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1 **Impact of human milk pasteurization on the kinetics of peptide release during *in vitro***
2 **dynamic digestion at the preterm newborn stage**

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10

11 Abbreviations: GRAVY, Grand Average of Hydropathy Value; MS, Mass spectrometry;

12 PHM, pasteurized human milk; RHM, raw human milk.

13

14 Keywords: digestion, human milk, pasteurization, peptidomics, preterm

15 Running title: Peptide release during preterm digestion of pasteurized human milk

16 Highlights :

- 17 • Pasteurization increased the pre-proteolysis of most of the β -casein sequence
18 • Pasteurization impacted the peptide release during digestion of preterm human milk
19 • Pasteurization impacted **some** bioactive peptide release before and during digestion

20

21 Number of words: 5510

22

23

24 **Abstract**

25 Holder pasteurization (62.5°C, 30 min) of human milk denatures beneficial proteins. The
26 present paper aimed to assess whether this can affect the kinetics of peptide release during
27 digestion at the preterm stage. Raw (RHM) or pasteurized (PHM) human milk were digested
28 in triplicates using an *in vitro* dynamic system. Mass spectrometry and multivariate statistics
29 were conducted. Pre-proteolysis occurred mostly on β -casein, for which cumulative peptide
30 abundance was significantly greater in PHM over 28% of the hydrolysed sequence. Eight
31 clusters resumed the kinetics of peptide release during digestion, which differed on seven
32 clusters (69% of the 1134 peptides). Clusters associated to the heat-denaturated proteins,
33 lactoferrin and bile salt-stimulated lipase, presented different kinetics of release during
34 digestion, unlike that for β -casein. Some bioactive peptides from β -casein presented
35 significant different abundances between PHM and RHM before digestion (1-18, 185-211) or
36 in the intestinal phase (154-160, 161-166). Further physiological consequences should be
37 investigated.

38

39 **1. Introduction**

40 Human milk is considered as the ideal food for neonates, supporting not only their nutrition
41 but also their optimal growth and development (Victora, Bahl, Barros, França, Horton,
42 Krasevec, et al., 2016). When vulnerable hospitalized neonates such as preterm infants
43 receive donor human milk, it has to be first pasteurized (62.5 °C, 30 min) in the milk bank for
44 sanitary reasons (Wight, 2001). Together with the associated freeze/thaw cycles,
45 pasteurization induces some protein denaturation, which reduces the content of some native
46 proteins and thus affect their bioactivity, such as for immunoglobulins (loss of 20 to 100%),
47 lysozyme (loss of 20 to 69%), lactoferrin (loss of 35 to 90%), and bile salt-stimulated lipase
48 (complete loss) (O'connor, Ewaschuk, & Unger, 2015; Peila, Moro, Bertino, Cavallarin,
49 Giribaldi, Giuliani, et al., 2016). These physical treatments also impact the human milk
50 microstructure, as previously observed (De Oliveira, Bourlieu, Ménard, Bellanger, Henry,
51 Rousseau, et al., 2016a; De Oliveira, Deglaire, Ménard, Bellanger, Rousseau, Henry, et al.,
52 2016b). In the latter studies, we have demonstrated that human milk pasteurization affected its
53 proteolysis kinetics, at different extent, during *in vitro* gastro-intestinal digestion at the
54 preterm and term stages (De Oliveira, et al., 2016a; De Oliveira, et al., 2016b), and during *in*
55 *vivo* gastric digestion in preterm neonates (De Oliveira, Bellanger, Ménard, Pladys, Le Gouar,
56 Dirson, et al., 2017). More precisely, at the preterm stage, pasteurization did not significantly
57 impact the kinetics of disappearance of the major proteins in the gastric phase, but during the
58 intestinal phase, amino acid bioaccessibility was selectively modulated (De Oliveira, et al.,
59 2016a). Knowledge on the kinetics of peptide release during digestion is crucial for a better
60 understanding of the potential peptide bioactivity released in the gastrointestinal tract and on
61 the amino acid absorption rate, the latter of which has been demonstrated to be an
62 independent factor of modulation of the postprandial regional metabolism, at least in adult
63 (Deglaire, Fromentin, Fouillet, Airinei, Gaudichon, Boutry, et al., 2009). To date, only our

64 study has investigated and showed that there was an impact of milk pasteurization on the
65 kinetics of peptide release during *in vitro* dynamic digestion of term milk at the term newborn
66 stage (Deglaire, De Oliveira, Jardin, Briard-Bion, Emily, Ménard, et al., 2016). We assume
67 that such an impact should also be found for preterm milk digested in the preterm newborn
68 condition but certainly at a different extent given the digestive immaturity of the preterm
69 newborn combined to a higher protein content in the preterm human milk. The objective of
70 the present paper was to verify this hypothesis. The kinetics of peptide release was followed
71 during gastro-intestinal digestion of a pool of raw or pasteurized preterm human milk at the
72 preterm newborn stage. Digestion was simulated using an *in vitro* dynamic model, where
73 digestive enzymes, pH and digestive compartment emptying were continuously controlled.
74 Nature and abundance of peptides were determined by mass spectrometry and data analyzed
75 by multivariate statistics.

76 2. Material and methods

77 All chemicals and enzymes were from commercial origin (Sigma-Aldrich, Saint Quentin
78 Fallavier, France), except for the Rabbit Gastric Extract, obtained from GERME SA
79 (Marseille, France).

80 2.1 Human milk collection and digestion

81 Collection of Human milk has been previously detailed by De Oliveira et al. (2016a). Briefly,
82 mature human milk samples were obtained frozen from five women (26-34 years old) on
83 average 6.6 ± 2.4 weeks after preterm delivery *via* a donor milk bank (University Hospital
84 Center, Rennes, France). The gestation length was in average 29.8 ± 3.0 weeks of
85 amenorrhea, ranging from 26 up to 34 weeks of gestation. After milk thawing (at 4°C, over 16
86 h) and pooling, half of the pool was pasteurized (62.5 °C, 30 min). Raw (RHM) and
87 pasteurized (PHM) human milk were then stored at -20°C. Milk was analyzed by a Human
88 Milk Analyzer (Miris, AB, Uppsala, Sweden) and consisted of 35.7 ± 0.7 g/L of fat, $13.2 \pm$
89 0.8 g/L of proteins and 78.3 ± 2.3 g/L of carbohydrates (De Oliveira, et al., 2016a).
90 Gastrointestinal digestion was performed on 100 mL of whole milk, as RHM or PHM, in a bi-
91 compartmental *in vitro* dynamic system (DIDGI®, INRA, France) **simulating the preterm**
92 **newborn digestive conditions, such as reviewed in Bourlieu et al. (2014). The rationale of the**
93 **parameters used in the present model has been detailed in De Oliveira et al. (2016a). Briefly,**
94 fluid secretions, pH and emptying were continuously monitored. The transit time of the
95 chyme followed the Elashoff equation with $t_{1/2} = 36$ min and $\beta = 1.15$ for the gastric emptying
96 and $t_{1/2} = 200$ and $\beta = 2.2$ for the intestinal emptying (De Oliveira, et al., 2016a). The gastric
97 pH followed the equation: $pH = -0.0155 \times \text{time (min)} + \text{milk pH}$. The intestinal pH was
98 maintained at 6.2. Gastric enzymes were added as rabbit gastric extract (pepsin and lipase:
99 120 and 8.6 U/mL of gastric content, respectively) and intestinal enzymes as porcine
100 pancreatin (trypsin, chymotrypsin and lipase: 16, 2 and 59 U/mL of intestinal content) in

101 addition to bovine bile salts. Enzyme units were determined based as a function of the mean
102 body weight, estimated at 1.9 kg for a preterm newborn at a gestational age of 28 weeks and a
103 postnatal age of four weeks (De Oliveira, et al., 2016a).

104 Digestions were conducted in triplicate for each matrix over 3 hours with digesta sampling
105 every 30 or 60 min. Samples were stored at -20°C after addition of the antiprotease
106 phenylmethylsulfonyl fluoride (PMSF) to avoid further proteolysis.

