Pro_{12}, Leu_{21}-Arg_{23} and Arg_{22}-Phe_{23}. The common cleavage bonds in this region were displayed at Glu_{14}-Val_{15}, Leu_{16}-Asn_{17}, Glu_{18}-Asn_{19} and Leu_{20}-Leu_{21}. No peptides were found from region 114-164, which is the phosphorylated region of this protein. At the end point of digestion (405 min), the identification of peptides throughout the protein sequence was more homogeneous. Peptides from domains 56-62 and 121-143, were identified for the first time at 405 min in unheated and heat treated milk. In addition, the number of peptides identified in non-heated samples from regions 80-190 and 180-199 was twice those found in heated milk. Among them, f180-191 only found in unheated milk, was also detected in the stomach of humans after the ingestion of yogurt (Chabance et al., 1998).

Peptides from α_{S2}-casein and κ-casein were identified in heat treated and non-heated milk digests at different digestion time points. As expected, the sequences identified from this protein represented 14% and 16%, respectively, of the total peptide content, due to their lower abundance compared with β-casein

and α_{S1}-casein. In addition, α_{S2}-casein and κ-casein may show higher resistance to *in vitro* digestion compared to other caseins (Dupont et al., 2010a). Again, differences between heated and non-heated samples at the end of digestion were found. For instance, in heat treated milk, α_{S2}-casein gave rise to five sequences from region 99-123, among others, two sequences containing 23 (2718 Da) and 24 residues (2831 Da). However, in the non-heated samples, only one peptide (f107-123) was detectable from this domain, which had been hydrolyzed into smaller sequences, already identified at 50 min. Interestingly, only one peptide was found from C-terminal end of the protein in unheated milk, whereas four sequences were detected in heat treated samples, for instance f183-206 and f183-207, containing 23 and 24 residues, respectively. Both peptides had previously shown antibacterial activity (Table 1) (Recio, & visser, 1999).

No peptides from β-Lg were found at 4 min nor in heated neither in unheated milk. In the present work, this protein was found to be rapidly hydrolyzed in heated milk, and resistant in the unheated one. After 405 min of digestion, several peptides from region 75-82 were identified in heated milk, but also some fragments were present in the non-heated samples, in agreement with the slight decrease of the intact protein observed at 315 min in the latter (Figure 1A). In non-heated milk, this protein may be partly denatured due to the drying process undergone by the powder, and this would explain the release of some peptides despite its known resistance to pepsin digestion. In addition, peptides from the domain comprised between residues 95-149 were abundant at this point in both samples (seven and nine peptides in heat treated and unheated milk, respectively). Among these sequences identified in unheated milk, it is

noteworthy that 4 of them (f123-130/131/132/133), belong to the region 125-135, which was reported to be especially resistant to digestion (Picariello et al., 2010).

To summarize, Table 1 shows the identified peptides physiologically relevant either concerning their potential bioactivity, their previous *in vivo* detection after dairy products ingestion or with allergenic implications as previously reported IgE epitopes. Table 1 also shows differences in regard to their time of release. For instance, it is noteworthy the presence of the antihypertensive peptides from α_{s1}-casein f143-149 (AYFYPEL) and 90-94 (RYLGY), the opioid peptide fragments f90-95 from α_{s1}-casein (RYLGYL) and f33-38 from k-CN (SRYPSY); and the antibacterial sequences from α_{s2}-casein f183-207 (VYQHQKAMKPWIQPKTKVIPYVRYL) and f186-206 (VYQHQKAMKPWIQPKTKVIPYVRY).

4.Conclusions

Heat treatment produced an increase of the resistance in casein fraction. However, β-Lg became more susceptible to hydrolysis. The impact of the heat treatment on the peptidome released from milk proteins during gastric dynamic digestion was evaluated. The homology values between samples, point out the difference in the identity of peptides formed during digestion of heated and unheated milk. The peptide homology at the end of digestion (48%) suggests an impact of heat treatment on the identity of peptides generated from the two milk samples during digestion. These differences in the identity of peptides and also in the time of peptide release revealed by MS analyses may have implications in health, in regard to bioactivity or formation/resistance of potential epitopes. Nevertheless, also some common traits after digestion could be found. Resistant

420 regions could be confirmed through the analyses, such as the region comprised
421 between the residues 76-93 of β-casein, where several binding epitopes are
422 included. Also, β-casein regions 128-140 and 190-209 were found to be
423 resistant.

