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Effect of pasteurisation and high-pressure processing on selected bioactive components in human milk – An experimental study

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Abstract: High-pressure processing (HPP) represents a promising alternative to conventional Holder pasteurisation (HoP) used by human milk banks worldwide. The objective of this study was to identify whether the HPP would achieve the same or better retention of the content of selected analytes than the HoP. Samples collected from 15 breast milk donors were processed in four ways: *i*) no treatment; *ii*) HoP; *iii*) HPP in cycles (350 MPa, 4 cycles); *iv*) continuous HPP (350 MPa, 20 min). The content of secretory immunoglobulin A (sIgA), lactoferrin and lysozyme was determined using commercially available ELISA kits, and the lipase activity was assessed using an A-lipase activity assay kit. Data were compared statistically using paired *t*-tests. HoP significantly reduced the content of lysozyme and lactoferrin as well as lipase activity ($P < 0.001$). Cycled HPP significantly decreased lipase activity ($P = 0.002$), while continuous

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HPP led to a significant decrease in lysozyme content (P = 0.001) and lipase activity (P = 0.014). Cycled HPP showed high retention of pretreatment levels of lysozyme – median 99 (88; 99%), lactoferrin – 84 (66; 105%), and sIgA content – 83 (28; 117%). Among the studied treatment regimens, the best preservation of initial levels of bioactive components was achieved using HPP at 350 MPa in cycles.

Keywords: bioactive components; breast milk; high-pressure processing; Holder pasteurisation; Milk Bank

Human milk (HM) contains hundreds to thousands of bioactive molecules that play an important role in protecting newborns from infection and inflammation and in enhancing immune maturation, organ development, and healthy microbial colonisation of the intestinal tract (Ballard and Morrow 2013). Freshly expressed fortified human milk represents the best possible way to feed preterm neonates. In cases when the mother's own milk is unavailable, donor breast milk provided by human milk banks has become a standard option worldwide for both term and preterm babies (Younger Meek and Noble 2020). This substitution for breastfeeding is especially important for premature babies in neonatal intensive care units. Clinical studies on HM substitution have shown a reduction in the incidence of necrotising enterocolitis, sepsis, and other infections in premature and high-risk infants (Arslanoglu et al. 2010). To prevent the transmission of viral and bacterial infections through donor breast milk, human milk banks carry out pasteurisation using international recommendations (Weaver et al. 2019) and meet national legislative requirements. Low temperature/long-time thermal pasteurisation, known as Holder pasteurisation (HoP) (62.5 °C for 30 min), is the method most commonly used to treat donor breast milk. This procedure, is usually followed by rapid chilling with subsequent fast freezing and storage at a temperature below –18 °C.

Disadvantage of HoP is its negative effect on the content of many, but not all, nutritional and protective bioactive substances in HM, but it is commonly accepted that the loss of bioactive substances due to HoP is not a significant reason to avoid the use of pasteurised human milk for both term and preterm babies (Arslanoglu et al. 2010; Moro and Arslanoglu 2011). Nonetheless, new research needs to focus on finding gentler processing methods that minimise the effect on bioactive substances in human milk.

An alternative promising method for milk processing could be high-pressure processing (HPP). It was initially used in Japan (Douzals et al. 1996), and the HPP treatment process was defined by three variables (pressure, time of exposure, and temperature at which pressurisation occurred). HPP application in dairy processing was reviewed by Chawla et al. (2011). Viazis et al. (2007, 2008) were the first to apply the method to human milk banking and demonstrated its capability to inactivate four non-sporulating microbial strains, i.e. *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus agalactiae*. Simultaneously, they demonstrated that compared to HoP, levels of secretory immunoglobulin A (sIgA) and lysozyme were significantly higher after HPP.

A breakthrough in the application of HPP in human milk banking was presented by Demazeau et al. (2018), who were the first to introduce repeated cycles to the process. This approach proved capable of eliminating sporulating microorganisms. Repeated cycle processing was also better at preserving the bioactivity of important human milk components.

Our study examined the influence of HPP on the activity of sIgA, lysozyme, lactoferrin, and lipase, representing the spectrum of components investigated by other authors. The beneficial effects of these substances are well known, and an ideal human milk treatment method should not lower their content.

MATERIAL AND METHODS

In our experiment, we analysed the content of the bioactive substances sIgA, lactoferrin, lysozyme and lipase activity in 60 aliquots of breast milk from 15 donors (mean age 34 ± 4.84 years). Donors provided milk in the period of stable lactation (from the second to the sixth month of lactation), which means we can exclude any colostrum bias. The donors included in the study gave written informed consent, and the study was approved by the ethics committee of University Hospital Hradec Králové (approval No. 202210 P04). We divided each fresh breast milk sample into 4 aliquots. In the first aliquot (untreated milk), bioactive substances were analysed directly, while the others (aliquots 2–4) were subjected to specific treatment methods (Figure 1).

