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## High hydrostatic pressure processing of human milk preserves milk oligosaccharides and avoids formation of Maillard reaction products

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1 **High hydrostatic pressure processing of human milk preserves milk**  
2 **oligosaccharides and avoids formation of Maillard reaction**  
3 **products**

4  
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## Abstract

27

28

29 **Background & Aims:** High hydrostatic pressure (HHP) processing is a non-thermal  
30 method proposed as an alternative to Holder pasteurization (HoP) for the treatment of  
31 human milk. HHP preserves numerous milk bioactive components that are degraded  
32 by HoP, but no data are available for milk oligosaccharides (HMOs) or the formation of  
33 Maillard reaction products, which may be deleterious for preterm newborns.

34 **Methods:** We evaluated the impact of HHP processing of human milk on 22 HMOs  
35 measured by liquid chromatography with fluorescence detection and on furosine,  
36 lactuloselysine, carboxymethyllysine (CML) and carboxyethyllysine (CEL) measured  
37 by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), four  
38 established indicators of the Maillard reaction. Human raw milk was sterilized by HoP  
39 (62.5°C for 30 min) or processed by HHP (350 MPa at 38°C).

40 **Results:** Neither HHP nor HoP processing affected the concentration of HMOs, but  
41 HoP significantly increased furosine, lactuloselysine, CML and CEL levels in milk.

42 **Conclusions:** Our findings demonstrate that HPP treatment preserves HMOs and  
43 avoids formation of Maillard reaction products. Our study confirms and extends  
44 previous findings that HHP treatment of human milk provides safe milk, with fewer  
45 detrimental effects on the biochemically active milk components than HoP.

46 **Keywords:** human milk, high hydrostatic pressure, human milk oligosaccharides,  
47 Maillard reaction products, holder pasteurization

## 48 1. Introduction

49 The invention and refinement of high hydrostatic pressure (HHP) treatment was  
50 an important scientific innovation from the last century [1]. Because of its ability to  
51 inactivate many pathogens while leaving most organoleptic properties of foods  
52 unchanged, the use of HHP began to be adopted by the food industry at the end of the  
53 twentieth century for the sterilization and preservation of foodstuffs [1–3]. Several  
54 studies have demonstrated that HHP may be an innovative method to ensure the  
55 microbiological safety of breast milk donated to human milk banks (HMBs) [4–6]. Most  
56 HMBs currently sterilize human milk using the standard method of Holder  
57 pasteurization (HoP) performed by heating milk to 62.5°C for 30 minutes [7]. Owing to  
58 this heating, HoP partly degrades numerous heat-sensitive bioactive factors in milk  
59 such as immunoglobulins, lactoferrin, some vitamins, lysozyme, the bile salt-  
60 dependent lipase (BSSL) and several hormones [7,8]. Interestingly, recent studies  
61 have shown that HHP processing, in addition to its bacteriolytic effect, maintains  
62 human milk’s nutritional and immunological factors close to their levels in raw milk [4–  
63 6]. For instance, Demazeau *et al.* showed that HHP processing using four 5-minute  
64 cycles of a moderate pressure (350 MPa) at 38°C is effective in sterilizing human milk,  
65 but also maintains numerous bioactive factors such as lactoferrin, lysozyme and BSSL  
66 enzyme at levels close to raw milk [5]. To our knowledge, the effect of HHP on other  
67 important milk compounds such as human milk oligosaccharides (HMOs) so far  
68 remains unknown. HMOs are lactose-derived molecules constituting the third major  
69 group of soluble components in human milk [9,10]. A high abundance and diversity of  
70 over 160 different HMOs have been characterized in human milk, with the majority  
71 showing fucose and sialic acid moieties variously linked to different backbone  
72 structures composed of lactose and additional galactose and N-acetyl-glucosamine

73 moieties [11]. There is growing evidence that HMOs exert numerous beneficial effects  
74 on neonatal development, as well as the protection and promotion of health in infants  
75 [10,12]. For example studies have shown that HMOs are powerful prebiotics for several  
76 bacteria, primarily *Bifidobacterium* species, and that they also hinder the adhesion of  
77 pathogens to gut epithelial cells, directly affecting their maturation and development,  
78 as well as supporting immune functions [10,12]. Notably, HMOs are also thought to  
79 convey important benefits for preterm born infants [13]. The primary aim of this study  
80 was to investigate if HHP treatment of human milk left HMOs unaltered by measuring  
81 22 major HMOs before and after HHP processing, while also comparing HHP to the  
82 standard HoP treatment in this regard.

83 Our second objective was to investigate if HHP processing of human milk leads  
84 to the formation of Maillard reaction products (MRPs, also called glycation products).  
85 MRPs are formed by a complex set of chemical reactions involving reducing sugars  
86 and the amino groups of proteins. During milk processing (notably in production of  
87 infant milk formulas) the most abundant MRP formed is the Amadori product,  
88 lactuloselysine, which is indirectly quantified by the measure of furosine after acid  
89 hydrolysis of milk [14–16]. Although relatively stable in milk, lactuloselysine can  
90 undergo chemical modifications and generate advanced glycation end products  
91 (AGEs) such as N-epsilon-carboxymethyllysine (CML), N-epsilon-carboxyethyllysine  
92 (CEL) [14–16].