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References

Almaas, H., Cases, A-L., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., et al. (2006). In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes. *International Dairy Journal, 16*, 961-968.

Aymard, P., Durand, D., & Nicolai, T. (1996). The effect of temperature and ionic strength on the dimerisation of β-lactoglobulin. *International journal of biological macromolecules estructure function and interaction, 19*,213-221.

Bateman, L., Ye, A., & Singh, H. (2010). In vitro digestion of β-lactoglobulin fibrils formed by heat treatment at low pH. *Journal of Agricultural and Food Chemistry, 58*, 9800-9808.

Barbé, F., Ménard, O., Le Gouar, Y., Buffière, C., Famelart, M.-H., Laroche, B., et al., (2013). The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. *Food Chemistry, 136*, 1203-1212.

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63,* 147-156.

Benedé, S., López-Expósito, I., Giménez, G., Grishina, G., Bardina, L., Sampson, H. A., et al. (2014). In vitro digestibility of bovine β-casein with simulated and human oral and gastrointestinal fluids. identification and IgE-reactivity of the resultant peptides. *Food Chemistry, 143*, 514-521.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., et al., (2013). Sequential release of milk protein derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition, 97*, 1314-1323.

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huërou-Luron, I., et al. (2012). *In vivo* digestion of infant formula in piglets: Protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition, 108*, 2105-2114.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., et al., (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie*, *80*, 155-165.

Chiang, C. -C., Croom, J., Chuang, S. -T., Chiou, P. W. S., & Yu, B. (2008). Development of a dynamic system simulating pig gastric digestion. *Asian-Australasian Journal of Animal Sciences, 21*, 1522-1528.

Contreras, M. M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal, 19*, 566-573.

Coste, M., Rochet, V., Leonil, J., Molle, D., Bouhallab, S., & Tome, D. (1992). Identification of C-terminal peptides of bovine β-casein that enhance proliferation of rat lymphocytes. *Immunology Letters,33*, 41-46.

Deglaire, A., Bos, C., Tomé, D., & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition, 102*, 1752-1759.

Dupont, D., Boutrou, R., Ménard, O., Jardin, J., Tanguy, G., Schuck, P., et al. (2010a). Heat Treatment of Milk during Powder Manufacture Increases Casein Resistance to Simulated Infant digestion. *Food Digestion*, *1*, 28-39.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Role-Répécaud, O., Duboz, G., et al. (2010b). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition & Food Research*, *54*, 1677-1689.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., et al. (2010c). Comparative resistance of food proteins to adult and infant *in vitro* digestion models*. Molecular Nutrition & Food Research, 54*, 767-780.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modelling human gastric and small intestinal digestion. *Cell Press*, *30*, 591-600.

Guo, M.R., Fox, P.F., Flynn, A. (1995). Susceptibility of *β*-Lactoglobulin and Sodium Caseinate to Proteolysis by Pepsin and Trypsin. *Journal of Dairy Science, 78*, 2336–2344.

Guo, M. R., Fox, P.F., Flynn, A., & Kindstedt, P.S. (1996). Heat-induced Modifications of the Functional Properties of Sodium Caseinate. *International Dairy Journal, 43*, 473-483.

Guyomarc'h, F., Law, A. J. R., & Dalgleish, D. G. (2003). Formation of soluble and micelle-bound protein aggregates in heated milk. *Journal of Agricultural and Food Chemistry, 51*, 4652-4660.

Hur, S.J., Lim, B.O., Decker, E.A., & McClements, J. (2011). In vitro human digestion models for food applications. *Food Chemistry*, *125*, 1-12.