107 Ethical approval was granted by the Hospital Ethics Committee (n° 13-12) and donors gave
108 their informed written consent.

109 **2.2 Mass spectrometry**

110 *2.2.1 Peptide identification*

111 Mass spectrometry (MS) analysis was conducted as described previously (Deglaire, et al.,
112 2016). Briefly, a nano-RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer
113 (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source was used.
114 Digesta samples were diluted 100 times in the injection buffer and filtered (0.45 µm cut-off)
115 before concentration on a µ-precolumn pepMap100 (C18 column, 300 µm i.d. × 5 mm length,
116 5 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) and separation on
117 a PepMap RSLC column (C18 column, 75 µm i.d. x 150 mm length, 3µm particle size, 100 Å
118 pore size; Dionex).

119 Peptide separation was performed at a flow rate of 0.3 µL.min⁻¹ using solvents A [2% (v/v)
120 acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in HPLC gradient grade water] and
121 B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in HPLC gradient
122 grade water]. The elution gradient first rose from 5 to 35% solvent B over 87 min, then up to
123 85% solvent B over 3 min before column reequilibration. The mass spectra were recorded in
124 positive mode using the m/z range 250-2000. The resolution of the mass analyzer for m/z of
125 200 amu (atomic mass unit) was set in the acquisition method to 70 000 for MS and 17 500

126 for MS/MS. For each MS scan, the ten most intense ions were selected for MS/MS
127 fragmentation and excluded from fragmentation for 15 s.

128 Peptides were identified from the MS/MS spectra using the X!TandemPipeline software
129 (Langella, Valot, Balliau, Blein-Nicolas, Bonhomme, & Zivy, 2017) against a human milk
130 protein database (Molinari, Casadio, Hartmann, Livk, Bringans, Arthur, et al., 2012) to which
131 was added the common Repository of Adventitious Protein (<http://thegpm.org/crap>). The
132 possible post-translational modifications were serine or threonine phosphorylation,
133 methionine oxidation, lysine or arginine lactosylation, cyclisation of glutamine or glutamic
134 acid into pyroglutamic acid and acetylation of cysteine, serine, lysine or any amino acid in N-
135 ter position. Peptides identified with an e-value < 0.01 were automatically validated. The
136 peptide false discovery rate was less than 0.3%.

137 *2.2.1.1 Quantification of peptides*

138 Each identified peptide was quantified **by label-free MS** using the MassChroQ software
139 (Valot, Langella, Nano, & Zivy, 2011). A m/z width of 10 ppm was used to perform extracted
140 ion chromatograms (XIC) of peptides in time-aligned chromatograms and the area under the
141 curve was then quantified. When a peptide was measured with several charge states, all ion
142 intensities were summed.

143 *2.2.2 Biochemical characteristics of the peptides*

144 Several biochemical characteristics were determined for each peptide, particularly the amino
145 acids at cleavage sites in positions P1 (C-ter end) and P1' (N-ter end), the number of essential
146 amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine,
147 valine), the isoelectric point, the molecular weight and the Grand Average of Hydropathy
148 Value (GRAVY) as defined by the ExPASy "ProtParam tool" (Gasteiger, Hoogland, Gattiker,
149 Duvaud, Wilkins, Appel, et al., 2005). A positive GRAVY corresponds to a hydrophobic
150 peptide and a negative one to a hydrophilic peptide (Kyte & Doolittle, 1982).

151 Bioactive peptides were searched within the databases of BIOPEP (Minkiewicz, Dziuba,
152 Iwaniak, Dziuba, & Darewicz, 2008) and MBPDB (Nielsen, Beverly, Qu, & Dallas, 2017)
153 both accessed on January 2018. Only exact matching between sequences was considered.
154 Prediction of antimicrobial activities was examined using CAMPR3 based on the algorithms
155 of Random Forest (RF) and Support Vector Machines (SVM), previously shown to have a
156 good prediction performance (Gabere & Noble, 2017). A peptide sequence was determined to
157 have a greater likelihood of antimicrobial functionality with scores approaching 1. All
158 peptides with scores > 0.5 on both prediction methods were considered significant for
159 antimicrobial functionality.

160 **2.3 NH₂ quantification**

161 As described earlier (Deglaire, et al., 2016), the method was a fluorescent microplate analysis
162 based on the reaction of ortho-phthaldialdehyde (OPA) and dithiothreitol (DTT) with primary
163 amines resulting in 1-alkylthio-2-alkylisondole detected at 340 nm.

164 **2.4 Enzymes predicted to be active in milk before gastrointestinal digestion**

165 The peptide sequences as identified in milk before digestion were submitted to the online
166 software Enzyme Predictor (<http://bioware.ucd.ie>), which examined the cleavage patterns and
167 predicted the enzymes responsible for this profile based on the cleavage specificities of 35
168 enzymes (Vijayakumar, Guerrero, Davey, Lebrilla, Shields, & Khaldi, 2012). Enzymes were
169 then ranked according to their likelihood of having being used to generate the hydrolysate
170 thanks to the calculation of an odds ratio (OR). The same human enzymes as those previously
171 considered (Deglaire, et al., 2016) were included. Enzymes presenting only one potential
172 cleavage were not considered. Cleavage sites of cytosol aminopeptidase and carboxypeptidase
173 B2 were examined manually over β -casein, such as performed previously (Dallas, Smink,
174 Robinson, Tian, Guerrero, Parker, et al., 2015).

175 2.5 Statistical analyses

176 Data analyses were performed using the R software, version 3.3.1 (R Core Team, 2014). All
177 graphical representations were based on the log₁₀-transformed abundances
178 [log₁₀(abundance+1)]. An in-house program allowed the peptide mapping and their average
179 cumulative abundances onto the parent protein. For each digestion and each phase (milk prior
180 to digestion, gastric phase or intestinal phase), abundances of the peptides were averaged over
181 the different digestion times of the corresponding phase, summed amino acid by amino acid
182 and log₁₀-transformed [log₁₀(abundance+1)]. Missing abundances were set at 0. Log₁₀-
183 transformed abundances were averaged by type of milk in order to be plotted along the amino
184 acid sequence of the protein; they were compared between RHM and PHM for each amino
185 acid using a t-test.

186 For each peptide identified at least on two digestions of RHM or PHM (n = 1134), a two-way
187 analysis of variance was fitted (*lm* function; pasteurization, time and their interaction as
188 factors) on the transformed abundances of the 9 time points (before digestion, 3 time points in
189 the gastric phase and 5 in the intestinal phase) over the three digestions of RHM and PHM,
190 such as performed previously (Deglaire, et al., 2016). Abundance transformation consisted in
191 abundance scaling after log-transformation [log(abundance + 1)] followed by setting the
192 maximum abundance to 1. From the two-way analysis of variance, the “Pasteurization x
193 Digestion time” interaction coefficients, for which the sum equalized zero over time within
194 each milk and at each time for RHM and PHM, were used for subsequent analyses. In
195 particular, the eight independent interaction coefficients of PHM were used for the ascending
196 hierarchical clustering (*hclus* function, stats package) of the 1134 peptides based on the
197 minimum within-cluster variance Ward’s agglomeration. The number of clusters was
198 determined thanks to the bar heights at one of the most marked jump. The function
199 *heatmap.2* was used to display the heatmap and its dendrogram. Cluster characterization was

200 undertaken (*catdes* function, FactoMiner package) using the biochemical characteristics. A
201 Chi-square test was undertaken for qualitative variables (protein origin and cleavage sites),
202 and a one-way analysis of variance for quantitative variables (isoelectric point, molecular
203 weight, number of essential amino acids, GRAVY). V-test indicated whether the modality
204 category was significantly overrepresented ($v > 2$) or underrepresented ($v < -2$).