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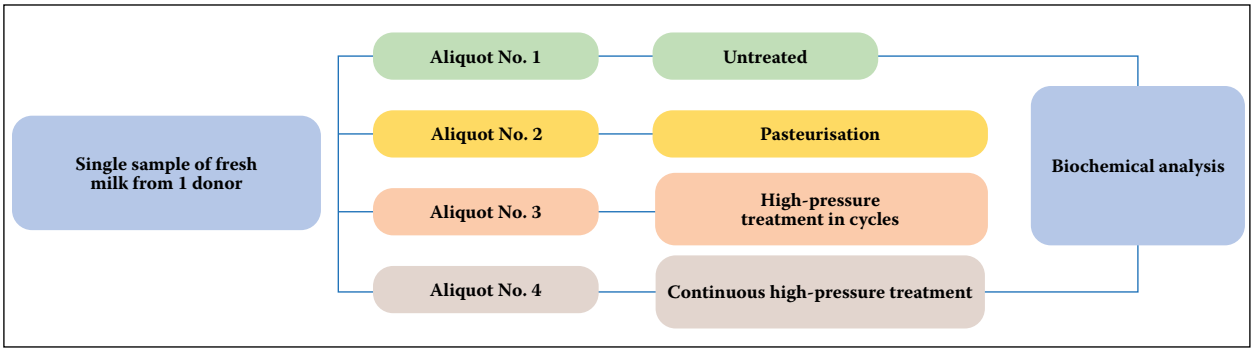

Figure 1. Design of the study; sample aliquots treated using pasteurisation (aliquot No. 2), cycled high-pressure treatment (aliquot No. 3), or continuous high-pressure treatment (aliquot No. 4) were analysed for the content of bioactive substances and compared with the untreated sample (aliquot No. 1)

Pasteurisation of milk samples

The second aliquot (aliquot No. 2) from the 15 milk samples was placed in 100 mL glass bottles and pasteurised at 62.5 °C for 30 min using conventional HoP, followed by chilling (Jandová et al. 2021).

High-pressure treatment

The third and fourth aliquots (aliquots No. 3 and No. 4) (100 mL) from the milk samples were placed in plastic bags (NUK, Czechia) and sealed. The bags were then treated using high-pressure isostatic press equipment (CYX 6/103, ŽĎAS joint-stock company, Czechia), with a chamber volume of 2 L and a chamber temperature of 38 °C. These samples were subjected to two high-pressure methods:

i) Aliquot 3 was treated with cycled HPP, with the pressure increased to maximum over approximately 5 min during each cycle. After reaching a pressure of 350 MPa (maximum), there was a 5 min holding period, which was followed by rapid depressurisation. Each cycle lasted 10 min and was repeated four times. The sample was then cooled to 5–8 °C and sent for analysis.

ii) Aliquot 4 was treated with continuous high pressure, with the pressure increased to the maximum over 5–6 min. After reaching a pressure of 350 MPa (maximum) at 38 °C, samples were held at these conditions for 20 min, which was followed by depressurisation and cooling to 5–8 °C. After cooling, the aliquots were sent for analysis of nutrients.

Determination of lysozyme content. The content of lysozyme was determined using a human lysozyme in vitro competitive enzyme-linked immunosorbent assay (ELISA) kit (Abcam plc., UK) designed for quantitative measurement of human lysozyme in milk; our procedure followed the manufacturer's instructions. The sensitivity of the test was 0.09 $\mu g \cdot mL^{-1}$, and the detection range was 0.093–6 $\mu g \cdot mL^{-1}$.

Determination of lactoferrin content. Lactoferrin content was determined using a human lactoferrin ELISA kit (CD Creative Diagnostics, USA) designed for the detection of lactoferrin in human milk; our procedure followed the manufacturer's instructions. The lower limit of test sensitivity was 0.13 $ng \cdot mL^{-1}$.

Determination of sIgA content. sIgA content was determined using an sIgA ELISA kit (CD Creative Diagnostics, USA) following the manufacturer's instructions. The test sensitivity was < 1.875 $ng \cdot mL^{-1}$; the detection range was 3.125–200 $ng \cdot mL^{-1}$.