Iametti, S., Cairoli, B. De Gregory, & F. Bonomi. (1995). Modifications of hight-order structures upon heating of β-Lactoglobulin: dependence on their protein concentration. *Journal of Agricultural & Food Chemistry, 43*,53-58.

Jean, K., Renan, M., Famelart, M-H., & Guyomarc'h, F. (2006). Structure and surface properties of the serum heat-induced protein aggregates isolated from heated skim milk. *International Dairy Journal, 16*, 303-315.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergeres, G., Portmann, R., & Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *The Journal of Nutrition*, *142*, 245-250.

Lahov, E., & Regelson, W. (1996). Antibacterial and immunostimulating casein-derived substances from milk: Casecidin, isracidin peptides. *Food and Chemical Toxicology, 34*, 131-145.

Langerholc, T., Maragkoudakis, P. A., Wollgast, J., Gradisnik, L., & Cencic, A. (2011). Novel and established intestinal cell line models - An indispensable tool in food science and nutrition. *Trends in Food Science & Technology*, *22*, 11-20.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., & Klee, W. A. (1983). Opioid activities and structures of α-casein-derived exorphins. *Biochemistry, 22*, 4567-4573.

Loveday, S.M., Wang, X.L., Rao, M.A., Anema, S.G., & Singh, H. (2012). β-Lactoglobulin nanofibrils: Effect of temperature on fibril formation kinetics, fibril

morphology and the rheological properties of fibril dispersions. *Food Hydrocolloids*, *27*, 242-249.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., & Recio, I. (2013). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *International Dairy Journal, 32*, 13-19.

Marteau, P., Pochart, P., Mahé, S., Crine, L., Huneau, J. F., & Tomé, D. (1991). Gastric emptying but not orocecal transit time differs between milk and yoghurt in lactose digesters. *Gastroenterology, 100*, A535.

Meisel, H. (1998). Overview on Milk Protein-derived peptides. *International Dairy Journal, 8,* 363-373.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., et al. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food chemistry, 145*, 1039-1045.

Miguel, M., Gómez-Ruiz, J. A., Recio, I., & Aleixandre, A. (2010). Changes in arterial blood pressure after single oral administration of milk-casein-derived peptides in spontaneously hypertensive rats. *Molecular Nutrition & Food Research, 54*, 1-6.

Mills, E.N.C., Sancho, A.I., Rigby, N.M., Jenkins, J.A., & Mackie, A., R. (2009). Impact of food processing on the structural and allergenic properties of food allergens. *Molecular Nutrition & Food Research, 53*, 963-969.

Minekus, M., Marteau, P., Havenaar, R., & Huis in Veld, J.H.J. (1995). A multicompartmental dynamic Computer-controlled Model Simulating the Stomach and Small Intestine. *Atla-Alternatives to Laboratory animals, 23*, 197-209.

Miranda, G., & Pelissier, J. P. (1983). Kinetic studies of in vivo digestion of bovine unheated skim-milk proteins in the rat stomach. *Journal of Dairy Science, 50*, 27-36.

Ong, L., Henriksson, A., & Shah, N. P. (2007). Angiotensin converting enzyme-inhibitory activity in cheddar cheeses made with the addition of probiotic lactobacillus casei sp. *Lait, 87*, 149-165.

Patel, H.A., Singh, H., Anema, S.G., & Creamer, L.K. (2006). Effects of Heat and High Hydrostatic Pressure Treatments on Disulfide Bonding Interchanges among

572 the Proteins in Skim Milk. *Journal or agricultural & Food Chemistry,* 54, 3409-
573 3420.
574 Peram, M., R., Loveday, S.M., Ye, A., & Singh, H. (2013). In vitro digestion of heat-
575 induced aggregates of β-Lactoglobulin. Journal of Dairy Science, 96, 63-74.
576 Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., et al.
577 (2010). Peptides surviving the simulated gastrointestinal digestion of milk
578 proteins: Biological and toxicological implications. *Journal of Chromatography B*,
579 *878*, 295-308.