205 A two-way analysis of variance (with pasteurization, time and their interaction as factors) was
206 fitted to the abundances of each bioactive peptide. When the interaction was statistically
207 significant, a *post-hoc* test was performed on the model residuals (*testinteractions* function,
208 *phia* package) using the p-value adjustment method of Benjamini & Hochberg (1995).
209 Statistical significance level was considered for p-value < 0.05 .

210 **3. Results**

211 **3.1 Identified proteins and peptides**

212 Twenty-seven human milk proteins (**Supplementary Material 1**) were at the origin of 1531
213 peptides (**as detailed in Supplementary Material 2**) identified in RHM and PHM prior to and
214 during gastro-intestinal digestion. Identified peptides contained 6 to 48 amino acids. Some
215 peptides (n = 162) presented modifications that were serine phosphorylation (n = 84),
216 methionine oxidation (n = 43), lactosylation of lysine (n = 8) or arginine (n = 2),
217 glutamine/glutamic acid cyclisation (n = 4), or acetylation (n = 21). A large proportion of the
218 modified peptides (73%) had a sequence that was also identified either without modification
219 or with a different modification.

220 **3.2 Proteolysis of human milk prior to gastrointestinal digestion**

221 Before digestion, 204 peptides were identified and derived at 82.3% from β -casein; the
222 remaining peptides derived from α_{s1} -casein (7.4%), osteopontin (4.9%) and polymeric
223 immunoglobulin receptor (4.4%). PHM and RHM presented 43 and 33 specific peptides,
224 respectively (at 84 and 70% from β -casein) and shared 128 common peptides. The average
225 abundances of all the peptides identified prior to digestion were plotted onto their parent
226 protein sequences, as shown in **figure 1A**. These peptides covered 95% of the β -casein
227 sequence in both RHM and PHM, with **28% of this hydrolysed sequence having a non-**
228 **transformed abundance significantly higher for PHM than for RHM (4 times; P < 0.05)**. The
229 greatest difference was observed **between the residues 136-138 residues with a PHM/RHM**
230 **ratio of 12.**

231 For α_{s1} -casein (figure 1A), peptides were identified over 53% and 71% of the sequence for
232 PHM and RHM, respectively. Non-transformed abundances were **significantly** lower for
233 PHM than for RHM **only between residues 84 and 109** (2 times; P < 0.05). **For osteopontin**
234 **and polymeric immunoglobulin receptor**, peptides were identified on a limited portion of the

235 sequence (figure 1A) and abundances were not significantly different between RHM and
236 PHM.

237 Based on their high odds ratio and total number of cleavages, plasmin (which shares the same
238 cleavage sites as trypsin) was predicted to be the most active enzyme in human milk
239 proteolysis prior to gastrointestinal digestion (Table 1), with a similar number of cleavages
240 between RHM and PHM (87 and 85, respectively). Cathepsin D was also assumed to be
241 responsible of a high number of cleavages (61 and 71 for RHM and PHM), but due to its large
242 specificity, the odds ratio was below 1. Cytosol aminopeptidase was assumed to have mainly
243 cleaved sites located on the C-ter side of β -casein for both RHM and PHM (Supplemental
244 material 3), while carboxypeptidase B2 mainly cleaved at the residue 96.

245 3.3 NH₂ release after digestion of human milk

246 The concentration of NH₂ released after gastric and intestinal digestion was not significantly
247 different ($P > 0.05$) between RHM and PHM, with average values of 47.5 ± 15.3 and $611.2 \pm$
248 14.7 mg of NH₂/L of milk at 90 min of gastric digestion and 180 min of intestinal digestion,
249 respectively.

250 3.4 Peptide release during gastro-intestinal digestion of RHM and PHM

251 *Peptidomic profiles of proteins*

252 The first eight proteins being at the origin of the identified peptides were included in Figure
253 1, showing the average cumulative peptide abundances onto the protein sequence during
254 digestion. The proportion of the sequence covered by peptides was at 100% during all the
255 digestion only for β -casein (figure 1A), while it decreased from the gastric to the intestinal
256 phase for polymeric immunoglobulin receptor and bile-salt stimulated lipase (-12 and -21%,
257 respectively) and increased for the other proteins (+13% for α_{s1} -casein up to +117% for α -
258 lactalbumin). During the gastric phase, only abundances from bile-salt stimulated lipase
259 significantly differed between RHM and PHM with greater abundances for RHM on 16% of

260 the hydrolysed sequence (**Figure 1B**). During the intestinal phase, this relationship was
261 opposite for bile-salt stimulated lipase with abundances being significantly higher for PHM (6
262 times higher for non-transformed abundances) on 59% of the hydrolysed sequence. A similar
263 relationship was observed for lactoferrin during the intestinal digestion (10 times significantly
264 higher for non-transformed abundances of PHM; 32% of the hydrolysed sequence), while for
265 α_{s1} -casein cumulative abundance was higher for RHM (6 times) but on a limited proportion of
266 the hydrolysed sequence (15%).

267 *Kinetics of peptide release during gastrointestinal digestion*

268 Among the 1531 peptides, 26 % of them were not included in the clustering analyses, because
269 they were detected exclusively in RHM (n = 172) or in PHM (n= 73), or sporadically in both
270 RHM and PHM (i.e. only detected on one out of the three digestions, n = 152). This did not
271 greatly affect the parent protein distribution (**Figure 2A**).

272 Eight clusters resumed the kinetics of release of the 1134 peptides (**Figure 2B**) and the
273 qualitative variable being the most significantly associated to the clustering was the parent
274 protein (p-value = 6.1 E-21). One cluster (n° 4; 31% of the peptides) included peptides
275 exerting similar kinetics along the entire gastro-intestinal digestion. These peptides were
276 originating mostly from β -casein and to a lesser extent from κ -casein, while there was a very
277 limited number of peptides from bile-salt stimulated lipase and lactoferrin (Table 2).

278 Clusters 6 and 8 (36% of the peptides) included peptides mainly produced during the gastric
279 phase and showed a lower abundance for PHM than for RHM (**Figure 2C**). In cluster 6,
280 leucine (cleavage specificity for pepsin) in position P1 was significantly more represented
281 unlike lysine and arginine (cleavage specificity for plasmin/trypsin) significantly less
282 represented than in the overall dataset; peptides had in average a higher molecular weight than
283 in overall (**Table 2**). Cluster 8 was significantly associated to bile salt-stimulated lipase.

284 Clusters 1 to 3 (20% of the peptides) included peptides mostly produced in the intestinal
285 phase, where the abundance was lower for PHM than for RHM **at some digestion time** (Figure
286 2C). In cluster 1, the proportion of α_{s1} -casein peptides and of arginine in position P1 was
287 greater than in overall (Table 2). In cluster 3, there was significantly less peptides from β -
288 casein than in overall and no peptides from bile salt-stimulated lipase (Table 2). Cluster 5 (5%
289 of the peptides) showed peptides produced only during the intestinal phase **at a constant level**
290 **for RHM while decreasing for PHM** (Figure 2C). These peptides were more originating from
291 lactoferrin (46% of the cluster peptides) and less from β -casein and α_{s1} -casein (24% of the
292 cluster peptides); **they contained** more lysine and arginine in position P1 than in overall
293 (Table 2). Cluster 7 (9% of the peptides) was the only cluster where PHM abundance was
294 higher during intestinal digestion (at 90 and 120 min). It included more peptides from bile
295 salt-stimulated lipase but less from β -casein and α -lactalbumin; their molecular weight was
296 lower than in overall (Table 2).