Determination of lipase activity. Bile salt–stimulated lipase activity was determined as described by Escuder-Vieco et al. (2021) with minor modifications. A lipase activity assay kit (Merck, Darmstadt, Germany) was used following the manufacturer's instructions. Sodium taurocholate hydrate was added to the lipase assay buffer (10 $mmol \cdot L^{-1}$). Whole-fat human milk samples were brought to room temperature, homogenised for 10 min on a rotating carousel shaker, and diluted with distilled water (1 : 250 $v \cdot v^{-1}$). The method was calibrated using glycerol solutions exactly as prescribed by the manufacturer. In each well, 50 µL of standard or diluted milk sample was mixed with 100 µL of reaction mix containing lipase assay buffer enriched with sodium taurocholate, peroxidase substrate, enzyme mix, and lipase substrate. Absorbance (at 570 nm) was determined using a microplate reader Epoch-SI reader (Agilent Technologies, USA), before and after 24 h of incubation at 37 °C. As specified by the manufacturer, one unit of lipase activity was defined as the amount of enzyme that would generate 1.0 µmol of glycerol per minute at 37 °C from triacyl glycerides.

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Statistical analysis

MS Excel 2016 (Microsoft Corp., USA) and NCSS 10 (2015, NCSS, LLC., USA) were used for statistical analysis. Data showing a normal distribution with more than 10 results in the test group are described using the mean and standard deviation ($x \pm SD$); other data are described using the median and the first and third quartiles (1st Q; 3rd Q). For reasons of convention, comparison of results with previous studies, and the difficulty of establishing reference values for analytes in breast milk due to interindividual variability between donors, the results are also presented in graphic form, comparing percentage changes of residual amounts related to untreated samples (100%). With respect to the structure of the test data, we opted to use the paired *t*-test. Due to multiple comparisons, we performed Bonferroni correction of the significance level ($\alpha = 0.0083$). For the boxplot visualisations, we applied the conventional method based on the Inter-Quartile Range (IQR) to define the boundaries for identifying outliers. The whisker boundaries were set at 1.5 times the IQR beyond the first and third quartiles. Values exceeding 3 times the IQR from the quartiles were considered beyond the severe outlier boundary. This approach corresponds to the standard implementation provided in the NCSS software.

RESULTS

Lysozyme content determination. We determined lysozyme content in 60 breast milk aliquots (untreated or treated using HoP and two HPP methods). When compared to the initial median of lysozyme content in untreated milk 485 (290; 590) mg·L^{-1}, we found a statistically significant decrease in the median concentration of lysozyme in HoP-treated milk [305 (150; 390) mg·L^{-1} ($P < 0.001$)] and continuous HPP–treated milk [395 (255; 480) mg·L^{-1} ($P = 0.001$)]. Cycled HPP caused no statistically significant change in lysozyme concentration. We demonstrated that cycled HPP resulted in smaller losses of the analyte being determined than the method of continuous HPP ($P = 0.004$). Figure 2 shows relative changes in lysozyme concentration after HoP, cycled HPP, and continuous HPP relative to initial values in untreated fresh milk (100%).

Lactoferrin content determination. We determined the lactoferrin content in 60 breast milk aliquots (untreated or treated using HoP and two HPP methods). When compared to the initial lactoferrin mean in untreated milk (2.64 ± 1.06 g·L^{-1}), there was a significant decrease ($P < 0.001$) in samples treated using HoP (0.29 ± 0.11 g·L^{-1}). Neither HPP method led to a statistically significant change in lactoferrin concentration. There was no statistically significant difference ($P = 0.440$) between the two HPP methods. Lactoferrin levels after HoP, cycled HPP, and continuous HPP, relative to the initial values in untreated fresh milk (100%), are shown in Figure 3.

Lipase activity determination. We determined lipase activity in 60 breast milk aliquots (untreated or treated using HoP and two HPP methods). When compared to the initial mean lipase activity in un-

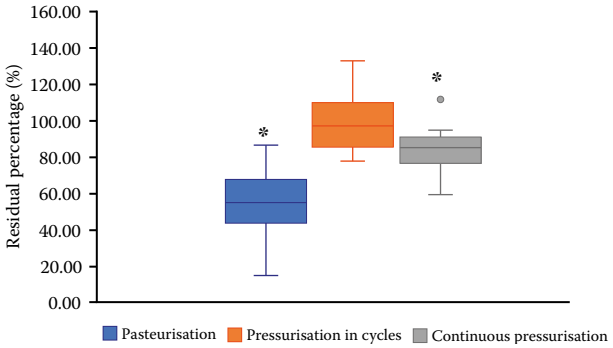

Figure 2. Residual lysozyme level (%) after Holder pasteurisation, cycled high-pressure processing (HPP), and continuous HPP

The asterisk indicates statistically significant results; both pressurisation methods are gentler and better preserve the original lysozyme content; the gray point, at approximately 115% in the continuous pressurisation part of the graph, represents an outlier; initial values in untreated fresh milk represent 100%