580 Pihlanto-Leppälä, A., Rokka, T. y Korhonen, H. (1998). Angiotensin I converting
581 enzyme inhibitory peptides from bovine milk proteins. *International Dairy Journal.*,
582 8, 325-331.
583 Recio, I., & Visser, S. (1999). Identification of two distinct antibacterial domains within
584 the sequence of bovine α(s2)-casein. *Biochimica Et Biophysica Acta - General*
585 *Subjects, 1428*, 314-326.

586 Recio, I., López-Expósito, I.Quirós, a., Hernández-Ledesma, B., Gómez-Ruiz, J. A.,
587 Miguel, M., Amigo, L., Ramos, M., & Aleixandre, A., & Contreras, M. (2006).
588 Bioactive peptides identified in casein hydrolysates produced by enzymatic
589 hydrolysis and process of production. *WO Pat*, 131586.

590 Rizzello, C. G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M. D., & Zambonin, P.
591 G. (2005). Antibacterial activities of peptides from the water-soluble extracts of
592 italian cheese varieties. *Journal of Dairy Science, 88*, 2348-2360.

593 Robert, M.C., Razaname, A., Mutter, M. & Juillerat, M. A. (2004). Peptides derived
594 from sodium caseinate hydrolysates produced by Lactobacillus helveticus
595 NCC2765. *Journal of Agricultural & Food Chemistry,* 52, 6923-6931.
596 *Savoie, L., Gauthier, S. F., Marin, J., Pouliot, Y. (2005).* In vitro determination of the
597 release kinetics of peptides and free amino acids during the digestion of food
598 proteins. *Journal of AOAC International*, *88*, 935–948.
599 Scanff, P., Yvon, M., Thirouin, S., Pelissier, J.-P. (1992). Characterization and
600 kinetics of gastric emptying of peptides derived from milk proteins in the
601 preruminant calf. *Journal of Dairy Research, 59*, 437-447.
602 Shandilya, U. K., Kapila, R., Haq, R. M., Kapila, S., & Kansal, VK. (2013). Effect of
603 thermal processing of cow and buffalo milk on the allergenic response to caseins

and whey proteins in mice. *Journal of the Science of Food & Agriculture, 93*, 2287-2292.

Shimizu, M. (2010). Interaction between food substances and the intestinal epithelium. *Bioscience, Biotechnology and Biochemistry, 74*, 232-241.

Sánchez-Rivera, L., Recio, I., Ramos, M., & Gómez-Ruiz, J. Á. (2013). Short communication: Peptide profiling in cheeses packed using different technologies. *Journal of Dairy Science*, *96*, 3551-3557.

Sanchez-Rivera, L., Martínez-Maqueda, D., Cruz-Huerta, E., Miralles, B., & Recio, I. (2014a). Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. Food Research International, *63*, 170-181.

Sánchez-Rivera, L., Diezhandino, I., Gómez-Ruiz, J.A., Fresno, J.M., Miralles, B., & Recio, I. (2014b). Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. *Electrophoresis,* 35, 1627-1636.

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *Journal of Nutritional Biochemistry, 11*, 128-131.

Svedberg, J., Haas, J, D., Leimenstoll, G., Paul, F., & Teschemacher, H. (1985). Demonstration of/3-Casomorphin Immunoreactive Materials in *In Vitro* Digests of Bovine Milk and in Small Intestine Contents After Bovine Milk Ingestion in Adult Humans. *Peptides, 6*, 825-830.

Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., & Scharpe, S. (1995). Proline motifs in peptides and their biological processing. *The Journal of the Federation of American Societies for Experimental Biology, 9*, 736-744.

Wada, Y., & Lönnerdal, B. (2014). Effects of different industrial heating processes of milk on site-specific protein modifications and their relationship to in vitro and in vivo digestibility. *Journal of Agricultural and Food Chemistry, 62*, 4175-4185.

Wickham, M., Faulks, R., & Mills, C. (2009). In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Molecular Nutrition & Food Research, 53*, 952-958.

