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Metabolic hormones in human breast milk are preserved by high hydrostatic pressure processing but reduced by Holder pasteurization

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25

26

Abstract

27 In human milk banks (HMBs), donor milk (DM) is commonly sterilized by Holder
28 pasteurization (HoP). High hydrostatic pressure (HHP) processing is an innovative,
29 alternative method for DM sterilization. We evaluated the impact of HHP processing
30 on the concentration of seven metabolic milk hormones. Eight samples of raw DM were
31 aliquoted. One aliquot was sterilized by HoP (62°C for 30 min), and another was
32 processed by HHP (350 MPa at 38°C). Compared with raw DM, HoP milk displayed
33 reduced concentrations of insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1, though
34 adiponectin levels were unchanged. HHP processing maintained the levels of insulin,
35 nesfatin-1, cortisol and leptin at their initial levels in raw DM, reduced apelin and
36 adiponectin levels, but increased GLP-1 level. Sterilization of DM by HHP thus
37 preserves the main metabolic hormones in human milk, underlining the interest of this
38 method for use in HMBs.

39

40 **Key-words:** high hydrostatic pressure, holder pasteurization, human milk, hormones

1. Introduction

During the last decade, evidence has steadily accumulated for the importance of bioactive breast milk (BM) hormones as factors in the health of newborns and the development of infants. Experimental and clinical studies have shown that BM hormones could act directly on the newborn intestine or be absorbed and transferred into the newborn's blood circulation to reach other organs (Victora et al., 2016; Badillo-Suárez et al., 2017; Savino & Liguori, 2008; Schueler et al., 2013). For instance, studies in rodents have demonstrated that BM leptin can be absorbed into the newborn's blood via the small intestine, and that it controls the establishment of hypothalamic neural networks involved in food intake (Casabiell et al., 1997; Bouret et al., 2004). Similarly, BM insulin was shown to reach the newborn's hypothalamus and modulate neural connections (Vogt et al., 2014), and was also implicated in gut maturation and in the reinforcement of the gut barrier (Shulman, 2002). So far, more than thirty peptidic and steroid hormones have been described in human BM (Schams, & Karg, 1986; Kratzsch et al., 2018). Their levels in BM are frequently correlated with those in maternal plasma and are modulated by maternal characteristics such as body mass index and diet (Schams, & Karg, 1986; Kratzsch et al., 2018; Andreas et al., 2014). Based on these findings, it was proposed that BM hormones may be part of a system controlling energy homeostasis and gut maturation in newborns, both directly and indirectly.

In preterm infants poor feeding is one of the main reasons for inadequate weight gain after birth. Immaturity of the gastrointestinal tract, feeding difficulties due to immaturity of sucking movements, low BM supply, and a lack of hepatic glycogen stores contribute to difficulty in meeting the nutritional requirements in preterm infants (McNelis et al., 2017). To ensure adequate growth of preterm babies, mother's BM is the best choice

for enteral feeding but, when unavailable, donor milk (DM) is often used (Boquien, 2018). DM is provided by human milk banks (HMBs) which collect, screen and store DM. In order to ensure microbial safety for preterm newborns, most HMBs sterilize DM using the standard method of Holder pasteurization (HoP), commonly performed by heating DM to 62.5°C for 30 minutes (Picaud, & Buffin, 2017). Although clinical practices demonstrate that many beneficial properties of DM still persist after HoP, this pasteurization is known to partly degrade numerous heat-sensitive bioactive factors in BM such as immunoglobulins, lactoferrin, some vitamins, lysozyme, the bile salt-dependent lipase (BSSL), and some milk hormones (Picaud, & Buffin, 2017; Escuder-Vieco et al., 2018; Ley et al., 2011). As these milk compounds are important for growth, development and immunity of premature babies, methods of DM sterilization such as high-temperature short-time (HTST) or ultra-violet (UV) pasteurization, as well as sterilization by high hydrostatic pressure (HHP) processing, have recently emerged as alternatives (Wesolowska et al., 2019).

The HHP process applies high pressures in a temperature and time-controlled manner. This method has been used for 25 years for sterilization in food processing and was recently adapted for DM sterilization (Wesolowska et al., 2019; Demazeau et al., 2018; Wesolowska et al., 2018). Demazeau *et al.* showed that HHP processing using four cycles of a moderate pressure (350 MPa) during 5 minutes at 38°C is effective for sterilizing DM and conserves lactoferrin, BSSL enzyme and lysozyme levels close to those in raw DM (Demazeau et al., 2018). The aim of the present study was to investigate if HHP treatment also conserved several key metabolic hormones in DM including six peptidic hormones (*i.e.* leptin, insulin, adiponectin, apelin, GLP-1 and nesfatin-1), as well as one steroid hormone (cortisol). These hormones were also measured in raw DM and in DM pasteurized by HoP for comparison.