297 **3.5 Bioactive peptides**

298 **The exact matching sequence search over the 1531 peptides resulted in 36 peptides having a**
299 **predicted antimicrobial activity; they were originating mostly from bile-salt stimulated lipase**
300 **(39% of them) and lactoferrin (19%) (data not shown). Eight peptides were found to have a**
301 **proved bioactivity according to the literature, such as indexed by BIOPEP and MBPDB**
302 **databases. They were all from β -casein, except for one peptide from lactoferrin (Table 3). The**
303 **antimicrobial peptide β -casein(185-211) had an abundance six times lower ($P < 0.05$) for**
304 **PHM prior to digestion but its abundance was then similar ($P > 0.05$) between RHM and**
305 **PHM during the first hour of gastric digestion before being null for both types of milk. Two**
306 **angiotensin-converting enzyme inhibitor peptides [β -casein(154-160) and β -casein(161-166)]**
307 **were present only in the intestinal phase and had abundances significantly higher ($P < 0.05$)**
308 **for PHM than for RHM. Non-transformed abundances were two times higher for β -**

309 casein(154-160) in PHM during the intestinal digestion (except at 60 min) and four times
310 higher for β -casein(161-166) in PHM only at 180 min of digestion. An additional bioactive
311 peptide, not reported within the bioactive peptide databases, was the β -casein(1-18), a
312 caseinophosphopeptide (CPP) homologue (Wada & Lonnerdal, 2015), which was present in
313 the human milk prior to digestion in a lower proportion in PHM than in RHM (3 times lower).
314 This difference was maintained along the gastric digestion, while the peptide was hydrolysed
315 rapidly in the intestinal phase.

316 **4. Discussion**

317 The present paper demonstrates that pasteurization **impacted selectively** the peptide release
318 from preterm human milk prior to digestion and during gastro-intestinal digestion at the
319 preterm newborn stage, **mainly depending on the protein origin**. This is the first time that a
320 complete view of the peptides identified and released during digestion is provided, from semi-
321 quantitative peptidomic maps up to peptide clustering based on their kinetics of release.

322 **4.1 Human milk pre-proteolysis**

323 Before digestion, endogenous peptides were detected in human milk and originated
324 essentially from β -casein, and to a lesser extent from α_{s1} -casein, osteopontin and polymeric
325 immunoglobulin receptor, such as observed previously (Dallas, Guerrero, Khaldi, Castillo,
326 Martin, Smilowitz, et al., 2013; Deglaire, et al., 2016; Dingess, De Waard, Boeren, Vervoort,
327 Lambers, Van Goudoever, et al., 2017; Ferranti, Traisci, Picariello, Nasi, Boschi, Siervo, et
328 al., 2004; Khaldi, Vijayakumar, Dallas, Guerrero, Wickramasinghe, Smilowitz, et al., 2014;
329 Nielsen, Beverly, & Dallas, 2017). This confirms that indigenous milk proteases (e.g.
330 plasmin, carboxypeptidase, cytosol aminopeptidase, kallikrein) can hydrolyse human milk
331 proteins, particularly β -casein, within the mammary gland (Demers-Mathieu, Nielsen,
332 Underwood, Borghese, & Dallas, 2017; Nielsen, Beverly, & Dallas, 2017) but also during
333 milk processing (freeze-thaw cycle, pasteurization), as qualitative and quantitative differences
334 occurred on the same pool of milk differing only by their processing (raw or pasteurized).

335 In the present study, plasmin was predicted, to be the most active protease in human milk,
336 such as reported previously (Deglaire, et al., 2016; Nielsen, Beverly, & Dallas, 2017). The
337 number of cleavages from plasmin between PHM and RHM was not different, unlike in our
338 previous study (Deglaire, et al., 2016), however PHM abundance was greater at 6 out of the
339 11 Lys residues (plasmin cleavage specificity) of the β -casein sequence. As discussed
340 previously (Deglaire, et al., 2016), pasteurization may have activated the milk plasmin system

341 through inactivation of plasminogen activator inhibitors and plasmin inhibitors (Ismail &
342 Nielsen, 2010). Besides, denaturation of lactoferrin by pasteurization, as confirmed by reverse
343 HPLC (signal decreased by 3 after pasteurization; unpublished data), may have contributed to
344 this activation, as it acts as a natural inhibitor of plasminogen activation (Zwirzitz, Reiter,
345 Skrabana, Ohradanova-Repic, Majdic, Gutekova, et al., 2018). It should be noted that the
346 arginine and lysine residues are also cleavage specificities of kallikrein and thrombin, which
347 have been shown to be active in preterm human milk (Demers-Mathieu, Nielsen, Underwood,
348 Borghese, & Dallas, 2017). In addition, the arginine and lysine residues are the cleavage sites
349 for trypsin that has been shown to be potentially present in human milk but not active
350 (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2017).

351 A greater abundance for PHM was observed between the β -casein residues 130-145, although
352 this was statistically significant only between residues 136-138. This area includes the
353 hydrophobic amino acids that are leucine, valine, isoleucine, which are cleavage sites for both
354 cathepsin D and elastase (Vijayakumar, Guerrero, Davey, Lebrilla, Shields, & Khaldi, 2012).
355 However, cathepsin D, which is an acid protease, is mostly present in human milk as
356 procathepsin D, i.e. the inactive zymogen (Vijayakumar, Guerrero, Davey, Lebrilla, Shields,
357 & Khaldi, 2012). On the contrary, elastase has been shown to be active in human milk
358 recently (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2017) and may have
359 been more active in PHM in this area due to the heat-induced structural changes of β -casein
360 (Guyomarc'h, Law, & Dalgleish, 2003) unmasking resistant area and possibly compensating
361 for the loss of activity likely induced by heat treatment. For the same reason, cystosol
362 aminopeptidase may also have been more active in PHM particularly at the residues 136 and
363 137 (Supplemental Material 3), where the abundance was 12 times greater for PHM. Because
364 cystosol aminopeptidase is a minor protease in milk, particularly in bovine milk, few
365 information are available on the impact of heat treatment on its activity (O'Mahony, Fox, &

366 Kelly, 2016). Some pepsin and chymotrypsin activities have been predicted in the present
367 paper, likely due to their overlapping specificity with elastase (Vijayakumar, Guerrero,
368 Davey, Lebrilla, Shields, & Khaldi, 2012) as they have not been shown to be present in
369 human milk (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2018).

370 Overall, as reported earlier (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas,
371 2017), the present work suggests that indigenous enzymes, such as plasmin, elastase and
372 probably cytosol aminopeptidase, were more active in pasteurized milk. As a result, some
373 peptides were more actively produced (i.e. more abundant) in PHM or, on the contrary, more
374 extensively degraded, such as observed for the antimicrobial peptide, β -casein(185-211). The
375 latter was less abundant in PHM prior to digestion, maybe due to a greater activity of cytosol
376 amino peptidase in this area (Supplemental Material 3). Nevertheless, this does not indicate
377 whether the proteolysis degree prior to digestion was different between RHM and PHM.

378 Pre-proteolysis of β -casein may have contributed to its further digestion, in particular on the
379 first 20 amino acids where the cumulative abundance remained similar before and after 30
380 min of gastric digestion. Indigenous enzymes, in particular the acid enzyme cathepsin D,
381 could have contributed to gastric digestion although a minimal contribution has been
382 previously assumed (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2018).

383 **4.2 Human milk digestion**

384 The distribution of the protein origin of the common peptides was similar to that observed in
385 our previous study at the term stage (Deglaire, et al., 2016), with the major protein of human
386 milk, β -casein, generating 42% of the peptides, whilst α -lactalbumin, the other major protein
387 in human milk, generating only 3% of the peptides. This can reflect its proteolysis resistance
388 as observed by SDS-PAGE (De Oliveira, et al., 2016a) but also probably the non-
389 identification of the peptides engaged in a disulphide bond (four out of 123 amino acids of the
390 protein sequence) with our non-reducing analysis. Such as observed previously (Deglaire, et

391 al., 2016), despite being minor proteins in terms of concentrations, bile-salt stimulated lipase
392 and osteopontin generated almost the same number of peptides as lactoferrin and α_{s1} -casein.
393 Nevertheless, this is not indicative of the proteolysis degree of these proteins.