Yamamoto, N., Akino, A., & Takano, T. (1994). Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from lactobacillus helveticus CP790. *Journal of Dairy Science, 77*, 917-922.

Yoshikawa, M., Tani, F., Yoshimura, T., & Chiba, H. (1986). Opioid peptides from milk proteins. *Agricultural and Biological Chemistry, 50*, 2419-2421.

Yvon, M., & Pelissier, J. P. (1987). Characterization and kinetics of evacuation of peptides resulting from casein hydrolysis in the stomach of the calf. *Journal of Agricultural and Food Chemistry, 35*, 148-156.

Zeece, M., Huppertz, T., & Kelly, A. (2008). Effect of high-pressure treatment on in-vitro digestibility of β-lactoglobulin. *Innovative Food Science and Emerging Technologies, 9*, 62-69.

Figure Captions

Figure 1. SDS-PAGE electrophoretic run of unheated (1A) and heat treated milk (1B). Lane numbers represent the time of digestion (min). MM= molecular marker.

Figure 2. Evolution of β-Lg (2A) and caseins (2B) over time of dynamic gastric digestion. Mean values ± SEM (n=3) are expressed as the percentage (%) of the remaining protein in respect of the initial value (before digestion). Significant differences between UH (unheated milk) and HT (heat treated milk) at the same time of digestion were found at $p \leq 0.001$ (*).

Figure 3. Peptidomic profile from β-casein after dynamic digestion of unheated (3A) and heat treated milk (3B), analyzed by MALDI TOF/TOF and ion trap. The lines in light gray indicate 4 min of digestion, dark gray lines 50 min, and black lines the end point of digestion (405 min). The peptides in the figure were identified in at least two of the three digestion replicates in each sample (A and B) and each time point injected in the ion trap instrument. The peptides identified by MALDI TOF/TOF in the figure were selected from one of the digestion replicates.

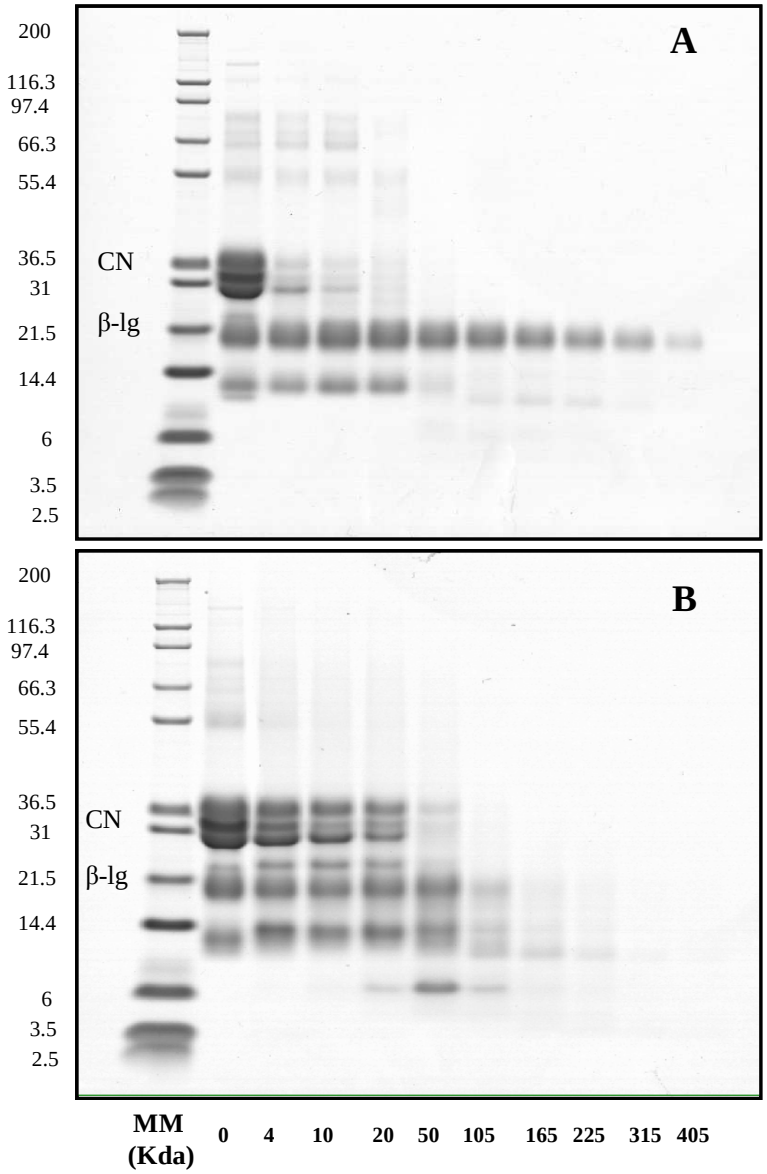
Figure 4. Peptidomic profile from α_{s1}-casein after dynamic digestion of unheated (3A) and heat treated milk (3B), analyzed by MALDI TOF/TOF and ion trap. The lines in light gray indicate 4 min of digestion, dark gray lines 50 min, and black lines the end point of digestion (405 min). The peptides in the figure were identified in at least two of the three digestion replicates from each sample (A and B) and each time point injected in the ion trap instrument. The peptides identified by MALDI TOF/TOF in the figure were selected from one of the digestion replicates.