2. Materials and Methods

2.1. Milk collection and processing

Sample collection, preparation, and the experimental design of the present study are shown in Figure 1. Frozen DM samples from 11 donors were provided by the regional HMB (Lactarium Régional de Lille, Jeanne de Flandre Children's Hospital, CHU Lille). Donors provided written, informed consent for the use of their milk for this research purpose. After thawing of milk samples, 8 different batches of DM were created under sterile conditions by mixing various volumes (from 10 to 30 ml) of all BM samples, primarily in order to homogenize DM composition between batches (BM hormones levels display high level of inter-individual variation).

Three aliquots of DM were prepared for each batch: one fraction was stored at -80°C without any other treatment (raw milk sample); one fraction was subjected to HoP according to the standard pasteurization protocol (62.5°C for 30 min) in our regional HMB; the last fraction was subjected to HHP processing as previously described (Demazeau et al., 2018). Briefly, the set of HHP parameters was as follows: pressure = 350 MPa, temperature = 38°C, VA (application rate) = 1 MPa.s⁻¹, number of cycles = 4 cycles, duration of each cycle = 5 min and a latency time with normal pressure between each cycle of 5 min. After HoP and HHP treatments, a microbiological analysis was performed to verify the efficacy of sterilization (Department of Microbiology, Centre de Biologie Pathologie, CHU de Lille). Sterilized samples were then stored at -80°C until analysis for milk hormonal content.

2.2. Quantification of milk hormones

The concentrations of hormones were determined in each milk fraction using whole milk. To check for potential matrix inhibition, each assay was optimized for linear-range detection of each hormone. Six abundant peptidic milk hormones and one steroid hormone were quantified using commercial ELISA kits: leptin (DLP00, R&D Systems, Abingdon, UK), insulin (HUEB0185, ELISA Genie, Dublin, Ireland), adiponectin (RD191023100, BioVendor, Brno, Czech Republic), apelin-12 (EKE-057-23, Phoenix Pharmaceuticals, Strasbourg, France), GLP-1 (HUF100805, ELISA Genie, Dublin, Ireland), nesfatin-1 (EK-003-26A, Phoenix Pharmaceuticals, Strasbourg, France) and cortisol (RE62111, IBL, Männedorf, Switzerland).

2.3. Statistics

Data are presented as mean \pm SEM. Statistical analysis were performed with GraphPad Prism 7.0. software (San Diego, USA). Grubb's test was used to detect any outliers. Normality of variables was evaluated by a D'Agostino-Pearson test. If necessary, a log₁₀ transformation was applied to reach normality. Statistical differences were then tested by paired T-test or Wilcoxon test according to sample normality assessment results. A *p* value <0.05 was considered significant.

3. Results

The microbiological analyses of milk samples treated by HoP and HHP confirmed their efficacy as sterilization methods and the microbial safety for nutritional use of DM for preterm newborns (data not shown). Effects of HoP and HHP treatments on DM hormones levels are reported in Figure 2. Compared to raw milk, HoP treatment reduced dramatically insulin (-63%, Fig. 2A), nesfatin-1 (-41%, Fig. 2B), cortisol (-11%, Fig. 2C), leptin (-100%, Fig. 2D), apelin (-41%, Fig. 2E) and GLP-1 (-83%, Fig. 2F).

However, adiponectin levels were not different between raw and HoP-treated milk fractions (Fig. 2G). Conversely, HHP-processed fractions did not show any significant reduction in levels of insulin (Fig. 2A), nesfatin-1 (Fig. 2B), cortisol (Fig. 2C) or leptin (Fig. 2D) compared with raw DM. However, HHP-treatment reduced milk apelin (-20%, Fig.2E) and adiponectin (-50%, Fig. 2G) levels compared with raw DM, while GLP-1 levels were increased (+64%, Fig.2F). Hence, a comparison between HoP and HHP treatments showed that HHP processing significantly increased concentrations of insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1. Only milk adiponectin was reduced by 52% by HHP processing compared with its level in pasteurized HoP-treated milk (Fig. 2G).