93 The nutritional and biological consequences of dietary intake of AGEs and other  
94 MRPs have been debated for more than 2 decades [17]. When considering the  
95 nutritional value of proteins, many subscribe to the view that the glycation of lysine in  
96 milk reduces its bioavailability [18]. This has given rise to the term “blocked lysine” to  
97 describe a dietary amino acid which is unavailable for the anabolism of new proteins

98 in vivo [19]. Although a reduced digestibility of glycated proteins and a consequent  
99 reduction in the bioavailability of some amino acids are commonly described in in vitro  
100 digestive systems, these observations have never been confirmed in animal or human  
101 studies [20]. With regard to the potential health consequences of dietary exposure to  
102 AGEs and other MRPs, some clinical studies of healthy and pathological adults  
103 suggest that high exposure to MRPs could increase insulin resistance, oxidative stress  
104 and inflammation [21,22]. While the health consequences of dietary MRPs in early  
105 childhood are the subject of significant discussion, they remain incompletely  
106 understood and poorly studied. The effects of MRPs on perinatal metabolic  
107 programming remain at the hypothesis stage, despite them sometimes being called  
108 “glycotoxins” [23]. Nevertheless, the negative effect of dietary AGEs on kidney and  
109 pancreatic function during early life has been reported both by ourselves and others  
110 who have tested different forms of purposely high AGE-exposure diets in animals  
111 [24,25]. However, the few published studies on infants report no apparent effect of  
112 dietary MRPs on the kidney function of preterm infants [26], nor on the insulin  
113 resistance or oxidative stress of full-term term infants [27].

114 In the absence of conclusive scientific evidence on the toxicity or otherwise of  
115 dietary MRPs or AGEs, a precautionary approach dictates that infants should be fed  
116 with milk containing the lowest possible amount of chemically modified proteins. Thus,  
117 the second objective of this study was to investigate if HHP processing of human milk  
118 could minimize the glycation of proteins. We analyzed HHP-processed milk for furosine  
119 (a surrogate marker for lactuloselysine) and CML, two established indicators of the  
120 early- and advanced-stages of the Maillard reaction, respectively [14,16], and the less-  
121 frequently studied CEL in raw milk and milk samples treated by the standard HoP  
122 method were also analyzed for these compounds.

123

## 124 **2. Materials and Methods**

### 125 **2.1. Milk collection and HoP and HHP processing**

126 Frozen donor milk (DM) samples from 11 donors were provided by the regional  
127 HMB (Lactarium Régional de Lille, Jeanne de Flandre Children's Hospital, CHU Lille).  
128 Donors provided written informed consent for the use of their milk for this research  
129 purpose. After thawing of milk samples, 8 different batches of DM were created under  
130 sterile conditions by mixing various volumes (from 10 to 30 ml) of all BM samples to  
131 homogenize DM composition among batches. Three aliquots of milk samples were  
132 prepared from each batch: one fraction was stored at -80°C without any other treatment  
133 (raw milk sample); one fraction was subjected to HoP according to the standard  
134 pasteurization protocol in our regional HMB (62.5°C for 30 min); the last fraction was  
135 subjected to HHP processing as previously described [5]. Briefly, the HHP parameters  
136 were as follows: 350 MPa pressure, 38°C temperature, 1 MPa.s<sup>-1</sup> VA (application rate),  
137 4 cycles of 5 min each, and a latency time with normal pressure between each cycle  
138 of 5 min. After HoP and HHP processing, samples were stored at -80°C until analysis.

### 139 **2.2. Quantification of HMOs**

140 We quantified the 22 major HMOs listed and illustrated in Table 1 and Figure 2  
141 using a previously described and validated method [28]. All milk samples were shipped  
142 on dry ice to Neutron Spa. (Italy) for HMO quantification by liquid chromatography with  
143 fluorescence detection after labelling with 2-aminobenzamide (2AB) as described by  
144 Austin & Benet (2018) [28]. Ten HMOs were quantified using genuine HMO standards  
145 with known purity assessed using quantitative nuclear magnetic resonance  
146 spectroscopy (Elicityl, France), namely 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL),

147 Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), 3'-sialyllactose (3'SL), 6'-  
148 sialyllactose (6'SL), Lacto-N-fucopentaose-I (LNFP-I), LNFP-V and Lacto-N-  
149 neofucopentaose (LNnFP). The remaining HMOs were quantified against maltotriose  
150 of known purity (Sigma-Aldrich, Germany), assuming equimolar response factors. The  
151 full names and abbreviations of all quantified HMOs are shown in the legend of Table  
152 1.