394 In the present study, the proportion of peptides predominantly released in the gastric phase
395 (36% of the peptides in 2 clusters) was lower than in the intestinal phase, while the opposite
396 was true in the term study (Deglaire, et al., 2016). This is certainly resulting from the
397 decreased level of pepsin (2 times lower) combined to a greater protein content (1.2 times
398 higher) and a faster gastric emptying ($t_{1/2}$ of 36 min) for the preterm model compared to the
399 term one ($t_{1/2}$ of 47 min) (Bourlieu, Menard, Bouzerzour, Mandalari, Macierzanka, Mackie, et
400 al., 2014).

401 In the gastric phase, peptide abundances were generally lower for PHM than for RHM,
402 particularly for peptides from bile salt-stimulated lipase, such as observed on both peptidomic
403 mapping and clustering. On the contrary, intestinal peptide abundances from bile-salt
404 stimulated lipase became in average higher for PHM than for RHM, as observed on the
405 protein sequence and in clusters 7 and 8 (gathering 50% of its peptides). These gastro-
406 intestinal kinetics of peptide release are in line with that previously at the term stage
407 (Deglaire, et al., 2016). This indicates a delay in the proteolysis of bile salt-stimulated lipase
408 from PHM unlike that for RHM, maybe due to some increased resistance to gastric
409 proteolysis due to some heat-induced structural changes, probably masking pepsin cleavage
410 sites. This heat-denaturation is already known to drastically inactivate this indigenous lipase
411 (Henderson, Fay, & Hamosh, 1998). Besides, it can be noted that no peptide was found from
412 the residue 526 until the C-ter end of bile-salt stimulated lipase. This region includes a high
413 number of proline and has been described, from residue 538, as a mucin-like flexible C-
414 terminal tail with a high level of glycosylation (Downs, Xu, Tang, Wang, 1994), probably
415 preventing it from proteolysis but also from MS/MS peptide identification.

416

417 Regarding lactoferrin, data suggested a greater and faster release of its peptides in PHM,
418 resulting in the intestinal phase in peptides being less abundant in PHM than in RHM (cluster
419 5 and protein sequence in supplemental Material 4). This can indicate a lower resistance to
420 intestinal proteolysis for the heat-denaturated lactoferrin, unlike that for the native lactoferrin,
421 a globular protein, known to be resistant to proteolysis such as discussed earlier (Deglaire et
422 al., 2016). The concentration of native lactoferrin reaching the intestinal epithelium is thus
423 likely to differ between PHM and RHM, which can modulate its bioactivity. As reported by
424 Buccigrossi et al. (2007), high lactoferrin concentrations induced epithelial cell proliferation
425 while low lactoferrin concentrations stimulated intestinal differentiation. In addition, the
426 greater persistence of intact peptides in the gastro-intestinal tract may have physiological
427 consequences, as it is known to be important for gut-associated-lymphoid tissue (GALT)
428 education and microbiota shaping (Chatterton, Nguyen, Bering, & Sangild, 2013; Newburg &
429 Walker, 2007). It should be noted that the end of our intestinal digestion may not be fully
430 representative of the *in vivo* condition, as brush border peptidases, not included in our model,
431 can further hydrolyse the present peptides. Thus, pasteurization may impact the regulation of
432 the early postnatal intestinal development, through modulation of lactoferrin proteolysis, but
433 further investigations are needed.

434 Regarding β -casein, data suggested that its peptide abundances were in average not different
435 between RHM and PHM and its kinetics of peptide release were opposite to those for
436 lactoferrin or bile salt-stimulated lipase. This is well in agreement with our previous
437 observations in the term study (Deglaire, et al., 2016). Nevertheless, there was no emerging
438 cluster significantly associated to β -casein with an increased gastric abundance for PHM in
439 the present study, while this was true in the term study for a limited proportion of the peptides

440 (11% of the total peptides, 17% of the β -casein peptides) (Deglaire, et al., 2016). The reasons
441 for this remain unknown.

442 Overall, the different kinetics of peptide release between PHM and RHM may have been
443 somewhat compensating, together with smaller and larger peptides not taken into account
444 here, as the overall degree of gastro-intestinal proteolysis (based on the primary amine
445 content) was not significantly different but the intestinal bioaccessibility of some amino acids
446 (lysine, phenylalanine, tyrosine) was increased by pasteurization (De Oliveira, et al., 2016a).

447 **4.3 Bioactive peptides**

448 The present bioactive peptides were identified only after exact matching sequence, although
449 other bioactive peptides can be encrypted within larger peptides and further released *in vivo*
450 by brush border enzymes not included in the present model. Most potential bioactive peptides
451 were predicted, but not experimentally demonstrated, as having an antimicrobial activity.
452 Only a limited number of bioactive peptides, mostly from β -casein, was based on a
453 demonstrated activity in the literature. Prior to digestion, two bioactive peptides, the
454 caseinophosphopeptide (CPP) homologue β -casein(1-18) (Wada & Lonnerdal, 2015) and the
455 antimicrobial peptide β -casein(185-211), were present in human milk as observed previously
456 (Deglaire, et al., 2016; Dingess, et al., 2017; Wada & Lonnerdal, 2015). Their abundance
457 were lower in PHM than in RHM prior digestion, unlike that observed in the term study
458 (Deglaire, et al., 2016). Whether this is due to the term vs. preterm condition remains
459 unknown. Both peptides were hydrolysed rapidly in the intestinal phase in both types of milk,
460 unlike that observed by Wada et al. (2015). This can result from milk variability but also from
461 the simpler and shorter model of digestion (static conditions) used by Wada et al. (2015).
462 Regarding lactoferrin, only one opioid antagonist peptide was released during the gastric
463 phase in a similar manner between RHM and PHM. No peptide was detected between the
464 residues 1-49 that includes the antimicrobial lactoferricin peptide (Zwirzitz, et al., 2018),

465 probably due to the presence of disulphide bonds between Cys¹⁹ and Cys³⁶ and between Cys⁹
466 and Cys⁴⁵ (Farnaud & Evans, 2003; Wada & Lonnerdal, 2015). However, looking at
467 individual bioactive peptides and at their abundances should be considered with care; further
468 confirmation would be required.

469 5. Conclusion

470 To conclude, the present study demonstrated that pasteurization impacted the human milk
471 peptidome prior to digestion mainly originating from β -casein and induced different kinetics
472 of peptide release during gastro-intestinal digestion mainly for the heat-denaturated proteins
473 (bile-salt stimulated lipase and lactoferrin), such as observed in the term stage. When
474 considering the overall proteolysis, there was no impact of pasteurization; however, it may
475 induce different concentrations of intact peptides within the gastro-intestinal, which may have
476 further physiological consequences.

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499 transformed abundances. (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$)

500 **Figure 2.** **A**) Protein origin of the peptides by cluster obtained during gastrointestinal
501 digestion of raw (RHM) and pasteurized (PHM) human milk, **B**) Heatmap of the “
502 Pasteurization x Digestion time ” interaction coefficients as classified by ascending
503 hierarchical classification , **C**) average abundances of the peptides by cluster. $n = 1134$
504 peptides; G: gastric phase, I: intestinal phase, digestion time in min.

505 **Supporting information**

506 **Supplemental material 1.** Proteins ($n = 27$) from which derived the identified peptides
507 before and during digestion of raw and pasteurized milk.

508 **Supplemental material 2.** Dataset of all the unique peptides ($n = 1531$) identified before and
509 during digestion of raw and pasteurized milk.

510 **Supplemental material 3.** Mapping of β -casein hydrolysis prior to digestion with cleavage
511 sites of plasmin, cytosol amino peptidase and carboxypeptidase B2.

512 **Supplemental material 4.** Mapping of the average peptide abundances of RHM and PHM
513 onto their parent proteins at 180 min of intestinal digestion. Peptide abundances, summed at each
514 amino acid, were averaged over the three digestion replicates of RHM or PHM at 180 min of intestinal digestion.