4. Discussion

Milk hormones are non-nutritive bioactive compounds that may exert short- and long-term beneficial health effects, as well as positively influence metabolic programming in both premature and full-term newborns (Victora et al., 2016; Badillo-Suárez et al., 2017; Savino & Liguori, 2008; Schueler et al., 2013; Bouret et al., 2004; Vogt et al., 2014). In this study, we compared the effect of HoP (the reference method for DM sterilization in HMBs) with an alternative sterilization method, HHP processing, on the preservation of seven important metabolic hormones. We showed that HHP processing better preserves the concentrations of insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1 in breast milk than HoP, and maintained these hormones at levels close to their initial levels in raw DM. We also observed that HHP process reduced milk adiponectin levels by 52%, whereas HoP did not affect this hormone in our study.

161 Few studies have characterized the effects of HoP on human breastmilk
162 hormones and, so far, they have focused on a few well-described hormones including
163 leptin, insulin and adiponectin (Escuder-Vieco et al., 2018; Ley et al., 2011; Vass et al.,
164 2020 Wesolowska et al., 2018). Although discrepancies due to samples preparation or
165 types of assay were reported, all these studies have shown that HoP reduced milk
166 hormonal content. For example, breastmilk insulin levels were reported to be reduced
167 after HoP treatment by 14% (Vass et al., 2020), 32% (Wesolowska et al., 2018) and
168 46% (Ley et al., 2011); milk leptin by 77% (Wesolowska et al., 2018), 87% (Vass et al.,
169 2020) and 90% (Escuder-Vieco et al., 2018); and milk adiponectin by 33% (Ley et al.,
170 2011; Wesolowska et al., 2018). In the present study, HoP treatment decreased insulin
171 by 63% and leptin by 100% but did not significantly affect adiponectin. In line with
172 previous observations, our study demonstrates that both insulin and leptin are very
173 sensitive components of human breastmilk and that their levels are drastically reduced
174 by HoP. Discrepancies between studies (Escuder-Vieco et al., 2018; Ley et al., 2011;
175 Wesolowska et al., 2018; Vass et al., 2020) could be related to differences in
176 pasteurizer performance (Picaud, & Buffin, 2017). For adiponectin, in accordance with
177 *Escuder-Vieco et al.* (Escuder-Vieco et al., 2018), we did not observe any effect of HoP
178 on this hormone in DM whereas two studies have reported a 33% reduction of
179 adiponectin level after HoP pasteurization (Ley et al., 2011; Wesolowska et al., 2018).
180 We also observed a small, but significant, 11% reduction in cortisol after HoP. Using
181 this same pasteurization method, Vass *et al.* (Vass et al., 2020) reported a 35%
182 reduction of this hormone after HoP, suggesting that this steroid hormone is also a
183 feature of DM sensitive to HoP. Finally, to the best of our knowledge, we report for the
184 first time that the levels of nesfatin-1, apelin and GLP-1 are reduced in HoP-treated
185 milk by 40%, 41% and 83%, respectively. This reinforces the idea that peptide

breastmilk hormones are largely degraded by HoP, probably leading to DM with reduced hormonal benefits for preterm newborns.

HHP processing was shown to protect numerous bioactive milk compounds from degradation (Picaud, & Buffin, 2017; Escuder-Vieco et al., 2018; Wesolowska et al., 2019; Demazeau et al., 2018; Wesolowska et al., 2018). We observed in this study that breastmilk insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1 are all preserved at close to raw DM levels after HHP sterilization. Surprisingly, milk GLP-1 levels were found to be increased after HHP processing. This could result from the influence of hydrostatic pressure on the physicochemical properties of human milk. Indeed, human milk is a very complex biological fluid that could be characterized simply as an emulsion of fat globules in an aqueous liquid with cells components. Although, the precise distribution of GLP-1 in human milk remains unknown, due to its peptide structure GLP-1 is probably mainly located in the emulsion phase of human milk, which consists of milk fat droplets or fat-associated proteins suspended in the aqueous fraction of the milk. Documented effects of high pressure on milk lipids are scarce: two studies have shown that while milk fat globules are still present after HHP, an alteration in globule size was observed (Demazeau et al., 2018; Mahara et al., 2014). Hence, our hypothesis is that HHP treatment of DM causes the release of GLP-1 from fat globules to the aqueous phase. This mechanism was also proposed by Wesolowska A *et al.* (Wesolowska et al., 2018), who observed that a high-pressure application (ranging from 400 to 600 MPa) increased milk leptin levels in the emulsion phase of DM.