### 153 **2.3. Quantification of the three MRPs: furosine, CML and CEL**

154 Samples were analyzed in duplicate by liquid chromatography with tandem mass  
155 spectrometric detection (LC-MS/MS) using a method adapted from Niquet-Léridon &  
156 Tessier (2011) [29]. From each sample of milk, an aliquot of 200 and one of 400µL  
157 were lyophilized overnight. To the lyophilized 200µL aliquots, 1mL of 6M HCl was  
158 added, while the lyophilized 400µL aliquots were first subjected to a reduction step  
159 using 500µL 0.1M NaBH<sub>4</sub> in 200mM borate buffer (pH 9.5, 2.5h at 20°C) followed by  
160 the addition of 500µL 12M HCl. Thereafter, all samples were hydrolyzed for 21h at  
161 110°C. The reduction step with sodium borohydrate (NaBH<sub>4</sub>) is considered necessary  
162 to block the Amadori product and to avoid *de novo* formation of CML during acid  
163 hydrolysis. Two hundred microliters of the hydrolysates were then evaporated to  
164 dryness by Speed-Vac (Thermo Fisher Scientific, Les Ulis, France), re-solubilized in  
165 200µL of 10mM nonafluoropentanoic acid (NFPA) and filtered (0.45µm). Three vials  
166 were prepared for subsequent analyses as follows: for the reduced filtered  
167 hydrolysates, 10x dilutions were prepared in 10mM NFPA containing (D<sub>2</sub>)-CML and  
168 (D<sub>4</sub>)-CEL (each at 0.1µg/mL) and 100x dilutions in 10mM NFPA containing (<sup>15</sup>N<sub>2</sub>)-  
169 lysine (10µg/mL); for the non-reduced filtered hydrolysate, 10x dilutions in 10mM NFPA  
170 containing (D<sub>4</sub>)-furosine (1µg/mL). Samples were analyzed on a Waters Acquity ultra-  
171 performance liquid chromatograph (LC) coupled to a Quattro Premier tandem mass



172 spectrometer (MS/MS; Waters, Saint-Quentin-en-Yvelines, France). A 10 $\mu$ L injection  
173 was made using the Acquity autosampler and target analytes were separated on a  
174 Hypercarb column (100 x 2.1mm, 5 $\mu$ m particle size – Thermo Fisher Scientific, Les  
175 Ulis, France) maintained at 10°C. Compounds were eluted using a binary gradient of  
176 an aqueous, ion-pairing solution (A – 10mM NFPA) and acetonitrile (B). The LC flow  
177 was 0.2mL/min and the gradient was as follows: 0 min 100% A; 10 min 77.5% A, 22.5%  
178 B; 10.1 min 40% A, 60% B held for 5 min; 15 min 100% A, held for 6 min. Heated  
179 electrospray ionization was in positive mode (3.75kV), desolvation temperature was  
180 400°C, source temperature 130°C, and desolvation and cone gas flow rates were 750  
181 and 50 L/h, respectively. Quantification was performed by isotopic dilution using the  
182 response ratios of labelled internal standards: the respective target molecule using 9  
183 concentration points to construct the calibration curves.

## 184 **2.4. Statistics**

185 Data are presented as mean $\pm$ SEM (standard error of the mean). Statistical  
186 analyses were performed with GraphPad Prism 7.0. software (San Diego, USA).  
187 Grubb's test was used to detect any outliers. Normality of variables was evaluated by  
188 a D'Agostino-Pearson test. If necessary, a log<sub>10</sub> transformation was applied to reach  
189 normality. Statistical differences were then tested by paired *t*-test or Wilcoxon test  
190 according to sample normality assessment results. A *p* value <0.05 was considered  
191 significant.

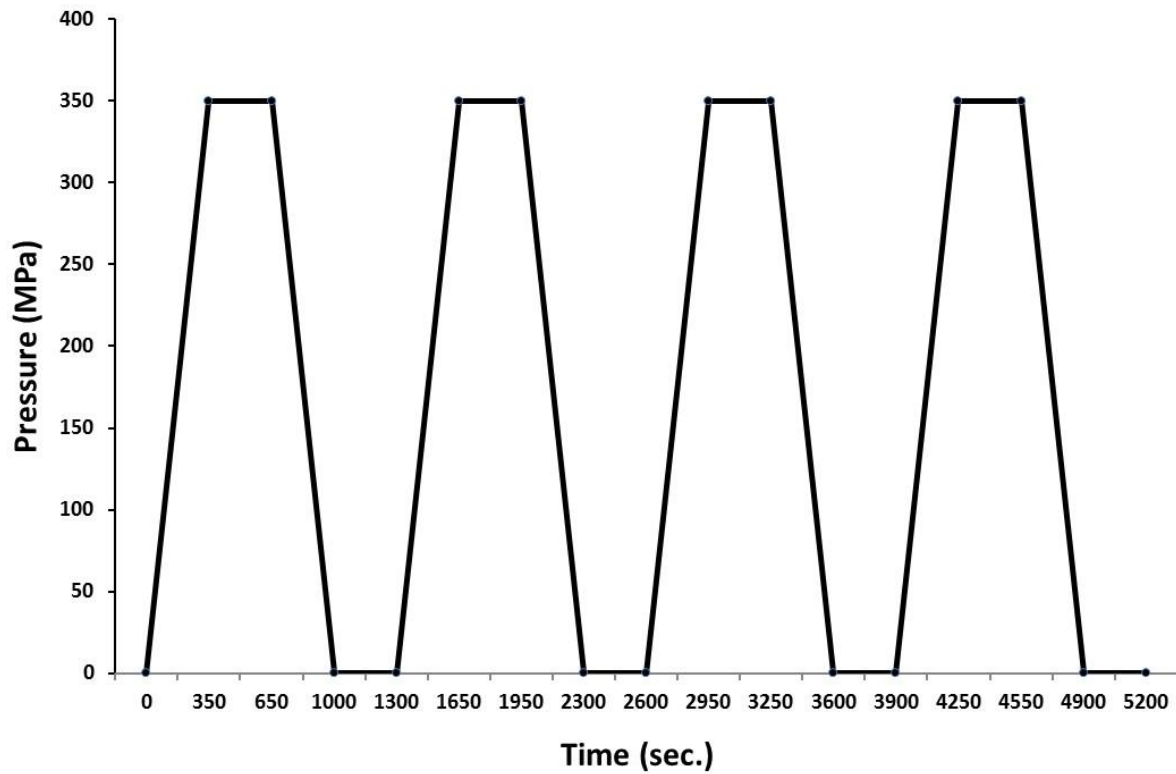
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## 193 **3. Results**