1. Identified peptides after gastric digestion of heated and unheated milk with previously reported bioactivity, and the time at which they were identified in each sample. Their presence in vivo in the gastrointestinal track within effluents gathered from humans or animals has been indicated.

Sequence	Protein fragment	Sample	In vivo	Described activity	Reference
AYFYPEL	α_{s1}-CN f143-149	HT(50, 405); UH(50, 405)	Human[1]; Calf[3]	AH	*Contreras, Carrón, Montero, Ramos, & Recio, 2009*
DAYPSGAW	α_{s1}-CN f157-164	HT(50, 405); UH(50, 405)	Calf[3]	ACE- I	*Pihlanto-Leppälä, Rokka, & Korhonen, 1998*
NLHLPLPLL	β-CN f132-140	HT(50, 405); UH(50, 405)	Human[2]	ACE- I	*Robert, Razaname, Mutter & Juillerat, 2004*
RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-CN f1-23	HT(4, 50)	Calf[3]	AB	*Lahov, & Regelson, 1996*
YFYPEL	α_{s1}-CN f144-149	HT(50, 405); UH(50, 405)	Human[1]	AX; MSE	*Suetsuna, Ukeda, & Och, 2000; Martínez-Maqueda, Miralles, Ramos, & Recio, 2013.*
RYLGY	α_{s1}-CN f90-94	HT(405); UH(50, 405)		AH	*Contreras et al., 2009*
RYLGYL	α_{s1}-CN f90-95	HT (405)		Opioid	*Loukas, Varoucha, Zioidrou, Streaty, & Klee, 1983*
VAPFPEVF	α_{s1}-CN f25-32	HT(405); UH(50, 405)	Human[1; 2]	ACE- I	*Contreras et al., 2009*
FVAPFPEVF	α_{S1}-CN f24-32	HT(50, 405); UH(50, 405)	Human[2]	ACE- I; AM	Ong, Henriksson, & Shah, 2007; Rizzelo et al., 2005
LLYQEPVLGPVRGPFPIIV	β-CN f191-209	HT(4, 50); UH(50)		ACE- I; IgE binding	*Yamamoto, Akino, &Takano, 1994; Benedé et al., 2014*
VVVPPFLQPEVM	β-CN f82 – 93	UH(405)		IgE binding	*Benedé et al., 2014*
YAKPVA	k-CN f61-66	HT(405)		AH	*Miguel, Gómez-Ruiz, Recio, & Aleixandre, 2010*
HPHPHLSF	k-CN f98-105	HT(4)		AH	*Miguel et al., 2010*
VYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-CN f183-207	HT 405)	Mini-pig[4]	AB	*Recio, & Visser, 1999*
VYQHQKAMKPWIQPKTKVIPYVRY	α_{S2}-CN f183-206	HT(405)	Mini-pig[4]	AB	*Recio, & Visser, 1999*
YQEPVLGPVRGPFPIIV	β-CN f193-209	HT(4, 50, 405); UH(4, 50, 405)	Calf[3]; Mini-pig[4]; Calves[5]	ACE-I; IM	*Ong et al., 2007; Coste et al., 1992*
LYQEPVLGPVRGPFPIIV	β-CN f192-209	HT(4, 50); UH(4, 50)	Mini-pig[4]	IM	*Coste et al., 1992*
PYVRYL	α_{S2}-CN f202-207	HT(4, 50, 405); UH(50)		AH	*Recio et al., 2006*
SRYPSY	k-CN f33-38	HT(50, 405); UH(405)		Opioid	*Yoshikawa, Tani, Yoshimura, & Chiba, 1986*