In this study, adiponectin was the only hormone whose levels were reduced after HHP processing. This finding is in accordance with the study of Wesolowska *et al.* who reported a drastic reduction of milk adiponectin following HHP process using various high pressures ranging from 200 to 600 MPa (Wesolowska et al., 2018). Adiponectin

is composed of multimer complexes of proteins in plasma (Kadowaki, & Yamauchi, 2005). High-molecular-weight (HMW) adiponectin, which is the most biologically active form, is a large multimer of 12–18 subunits (Lihn et al., 2005)). This HMW adiponectin is present in human milk (Bronsky et al., 2006; Martin et al., 2006). As postulated above for GLP-1, we here speculate that HHP may have modified the physicochemical properties of human milk and altered the structure of the bioactive HMW adiponectin leading to its partial degradation. Further study is needed to test this hypothesis.

Previous studies have shown that HHP better preserved numerous compounds in DM than HoP, such as immunoglobins, lactoferrin, BSSL enzyme, lysozyme or vitamins (Wesolowska et al., 2019; Demazeau et al., 2018; Wesolowska et al., 2018). The present findings demonstrate that hormonal components of DM are also largely preserved by this non-thermal method. We believe that preservation of milk hormones in sterilized DM is especially important in the context of feeding preterm newborns with DM, as these babies have specific nutritional needs and metabolism, and hormones are involved in the maturation of numerous physiological systems in the early postnatal period (McNelis et al., 2017; Boquien, 2018; Platt, 2014). For example, experimental data have shown that leptin in breastmilk participates in the neonatal regulation of food intake and, along with insulin, in hypothalamic development; while insulin has been also shown to be involved in the maturation of digestive mucosa (Schueler et al., 2013; Casabiell et al., 1997). Both leptin and insulin were recently associated with early changes in the infant intestinal microbiome (Lemas et al., 2016; Gotteland et al., 2017). Milk glucocorticoids have been implicated in the maturation of the gastrointestinal tract, energetic metabolism and in long-term metabolic programming (Badillo-Suárez et al., 2017; Rose et al., 2013; Van Der Voorn et al., 2020). Adiponectin has been proposed to regulate the carbohydrate-lipid metabolism in newborns (Savino, & Liguori, 2008;

Kratzsch et al., 2018), while nesfatin-1 may regulate food and water intake (Schalla, & Stengel, 2018; Aydin et al., 2009; Aydin, 2010). Breastmilk GLP-1 was proposed to be involved in infant satiety, glucose homeostasis and reduction of gastrointestinal motility (Müller et al., 2019; Schueler et al., 2013). Finally, oral apelin administration was shown to control intestinal maturation by modulating cell proliferation in the digestive mucosa in developing rodents (Antushevich et al., 2015).

5. Conclusion

In conclusion, we have demonstrated that our HHP protocol (4 cycles of a moderate pressure (350 MPa) during 5 minutes performed at 38°C) preserves the main metabolic hormones in human milk, reinforcing the potential of this new sterilization method to be used to treat DM in HMBs. Through the preservation of numerous important milk bioactive factors, HHP-treatment of DM could improve growth, neurodevelopment, intestinal maturation and microbiota establishment in preterm infants who receive it.

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The authors declare no conflict of interest.

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LM, LT and JL drafted the manuscript. LM, LT, EM, MDL and JL did the experiments and collected the data. DL, VP, DE and FG reviewed the paper. All authors approved the final version before submission.

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Figures legends

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421 **Fig. 1.** Descriptive diagram of the methodology used of this study

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423 **Fig. 2.** Concentrations of hormones in raw DM and in DM after Holder pasteurization
424 (HoP) and High hydrostatic pressure (HHP) processing. Data are presented as
425 mean±SEM. Asterisks correspond to level of statistical significance for paired
426 comparisons *P<0.05; **P<0.01; ***P<0.001

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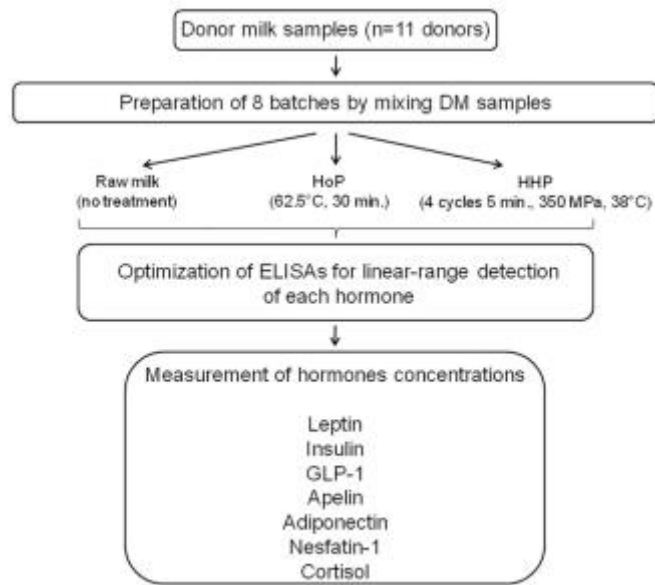