### 194 **3.1. Quantification of 22 HMOs in raw and treated milk samples.**

195 The kinetics of pressure application during the 90 minutes HHP processing are  
 196 shown in Figure 1.

197 **Figure 1.**



198

199 Using an internal probe in the tank of the HP system, we were also able to  
 200 measure the internal temperature during this process, which remained stable  
 201 throughout, ranging from 34 to 39°C. The effects of HoP and HHP treatments on HMO  
 202 concentrations are reported in Table1 and Figure 2.

203

204

205 **Table 1.**

HMOs (mg/kg milk)	Types	Raw			HoP			HHP		
		Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
2'FL	NF	1641	±	156	1618	±	140	1572	±	130

A-TETRA	NF	24.34	±	5.89	24.12	±	5.86	23.55	±	5.5
3FL	NF	696.4	±	84.7	698.5	±	89.3	694.4	±	89.5
LDFT	NF	131.9	±	20.5	131.3	±	19.7	128.9	±	18.4
LNFP-I	NF	793.4	±	89	786.9	±	83.5	775.8	±	80.7
LNFP-II	NF	644.6	±	59	648.8	±	63.7	646.7	±	65.1
LNFP-III	NF	277.5	±	11.7	275.9	±	10.6	271.7	±	11
LNFP-V	NF	64.84	±	6.99	65.25	±	7.27	65.1	±	7.45
LNnFP-V	NF	16.99	±	3.43	17	±	3.38	17.31	±	3.52
MFLNH-III	NF	367.7	±	24.1	366.3	±	23.8	362.9	±	25.7
LNDFH	NF	671.1	±	73.7	667	±	70.2	656.1	±	63.3
DFLNHa	NF	131.6	±	19.3	131.6	±	20	127.8	±	17.9
6'GL	NnF	30.89	±	2.38	30.71	±	2.37	31.16	±	2.53
LNT	NnF	1239	±	115	1241	±	122	1230	±	124
LNnT	NnF	223.3	±	25.3	221.9	±	24.7	220.3	±	25.3
LNHa	NnF	102.9	±	13.2	102.7	±	12.9	101.7	±	13.3
LNHb	S	41.17	±	5.76	41.06	±	5.35	40.73	±	5.56
3'SL	S	185.6	±	3.7	185.5	±	5.2	182.7	±	5.6
6'SL	S	580.1	±	24.6	579.3	±	29.1	576.4	±	29.4
LST b	S	66.65	±	6.78	66.35	±	7.07	66.32	±	7.3
LST c	S	257.4	±	26.9	258	±	27	255.3	±	28
DSLNT	S	291.3	±	31.1	291	±	32.4	288.4	±	32.8