Table 1: Predicted enzymes and counts for proteolysis of human milk as raw or pasteurized before gastrointestinal digestion¹

Enzymes	Raw human milk (n = 161 peptides)				Pasteurised human milk (n = 171 peptides)					
	Total number of cleavages	Number of expected cleavages within peptides	Number of proteins cleaved	Odds Ratio ²	Standard Error ³	Total number of cleavages	Number of expected cleavages within peptides	Number of proteins cleaved	Odds Ratio ²	Standard Error ³
Plasmin	87	137	5	4.7	1.2	85	137	5	4.8	1.2
Trypsin 1	87	137	5	4.7	1.2	85	137	5	4.8	1.2
Cathepsin D	61	769	4	0.4	1.2	71	788	3	0.5	1.1
Pepsin (pH1.3) 1	56	238	4	1.5	1.2	58	254	4	1.6	1.2
Chymotrypsin low 1	48	300	4	1.0	1.2	44	304	3	0.9	1.2
Elastase	45	413	2	0.6	1.2	39	428	3	0.6	1.2
Pepsin (pH1.3) 2	34	232	3	0.9	1.2	27	241	2	0.7	1.2
Proline endopeptidase	18	492	2	0.2	1.3	21	505	2	0.2	1.3

¹ Results obtained from the online software Enzyme Predictor. Cleavages from cytosol aminopeptidase and carboxypeptidase B2 were examined manually on β -casein and appear only in supplementatl figure 3.

² Odds ratio (OR) were calculated on the present enzymes as $OR = (a * d) / (b * c)$, where a is the total number of sites cleaved by enzyme “A” at the peptide termini (“total number of cleavages”), b is the total number of all sites cleaved by all enzymes except “A”; c is the total number of sites enzyme “A” could have cleaved within the current peptides (“number of expected cleavages within peptides”), and d is the number of sites all enzymes, aside from A, could have cleaved within the current peptides.

³ Standard errors (SE) were calculated as : $SE = \exp(1/a + 1/b + 1/c + 1/d)^{1/2}$ (Vijayakumar, Guerrero, Davey, Lebrilla, Shields, & Khaldi, 2012).

Table 2. Peptide biochemical characteristics significantly associated ($p < 0.05$) with clusters^{1,2}.

	Qualitative modalities					Quantitative modalities					
	Modality	Cl/Mod ³	Mod/Cl ⁴	Overall ⁵ (n= 1134)	v.test	p.value	Modality	Mean \pm SD in category	Mean \pm SD in overall (n= 1134)	v.test	p.value
Cluster 1	protein = CASA1	14.43	19.44	8.55	3.01	0.0027					
	P1' = M	19.23	6.94	2.29	2.24	0.0251					
	P1' = S	15.22	9.72	4.06	2.18	0.0290					
	P1 = R	11.02	19.44	11.20	2.13	0.0336					
	protein = FAS	40.00	2.78	0.44	2.08	0.0373					
	protein = TRFL	11.11	16.67	9.52	1.97	0.0483					
	P1 = T	0.00	0.00	4.14	-2.02	0.0429					
	P1' = K	0.00	0.00	4.59	-2.16	0.0304					
P1' = D	0.00	0.00	4.59	-2.16	0.0304						
Cluster 2	protein = AMY2B	42.86	2.86	0.62	2.28	0.0224	aliphatic index	94.41 \pm 42.47	87.58 \pm 37.07	1.98	0.0476
	P1' = L	13.82	20.00	13.40	1.98	0.0472	MW ⁶	1.75 \pm 0.67	1.92 \pm 0.8	-2.26	0.0235
	P1' = P	1.67	0.95	5.29	-2.31	0.0212	isoelectric point	5.4 \pm 1.55	5.85 \pm 1.99	-2.45	0.0143
Cluster 3	protein = BT1A1	22.22	12.50	2.38	3.39	0.0007					
	P1' = G	14.29	12.50	3.70	2.64	0.0082					
	protein = FAS	40.00	4.17	0.44	2.39	0.0168					
	P1' = K	11.54	12.50	4.59	2.25	0.0241					
	P1' = D	11.54	12.50	4.59	2.25	0.0241					
	protein = AMY2B	28.57	4.17	0.62	2.12	0.0344					
	P1 = F	9.21	14.58	6.70	1.98	0.0482					
	P1 = L	1.79	8.33	19.66	-2.12	0.0338	aliphatic index	76.84 \pm 42.38	87.58 \pm 37.07	-2.05	0.0403
	protein = CASB	2.74	27.08	41.89	-2.14	0.0324	GRAVY	-0.7 \pm 0.87	-0.42 \pm 0.74	-2.62	0.0088
	protein = CEL	0.00	0.00	7.50	-2.29	0.0219	isoelectric point	4.9 \pm 1.43	5.85 \pm 1.99	-3.37	0.0007
	P1' = L	0.66	2.08	13.40	-2.64	0.0084					
Cluster 4	protein = CASB	38.53	51.55	41.89	4.43	<0.0001					
	protein = CASK	55.81	6.76	3.79	3.36	0.0008					
	P1' = N	53.85	5.92	3.44	2.94	0.0033					
	P1' = H	54.84	4.79	2.73	2.72	0.0066					
	P1 = M	18.87	2.82	4.67	-2.04	0.0412	GRAVY	-0.52 \pm 0.76	-0.42 \pm 0.74	-3.04	0.0024
	P1 = F	17.11	3.66	6.70	-2.86	0.0042					
	protein = CEL	8.24	1.97	7.50	-5.23	<0.0001					
	protein = TRFL	6.48	1.97	9.52	-6.51	<0.0001					
Cluster 5	protein = TRFL	25.00	45.76	9.52	7.53	<0.0001					
	P1 = K	13.37	38.98	15.17	4.58	<0.0001					
	P1' = T	14.29	10.17	3.70	2.27	0.0232					
	P1 = R	9.45	20.34	11.20	2.10	0.0355					
	protein = CASA1	1.03	1.69	8.55	-2.11	0.0347	essential AA	5.07 \pm 1.57	7.02 \pm 3.65	-4.23	<0.0001
protein = CASB	2.95	23.73	41.89	-2.96	0.0031	MW	1.42 \pm 0.44	1.92 \pm 0.8	-4.97	<0.0001	
Cluster 6	P1 = D	65.00	5.70	1.76	4.34	<0.0001	essential AA	8.62 \pm 3.98	7.02 \pm 3.65	7.39	<0.0001
	P1 = L	29.15	28.51	19.66	3.63	0.0003	MW	2.24 \pm 0.85	1.92 \pm 0.8	6.63	<0.0001
	P1' = P	40.00	10.53	5.29	3.62	0.0003	isoelectric point	6.46 \pm 2.21	5.85 \pm 1.99	5.20	<0.0001
	P1 = M	33.96	7.89	4.67	2.42	0.0157	GRAVY	-0.29 \pm 0.63	-0.42 \pm 0.74	2.98	0.0030
	protein = ALBU	50.00	2.63	1.06	2.27	0.0232					
	P1' = K	9.62	2.19	4.59	-2.01	0.0440					
	P1' = N	7.69	1.32	3.44	-2.07	0.0383					
	protein = OSTP	10.87	4.39	8.11	-2.41	0.0160					
	P1 = Y	9.68	3.95	8.20	-2.77	0.0056					
	protein = CASK	4.65	0.88	3.79	-2.84	0.0046					
	P1 = R	11.02	6.14	11.20	-2.84	0.0045					
P1 = K	11.05	8.33	15.17	-3.37	0.0007						
Cluster 7	protein = CEL	20.00	18.68	7.50	3.66	0.0002					
	P1' = K	19.23	10.99	4.59	2.65	0.0082					
	P1' = G	19.05	8.79	3.70	2.33	0.0198	isoelectric point	5.4 \pm 1.61	5.85 \pm 1.99	-2.24	0.0254
	protein = LALBA	19.35	6.59	2.73	2.04	0.0414	essential AA	5.51 \pm 2.58	7.02 \pm 3.65	-4.14	<0.0001
	protein = CASB	6.11	31.87	41.89	-2.03	0.0425	MW	1.57 \pm 0.65	1.92 \pm 0.8	-4.39	<0.0001
Cluster 8	protein = CEL	32.94	15.91	7.50	4.17	<0.0001					
	P1' = T	28.57	6.82	3.70	2.19	0.0286					
	P1' = P	25.00	8.52	5.29	1.96	0.0498					
	P1' = G	2.38	0.57	3.70	-2.68	0.0073					

¹ Abbreviations: AA: amino acid, ALBU : serum albumin, AM2YB : α -amylase 2B, BT1A1 : butyrophilin subfamily 1 member A1, CASA1: α s1-casein, CASB : β -casein, CASK : K-casein, CEL : carboxyl ester lipase or bile salt-stimulated lipase, FAS: fatty acid synthase, LALBA : α -lactalbumin, OSTP: osteopontin, TRFL: lactoferrin.