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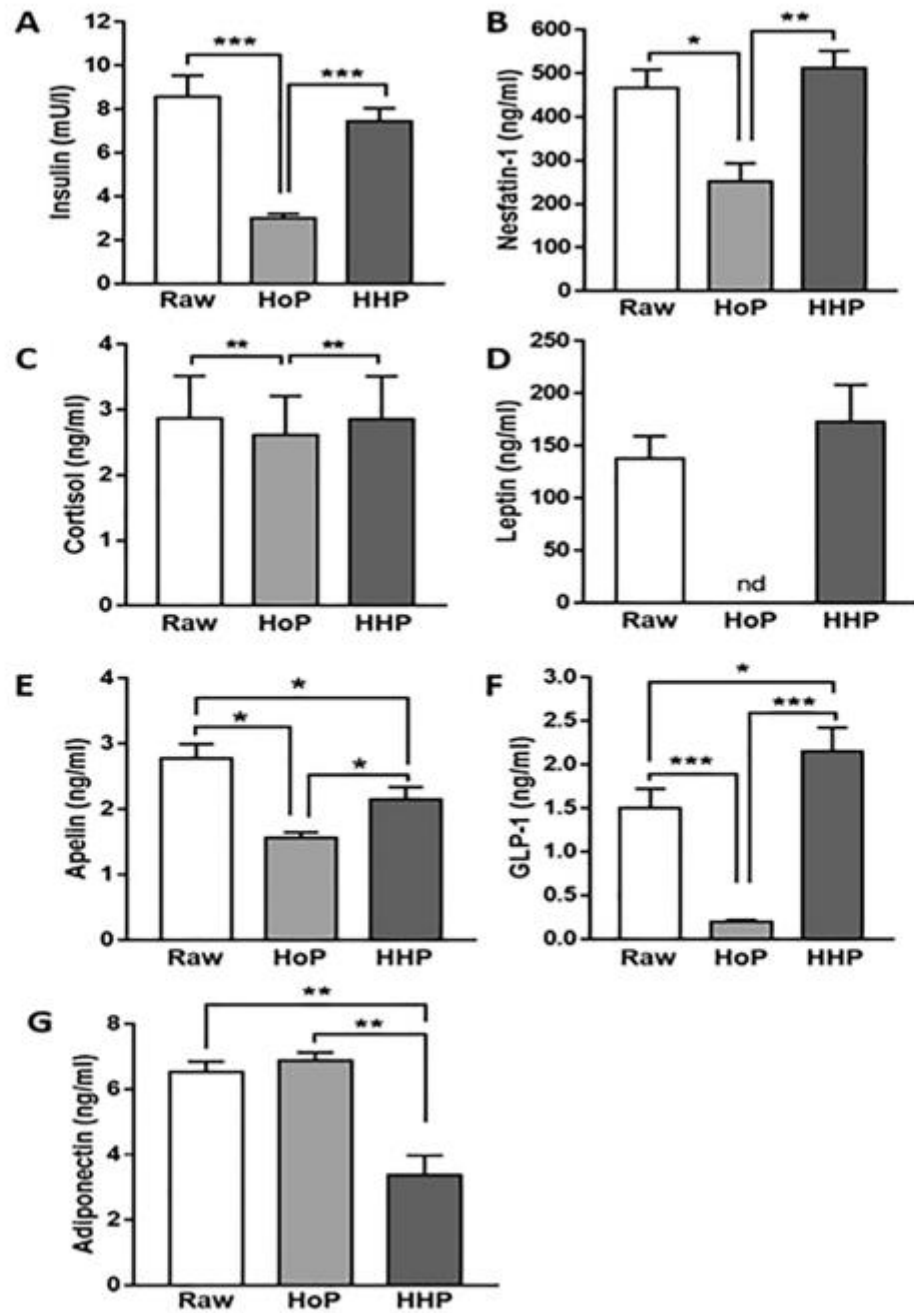


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