206  
207 *A-TETRA, A-tetrasaccharide; DFLNHa; difucosylated lacto-N-hexaose a; DSLNT, disialyllacto-N-*  
208 *tetraose; 2'/3FL, 2'/3-fucosyllactose; 6'GL, 6'-galactosyloligosaccharide; LDFT, Lactodifucotetraose*  
209 *(difucosyllactose); LNDFH, lacto-N-difucohexaose; LNFP-I/II/III/V, lacto-N-fucopentaose I/II/III/V; LNH*  
210 *a/b, lacto-N-hexaose a/b; LNnFP-V, Lacto-N-neofucopentaose-V; LNnT, lacto-N-neotetraose; LNT,*  
211 *lacto-N-tetraose; LST b/c, sialyl-lacto-N-tetraose b/c; MFLNH-III, Monofucosyllacto-N-hexose-III;*  
212 *3'/6'SL, 3'/6'-sialyllactose; NF, neutral fucosylated; NnF, Neutral non-fucosylated; S, sialylated (acidic).*  
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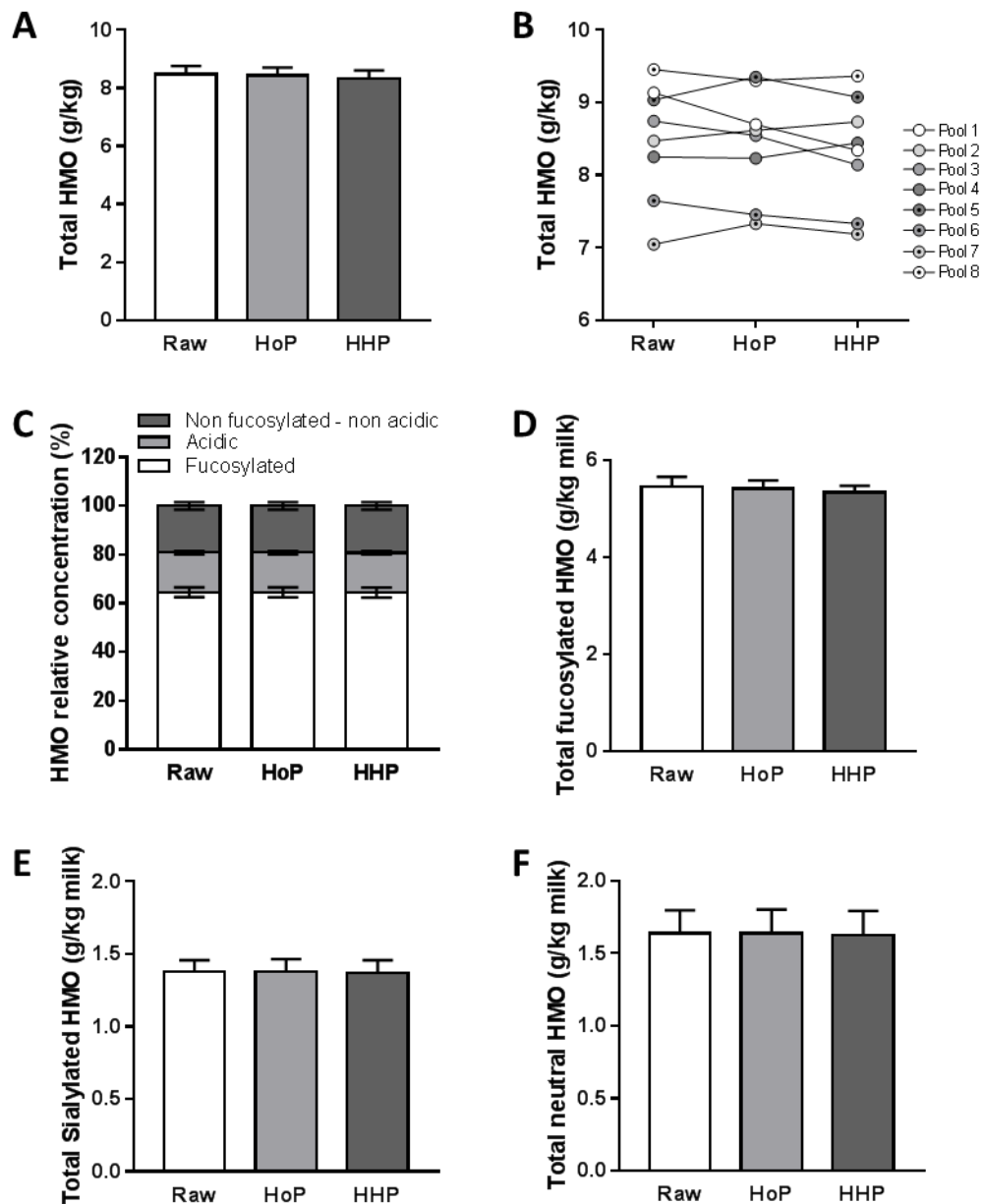
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222 **Figure 2.**

223



224

225 Neither sterilization process affected the concentration of the 22 major HMOs that we  
226 measured in milk batches from pooled human milk samples, demonstrating that these  
227 oligosaccharides were unaffected by thermal sterilization (HoP) or high pressure  
228 processing (HPP).

229 **3.2. Determination of 3 MRPs in raw milk and treated milk samples.**

230 Among the literature focusing on furosine, CML, CEL and other MRP levels in  
 231 milk, there is little consensus on the units of measurement. Hence, Table 2 presents  
 232 the concentrations of MRPs in µg/g protein, µg or ng/mL of milk and µmol/mol lysine  
 233 to facilitate a comparison of our data with those from the literature.