² Grey lines correspond to modalities that are negatively associated to a cluster

³ Cla/Mod: occurrence of the modality category in the cluster divided by its occurrence in the entire dataset

⁴ Mod/Cla: proportion of the modality category within the cluster

⁵ Overall proportion of this modality category within the entire dataset

⁶ MW: Molecular Weight, expressed in kDa.

Table 3. Bioactive peptides identified within the entire dataset (n = 1531) using an exact matching search within the databases of BIOPEP and MBPDB

Parent protein	Peptide position ¹	Peptide sequence	Activity	Pasteurization effect ²	Time effect ²	Pasteurization x Time effect ²
	51-58	YPFVEPIP	opioid agonist	NS	***	NS
	54-59	VEPIPY	immunomodulating	NS	***	NS
β-casein	105-117	SPTIPFFDPQIPK	stimulating proliferation	NS	***	NS
	125-130	HLPLPL	anti-amnestic	NS	***	NS
	154-160	WSVPQPK	ACE inhibitor/ antioxidant	***	***	***
	161-166	VLPIPQ	ACE inhibitor	NS	***	*
	185-211	QELLLNPTHQIYPVTQPLAPVHNPISV	antimicrobial	NS	***	**
Lactoferrin	319-324	YLGSGY	opioid antagonist	NS	***	NS

¹ Peptide position on the parent protein without taking into account the signal peptide.

² Effects were determined using a two-way analysis of variance with Pasteurization, Time and their interaction as factors.

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Figure 1
Deglaire

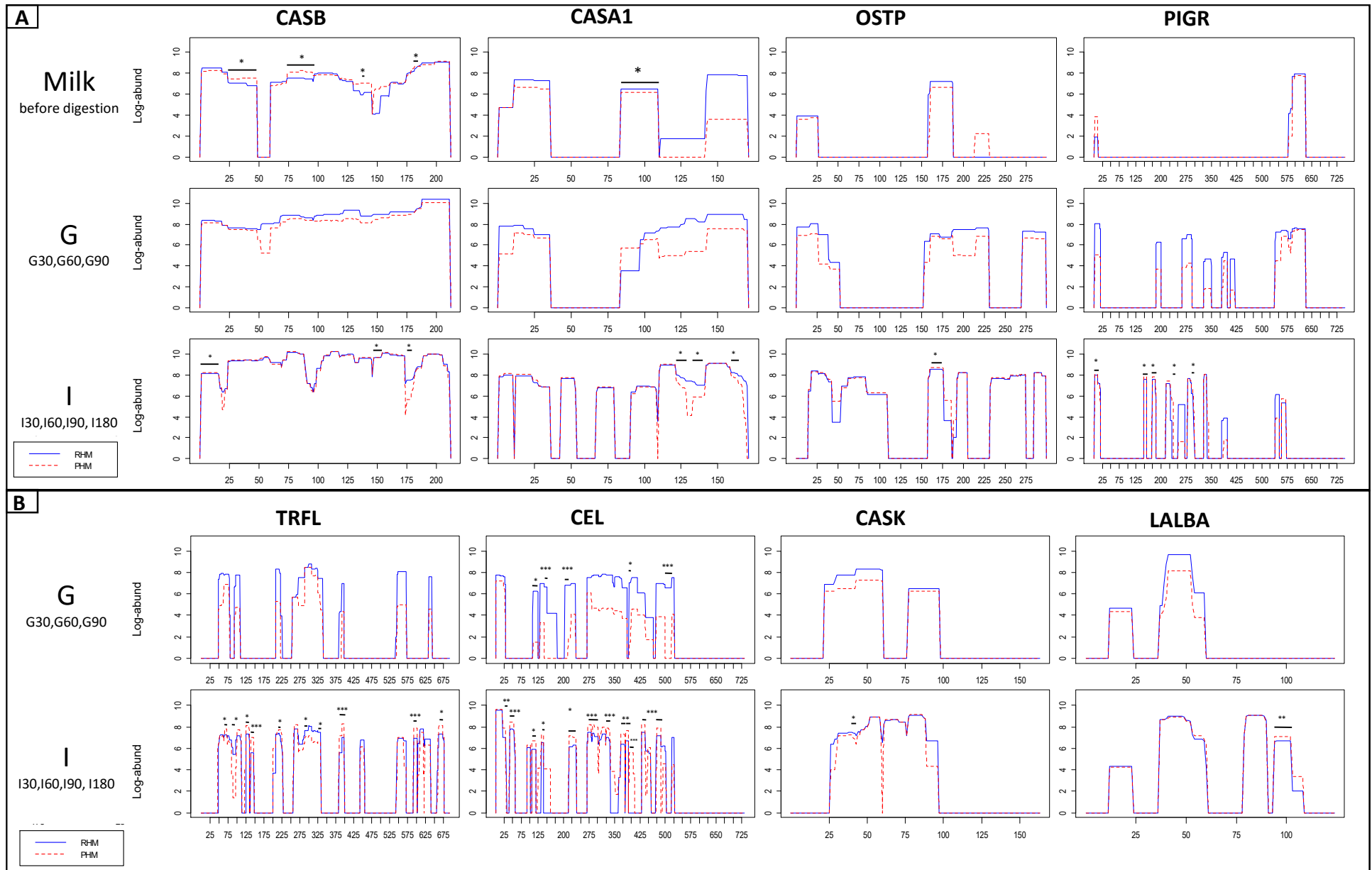
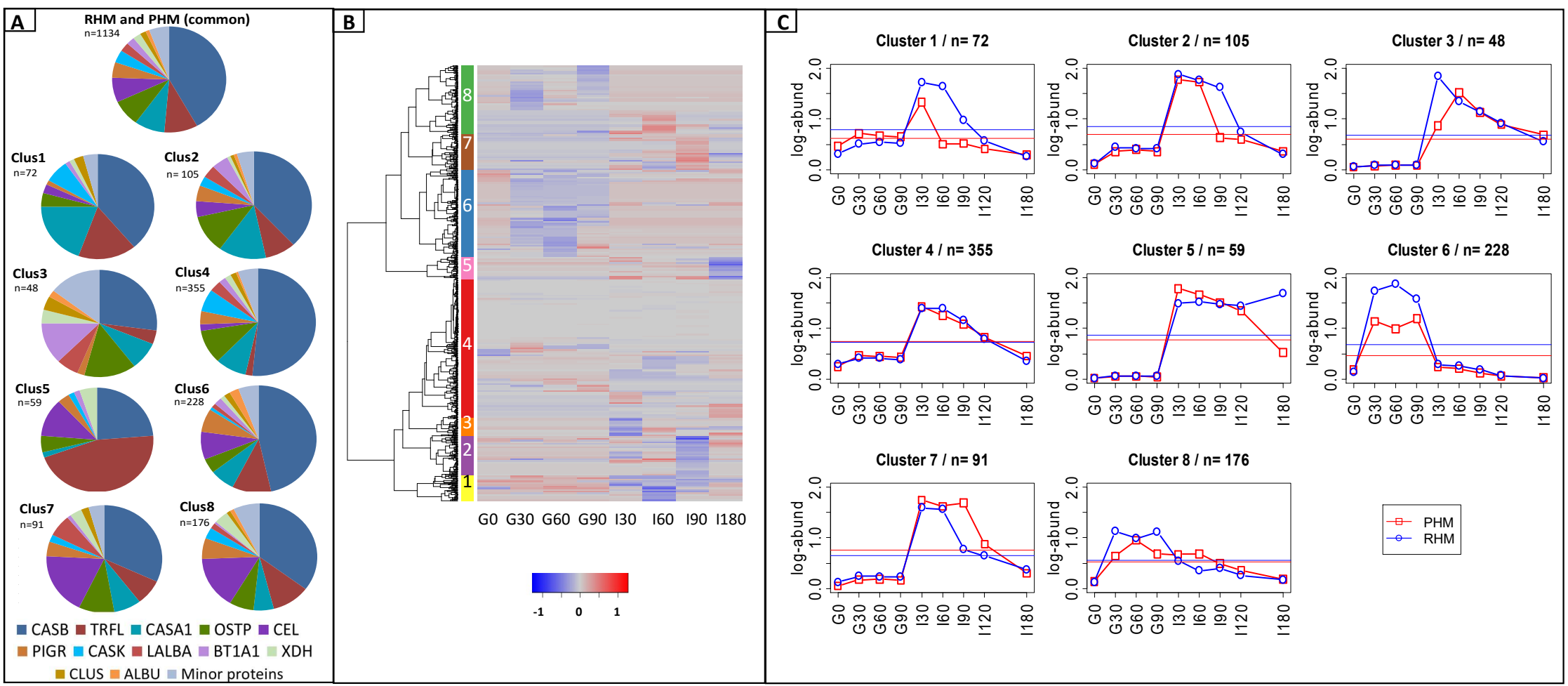