[1] Chabance et al., 1998; [2] Boutrou et al., 2013; [3] Yvon & Pelissier, 1987; [4]Barbé et al., 2014 ; [5]Scanff et al., 1992

ACE-I : Angiotensin I-converting enzyme- Inhibitor ; HT : Heat treated milk ; UH : Unheated milk ; MSE : Mucin secretion and expression; AH: antihypertensive; AB: antibacterial; AX: antioxidant; AM: antimicrobial; IM: immunomodulation


A
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
6
3.5
2.5
CN
β-lg
B
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
6
3.5
2.5
CN
β-lg
MM (Kda)
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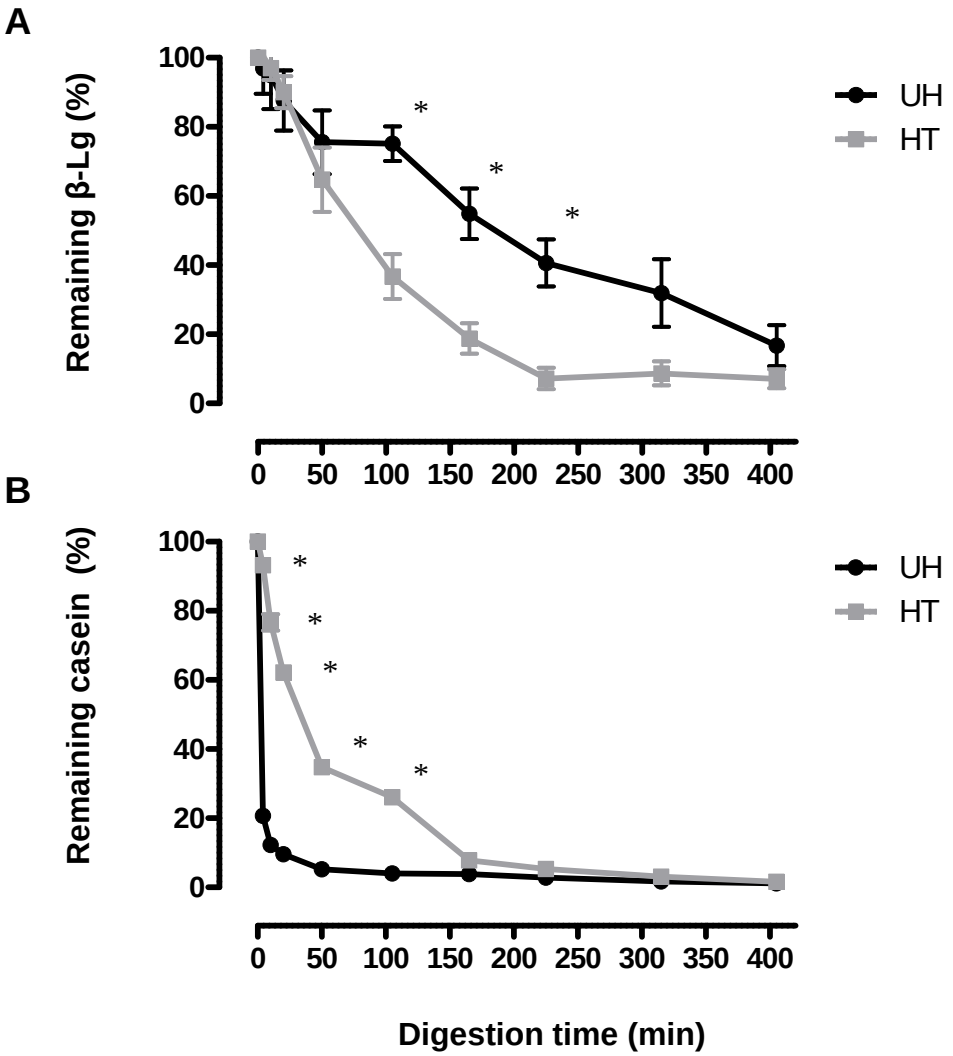
4
10
20
50
105
165
225
315
405