234 **Table 2.**

	Unit	RAW	HoP	HHP
		Mean ± SEM	Mean ± SEM	Mean ± SEM
<b>Furosine</b>	µg/g protein	69,8 ± 2,75	115 ± 2,98 <sup>***</sup>	69,8 ± 1,23 <sup>###</sup>
	µg/mL	1,52 ± 0,04	2,49 ± 0,10 <sup>***</sup>	1,47 ± 0,05 <sup>###</sup>
<b>Lactuloselysine</b>	µg/g protein	258 ± 10,2	424 ± 11,0 <sup>***</sup>	258 ± 4,57 <sup>###</sup>
	µg/mL	5,62 ± 0,16	9,20 ± 0,35 <sup>***</sup>	5,44 ± 0,19 <sup>###</sup>
	µmol/mol lysine	1863 ± 88,4	2971 ± 200 <sup>ns</sup>	1780 ± 35,0 <sup>#</sup>
<b>CML</b>	µg/g protein	3,95 ± 0,26	5,56 ± 0,36 <sup>*</sup>	3,92 ± 0,19 <sup>###</sup>
	ng/mL	86,3 ± 5,40	121 ± 10,2 <sup>ns</sup>	82,7 ± 5,20 <sup>###</sup>
	µmol/mol lysine	65,6 ± 4,51	94,5 ± 5,7 <sup>**</sup>	62,1 ± 2,58 <sup>###</sup>
<b>CEL</b>	µg/g protein	1,57 ± 0,15	3,04 ± 0,24 <sup>***</sup>	1,55 ± 0,16 <sup>###</sup>
	ng/mL	34,8 ± 3,98	66,6 ± 6,51 <sup>***</sup>	33,2 ± 4,05 <sup>###</sup>
	µmol/mol lysine	24,2 ± 2,21	48,6 ± 4,14 <sup>***</sup>	22,9 ± 2,11 <sup>###</sup>

235  
 236 *Lactuloselysine was estimated based on a 50% recovery of furosine from the lactuloselysine after acid*  
 237 *hydrolysis [30].*

238  
 239 Amadori products, including lactuloselysine, are the first stable compounds  
 240 formed through the Maillard reaction upon the rearrangement of the Schiff base formed  
 241 from the reaction between reducing carbonyls and an amino group. Lactuloselysine  
 242 undergoes transformation to furosine during acid hydrolysis as performed here during

243 sample preparation. This allows us to indirectly measure the concentration of  
244 lactuloselysine in raw milk, and in corresponding milk samples after HoP and HHP  
245 treatments, through the quantification of furosine. The recovery of furosine from  
246 lactuloselysine after acid hydrolysis is well characterized, and was estimated to be 50%  
247 under the conditions used here and described by Krause et al. [30]. Using this  
248 conversion factor, we report in Table 2 both the concentrations of furosine as measured  
249 and also the calculated lactuloselysine concentrations assuming 1 mole of furosine  
250 equals to 2 moles of lactuloselysine. This estimation enables a comparison of the  
251 number of lysine modified to lactuloselysine with the number of lysine modified to CML  
252 or CEL (all expressed in  $\mu\text{mol}/\text{mol}$  lysine). For furosine, we observed that HoP  
253 treatment drastically increased furosine levels ( $+64\%$ ;  $p<0.001$ ) in Table 2). HHP-  
254 processed milk fractions did not show any significant increase in furosine level  
255 compared with raw milk (Table 2). The concentration of lactuloselysine in raw and  
256 HHP-processed milks were estimated as  $1.86\pm 0.08$  and  $1.78\pm 0.03$   $\text{mmol}/\text{mol}$  lysine  
257 ( $p>0.05$ ), respectively. A significant increase was observed in the milk pasteurized by  
258 HoP ( $2.9\pm 0.2$   $\mu\text{mol}/\text{mol}$  lysine, namely 59% more than observed in milk subjected to  
259 HHP treatment,  $p<0.05$ ).

260 In the same milk samples, CML and CEL were also quantified. Compared with  
261 raw milk, HoP treatment significantly increased both CML and CEL levels: +41 to 44%  
262 for CML ( $p<0.05$ ) and +94 to 101% for CEL ( $p<0.001$ ), depending on the units  
263 compared (Table 2). HHP-processed milk fractions did not show any significant  
264 increase in CML and CEL concentrations compared with raw milk.

265

## 266 4. Discussion

267 Breast feeding has numerous health advantages for preterm infants [31–33].  
268 Mother’s milk is rich in diverse bioactive components, among them HMOs, one of the  
269 major components of breast milk by mass. HMOs are non-nutritive, bioactive  
270 compounds that have multiple short- and long-term health effects in newborns, as  
271 recently reviewed [10,12,13,34]. Basic research and clinical observations indicate that  
272 HMOs help to protect from neonatal infections, reduce susceptibility to intestinal  
273 diseases such as necrotizing enterocolitis (NEC), and may exert long-term beneficial  
274 effects on others tissues and organs such as the heart, adipose tissue and brain [35–  
275 39]. In the present study, aiming to analyze the effect of two sterilization processes of  
276 human milk on HMOs, we decided to pool donor milk samples from 11 women to obtain  
277 milk batches with similar HMOs composition to reduce a putative bias from maternal  
278 characteristics that may affect HMO composition, like the secretory status and time  
279 post-partum when the milk was collected [40,41].