Figure 2
Deglaire



	Qualitative modalities					Quantitative modalities					
	Modality	Cla/Mod ³	Mod/Cla ⁴	Overall ⁵ (n=1134)	v.test	p.value	Modality	Mean ± SD in category	Mean ± SD in overall (n=1134)	v.test	p.value
Cluster 1	protein = CASA1	14.43	19.44	8.55	3.01	0.0027					
	P1' = M	19.23	6.94	2.29	2.24	0.0251					
	P1' = S	15.22	9.72	4.06	2.18	0.0290					
	P1 = R	11.02	19.44	11.20	2.13	0.0336					
	protein = FAS	40.00	2.78	0.44	2.08	0.0373					
	protein = TRFL	11.11	16.67	9.52	1.97	0.0483					
	P1 = T	0.00	0.00	4.14	-2.02	0.0429					
	P1' = K	0.00	0.00	4.59	-2.16	0.0304					
	P1' = D	0.00	0.00	4.59	-2.16	0.0304					
Cluster 2	protein = AMY2B	42.86	2.86	0.62	2.28	0.0224		94.41 ±			
	P1' = L	13.82	20.00	13.40	1.98	0.0472	aliphatic index	42.47	87.58 ± 37.07	1.98	0.0476
	P1' = P	1.67	0.95	5.29	-2.31	0.0212	MW ⁶	1.75 ± 0.67	1.92 ± 0.8	-2.26	0.0235
Cluster 3	protein = BT1A1	22.22	12.50	2.38	3.39	0.0007	isoelectric point	5.4 ± 1.55	5.85 ± 1.99	-2.45	0.0143
	P1' = G	14.29	12.50	3.70	2.64	0.0082					
	protein = FAS	40.00	4.17	0.44	2.39	0.0168					
	P1' = K	11.54	12.50	4.59	2.25	0.0241					
	P1' = D	11.54	12.50	4.59	2.25	0.0241					
	protein = AMY2B	28.57	4.17	0.62	2.12	0.0344					
	P1 = F	9.21	14.58	6.70	1.98	0.0482					
	P1 = L	1.79	8.33	19.66	-2.12	0.0338		76.84 ±			
	protein = CASB	2.74	27.08	41.89	-2.14	0.0324	aliphatic index	42.38	87.58 ± 37.07	-2.05	0.0403
	protein = CEL	0.00	0.00	7.50	-2.29	0.0219	GRAVY	-0.7 ± 0.87	-0.42 ± 0.74	-2.62	0.0088
P1' = L	0.66	2.08	13.40	-2.64	0.0084	isoelectric point	4.9 ± 1.43	5.85 ± 1.99	-3.37	0.0007	

Cluster 4	protein = CASB	38.53	51.55	41.89	4.43	<0.0001
	protein = CASK	55.81	6.76	3.79	3.36	0.0008
	P1' = N	53.85	5.92	3.44	2.94	0.0033
	P1' = H	54.84	4.79	2.73	2.72	0.0066
	P1 = M	18.87	2.82	4.67	-2.04	0.0412
	P1 = F	17.11	3.66	6.70	-2.86	0.0042
	protein = CEL	8.24	1.97	7.50	-5.23	<0.0001
protein = TRFL	6.48	1.97	9.52	-6.51	<0.0001	

GRAVY	-0.52 ± 0.76	-0.42 ± 0.74	-3.04	0.0024
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Cluster 5	protein = TRFL	25.00	45.76	9.52	7.53	<0.0001
	P1 = K	13.37	38.98	15.17	4.58	<0.0001
	P1' = T	14.29	10.17	3.70	2.27	0.0232
	P1 = R	9.45	20.34	11.20	2.10	0.0355
	protein = CASA1	1.03	1.69	8.55	-2.11	0.0347
	protein = CASB	2.95	23.73	41.89	-2.96	0.0031

essential AA	5.07 ± 1.57	7.02 ± 3.65	-4.23	<0.0001
MW	1.42 ± 0.44	1.92 ± 0.8	-4.97	<0.0001

Cluster 6	P1 = D	65.00	5.70	1.76	4.34	<0.0001
	P1 = L	29.15	28.51	19.66	3.63	0.0003
	P1' = P	40.00	10.53	5.29	3.62	0.0003
	P1 = M	33.96	7.89	4.67	2.42	0.0157
	protein = ALBU	50.00	2.63	1.06	2.27	0.0232
	P1' = K	9.62	2.19	4.59	-2.01	0.0440
	P1' = N	7.69	1.32	3.44	-2.07	0.0383
	protein = OSTP	10.87	4.39	8.11	-2.41	0.0160
	P1 = Y	9.68	3.95	8.20	-2.77	0.0056
	protein = CASK	4.65	0.88	3.79	-2.84	0.0046
	P1 = R	11.02	6.14	11.20	-2.84	0.0045
	P1 = K	11.05	8.33	15.17	-3.37	0.0007

essential AA	8.62 ± 3.98	7.02 ± 3.65	7.39	<0.0001
MW	2.24 ± 0.85	1.92 ± 0.8	6.63	<0.0001
isoelectric point	6.46 ± 2.21	5.85 ± 1.99	5.20	<0.0001
GRAVY	-0.29 ± 0.63	-0.42 ± 0.74	2.98	0.0030

Cluster 7	protein = CEL	20.00	18.68	7.50	3.66	0.0002					
	P1' = K	19.23	10.99	4.59	2.65	0.0082					
	P1' = G	19.05	8.79	3.70	2.33	0.0198	isoelectric point	5.4 ± 1.61	5.85 ± 1.99	-2.24	0.0254
	protein = LALBA	19.35	6.59	2.73	2.04	0.0414	essential AA	5.51 ± 2.58	7.02 ± 3.65	-4.14	<0.0001
	protein = CASB	6.11	31.87	41.89	-2.03	0.0425	MW	1.57 ± 0.65	1.92 ± 0.8	-4.39	<0.0001
Cluster 8	protein = CEL	32.94	15.91	7.50	4.17	<0.0001					
	P1' = T	28.57	6.82	3.70	2.19	0.0286					
	P1' = P	25.00	8.52	5.29	1.96	0.0498					
	P1' = G	2.38	0.57	3.70	-2.68	0.0073					