A
Remaining β-Lg (%)
100
80
60
40
20
0
0 50 100 150 200 250 300 350 400
UH
HT
B
Remaining casein (%)
100
80
60
40
20
0
0 50 100 150 200 250 300 350 400
UH
HT
Digestion time (min)

A


RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAME
DIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEI
VPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAW
YYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

B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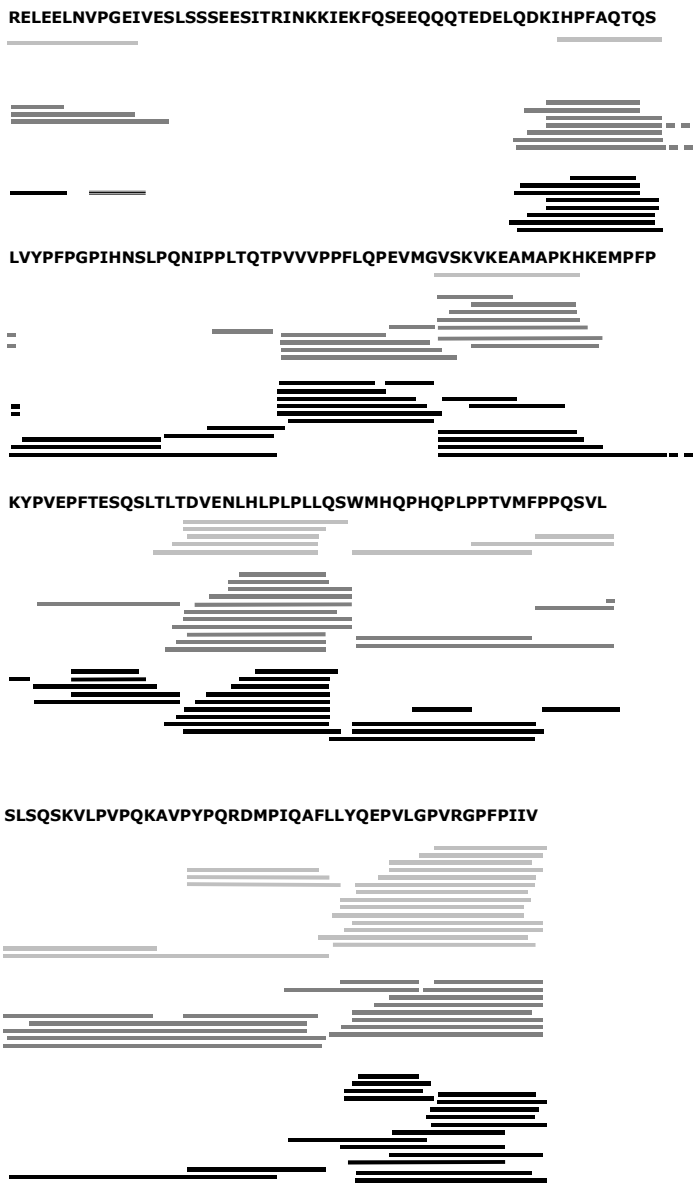
A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B


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