280 Donor milk samples are usually pooled in human milk banks (HMBs), though not  
281 always from different mothers, and then sterilized using the standard HoP protocol to  
282 ensure microbial safety as donor milk is destined mainly for preterm babies. In the  
283 present study, we observed that HoP maintained the 22 major HMOs at levels  
284 unchanged from their initial concentrations in raw milk. Since, the HMO quantification  
285 method employed a 2AB labeling step of the reducing ends, and does not work if  
286 terminal glucose is isomerized to fructose, we can also exclude the possibility that the  
287 HMOs changed their natural structure to any significant extent. To date, only three  
288 studies have investigated the effect of HoP on HMOs [42–44]. All these studies, in  
289 accordance with our observations here, found no effect of HoP on HMO levels  
290 demonstrating that these oligosaccharides are resistant to thermal degradation at  
291 62.5°C during 30 minutes. For the first time here, we report that an alternative

292 sterilization technique, namely HHP processing at 350 MPa, similarly had no effect  
293 upon HMOs levels. Our results confirm the preservation of the biological value of  
294 human milk with respect to HMOs, after either pasteurization by HoP or sterilization  
295 using HHP, and constitutes an important finding for the nutritional management of  
296 preterm infants.

297         One of the main biological effects of HMOs occurs in the colon where they are  
298 metabolized by gut microbiota [45]. Indeed, HMOs have been shown to stimulate  
299 growth of gut microbiota, specifically *Bifidobacterium* species, and increase their  
300 metabolic activity [46–49]. However, in addition to metabolizing HMOs, gut microbes  
301 are also able to use other milk components such as glycated proteins or peptides that  
302 have been incompletely enzymatically digested and absorbed in the upper and middle  
303 intestinal tract. This is the case for some MRPs, which are formed between reducing  
304 sugars and amino groups during heat treatment of milk [50]. The Maillard reaction on  
305 dietary proteins leads to the formation of an heterogenous group of early and advanced  
306 glycation products on lysine, arginine and some other amino acids. Each glycation  
307 product has a unique bioavailability [50]. For instance, the Amadori products (e.g.  
308 lactuloselysine in milk) seem to be poorly absorbed and mainly fermented by the gut  
309 microbiota [15,51,52]. On the other hand, dietary CML has a more complex  
310 biodistribution divided amongst microbiota digestion [53], intestinal absorption and  
311 transport into the circulation, accumulation by some organs and tissues [54], and partial  
312 elimination in the urine and feces [55]. A clear relationship has been found between  
313 dietary exposure to CML and its plasma concentration in babies fed infant formula or  
314 breast milk [27], while a weaker correlation was observed when CML was measured  
315 in babies' urine [48].



316 While the effects of the Maillard reaction on the overall digestibility of proteins is  
317 still debated, then, what is known for sure is that some AGEs and other MRPs reach  
318 the blood while others remain in the gut and are transferred to the colon where they  
319 are fermented. As a consequence, several studies suggest that the ingestion of MRPs  
320 influences the composition of the microbiota [41–45]. As described in the introduction,  
321 the deleterious effects (*e.g.* inflammation, insulin resistance, oxidative stress) of a  
322 chronic, deliberately very high exposure to AGEs and other MRPs in early life were  
323 observed only in animal models, and human studies remain too few to be conclusive.  
324 However, we believe that the precautionary principle obliges us to abide by the ALARA  
325 concept (as low as reasonably achievable).

326 Therefore, our second aim was to examine whether HoP and HHP processing of  
327 human milk differentially affected MRP formation. Data on MRPs in untreated human  
328 breast milk are scarce in the literature, which limits the comparison of our data with  
329 other studies. The mean level of furosine measured here in the 8 batches of raw DM  
330 was 69.8 +/-2.7 µg/g protein. This is in same order of magnitude as the levels found in  
331 human milk by Martysiak-Zurowska & Stolyhwo (2007) [56], while a concentration  
332 range of 35 – 55 µg/g protein was reported in untreated bovine milk by Henle et al.  
333 (1995) [57].

334 The mean concentration of CML in the 8 batches of untreated DM was 86.3 +/-  
335 5.4 ng/mL. Using an ELISA method, Dittrich et al. (2006) found a mean CML  
336 concentration of 137 +/-83 ng/mL of human breast milk, with concentrations ranging  
337 from 54 to 436 ng/mL [58]. Using a similar analytical method to our study, Delatour et  
338 al. (2009) reported a mean concentration of 84.1 +/-56.2 ng of CML/mL in human milk  
339 [59]. The data from our study and those mentioned above are thus consistent. The  
340 mean concentration of CEL found in the 8 batches of raw DM (34.8 +/-3.9 ng/mL) is

341 lower than for CML, but remains in the same order of magnitude. To our knowledge,  
342 no data for CEL in breast milk is available in the literature while a concentration of 144  
343 +/-51 ng/mL has been reported in untreated bovine milk [60]. As expected, the  
344 estimated concentration of lactuloselysine in raw human milk was 28 times higher than  
345 the concentration of CML, and 77 times higher than that of CEL. These results also  
346 indicate that 186 from 100 000 lysine moieties were modified into lactuloselysine in raw  
347 milk compared with only 6 and 2 of 100 000 lysine residues for CML and CEL,  
348 respectively. Moreover, due to the high temperature at which HoP was conducted,  
349 increased levels of furosine (and lactuloselysine), CML and CEL were generated by  
350 HoP compared to HHP processing that was performed at a much lower temperature  
351 close to 38°C.

352 The only data from the literature that can be compared with our data stem from  
353 the pasteurization of bovine milk. For furosine, Henle et al. observed a 1.35 times  
354 increase in the median concentration when bovine milk was pasteurized [57]. The HoP  
355 treatment used on the 8 batches of DM here increased the concentration of furosine  
356 about 1.6 times. Considering primarily publications that used a similar LC-MS/MS  
357 method to ours, very few comparisons can be presented on the effect of pasteurization  
358 on the formation of CML and CEL in bovine milk. One of the first to study the topic were  
359 Ahmed et al. in 2005, and they observed an increase of formation of CML and CEL in  
360 bovine milk in proportion to the severity of the heat treatment [60]. Conversely, Delatour  
361 et al. (2009) observed no significant difference in the concentration of CML between  
362 raw and pasteurized bovine milk samples, but observed a slight increase in UHT milk  
363 samples [59]. Assar et al. (2009) observed the same lack of difference between raw  
364 and pasteurized skimmed milk, but a slight increase when raw whole milk was  
365 compared with pasteurized whole milk [61]. In our study, the pasteurization conditions

366 used (HoP) clearly induced a significant increased formation of CML and CEL in  
367 human breast milk whereas the HHP treatment had no impact on the levels of these  
368 MRPs.

369

## 370 **5. Conclusion**

371 In conclusion, we have demonstrated that both HoP and HHP processing of  
372 human milk do not affect the amount or profile of HMOs present in raw human milk.  
373 However, our results showed that HoP increased the formation of three key MRPs in  
374 donor human milk, potentially affecting their nutritional quality by decreasing the  
375 proportion of milk proteins present in their native form. The increased concentration of  
376 MRPs in human milk treated by HoP raises concerns about the optimal nutritional  
377 environment and health of infants: this is important because human donor milk is  
378 mainly used for the feeding of vulnerable, preterm babies.

379 More broadly, the hypothesis that perinatal exposure to MRPs influences  
380 metabolic programming [23–25], the development of allergies, and susceptibility to  
381 other pathologies that affect health in adulthood, obliges us to strive for banked human  
382 milk with the lowest MRP content possible. However, our observation should be seen  
383 in perspective and it must be noted that the increase of MRPs in our HoP-treated  
384 human milks was nevertheless small when the mean concentration of CML is  
385 compared with that reported in some infant formulas (121 ng/mL compared with 200 –  
386 13 000 ng/mL in Delatour et al. (2009) [59]).

387 Finally, our study confirms and extends previous findings that HHP treatment of  
388 human milk provides bacteriologically safe milk, with fewer detrimental effects on the  
389 biochemically active milk components than HoP.

390

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400

401 **Conflict of interest:**

402 The authors declare no conflict of interest. NS is an employee of Société des Produits  
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404

405 **Authors' contribution:**

406 LM and JL drafted the manuscript. LM, MDL, SJO, MH, LT, EM and JL did the  
407 experiments and collected the data. NS, FT, MH, FG and DL reviewed the paper. All  
408 authors approved the final version before submission.

409

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599

## Figure and Table legends

600

601 **Fig. 1.** Descriptive diagram of the HHP methodology used in this study. Evolution of  
602 the pressure in the tank of the HHP system used in the present study.

603

604 **Fig. 2.** An additional representation of HMO concentrations in pooled samples (n=8)  
605 of raw human milk (Raw) and after pasteurization (HoP) or high hydrostatic pressure  
606 (HHP) processing reported in Table 1. Data are expressed as Mean±SEM. (A) Mean  
607 concentration of the 22 HMOs measured (total) in this study. (B) Individual variations  
608 of total HMOs in each pool of milk. (C) Relative concentration of HMO types and total  
609 concentration of fucosylated (D), acidic (E) and neutral non-fucosylated (F) HMOs in  
610 raw, HoP and HHP samples. Total and relative concentration were defined by the sum  
611 of fucosylated, acidic sialylated or neutral non-fucosylated HMOs. Grouping by types  
612 of HMOs is detailed in Table 1. Data are expressed as Mean±SEM. Differences  
613 between groups were tested by repeated measures Anova and Tukey's post-test.

614

615 **Table 1.** HMO concentrations in pooled samples (n=8) of raw human milk (Raw) and  
616 after pasteurization (HoP) or high hydrostatic pressure (HHP) processing. Data are  
617 expressed as Mean±SEM. Differences between groups were tested by repeated  
618 measures Anova and Tukey's post-test. No significant differences between groups  
619 were found.

620

621 **Table 2.** Milk levels of furosine, lactuloselysine, carboxymethyllysine (CML) and N-  
622 epsilon-carboxyethyllysine (CEL) in pooled samples (n=8) of raw human milk (Raw)  
623 and after pasteurization (HoP) or high hydrostatic pressure (HHP) processing. Data  
624 are expressed as Mean±SEM. Differences between groups were analyzed by Tukey's  
625 post-test following repeated measures Anova or Dunn's post-test following Friedman  
626 test. \*\*\*: p<0.001 Raw vs HoP; ###: p<0.001 HHP vs HoP; \*\*: p<0.01 Raw vs HoP; ##:  
627 p<0.01 HHP vs HoP; \*: p<0.05 Raw vs HoP; #: p<0.05 HHP vs HoP.