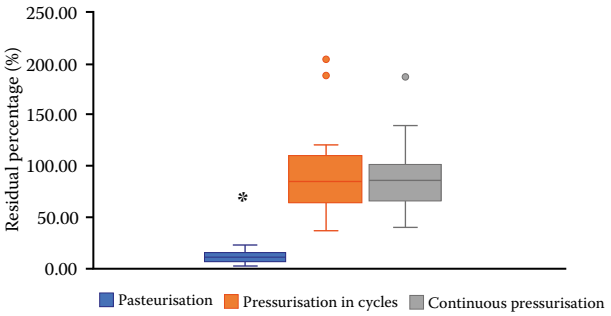

Figure 3. Residual lactoferrin levels (%) after Holder pasteurisation, high-pressure processing (HPP) in cycles, and continuous HPP

The asterisk indicates statistically significant results; the graph shows that HPP methods are gentler and lead to better preservation of lactoferrin content; the gray and orange points represent outliers; initial values in untreated fresh milk represent 100%

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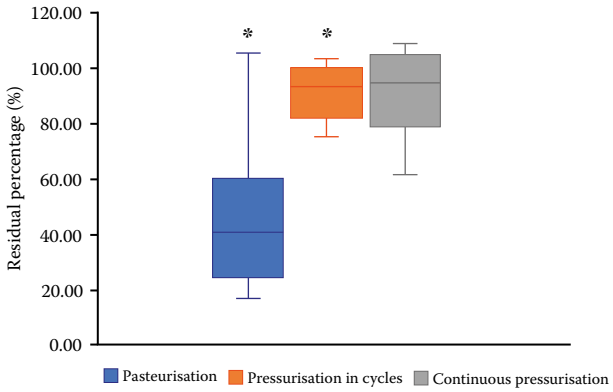

Figure 4. Residual lipase activity (%) after Holder pasteurisation, cycled high-pressure processing (HPP), and continuous HPP

The asterisk indicates statistically significant results; the graph shows that pressurisation methods are gentler and lead to higher retention of lipase activity; initial values in untreated fresh milk represent 100%

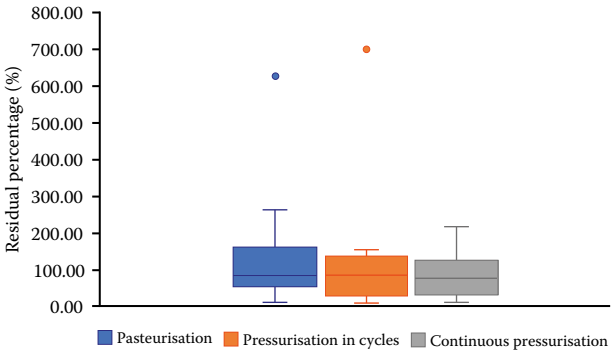

Figure 5. Residual secretory immunoglobulin A (sIgA) level (%) after Holder pasteurisation (HoP), cycled high-pressure processing (HPP), and continuous HPP

Blue and orange points are outliers; there was no significant decrease in sIgA values after HoP, cycled HPP, or continuous HPP; initial values in untreated fresh milk represent 100%

treated milk (7.35 ± 2.64 U·mL^{-1}), we found a statistically significant decrease in lipase activity after HoP (3.39 ± 2.12 U·mL^{-1}; $P < 0.001$) and cycled HPP (6.8 ± 2.73 U·mL^{-1}; $P = 0.002$). The two HPP methods had significantly better results ($P < 0.001$) than the HoP method. However, no statistically significant difference ($P = 0.240$) between the two HPP methods was found. Lipase activities after the HoP and HPP methods, relative to the initial values in untreated fresh milk (100%), are shown in Figure 4.

sIgA content determination. We determined the content of sIgA in 60 breast milk aliquots (untreated or treated using HoP and two HPP methods). In some samples, the value of sIgA in fresh, untreated milk was lower than after treatment by more than 200%; these data points were excluded from the data set. The median sIgA concentration was 364 (282; 837) mg·L^{-1} after HoP treatment, 400 (227; 514) mg·L^{-1} after cycled HPP, and 372 (211; 599.5) mg·L^{-1} after continuous HPP. There was no significant decrease in sIgA after HoP ($P = 0.053$), cycled HPP ($P = 0.053$), or continuous HPP ($P = 0.060$). No statistically significant difference ($P = 0.344$) between the two HPP methods was found. The relative sIgA levels after HoP and HPP methods, relative to the initial values in untreated fresh milk (100%), are shown in Figure 5.

Summary percentual retention rates after HPP and HoP. The summary retention times after treatment with individual methods are shown in Table 1.

DISCUSSION

In the daily operation of our Milk Bank, we use the HoP method (62.5 °C/30 min), which, however, is known to be ineffective against bacterial spores as well as to degrade bioactive substances important for the newborn's immune system. We examined pressurisation as a possible methodology for use

Table 1. Post-HPP and post-HoP treatment retention rates of analysed bioactive substances (%) – median and the first and third quartile $\tilde{x}$ (1stQ; 3rdQ)

Substance	HPP in cycles	Continuous HPP	HoP
Lysozyme	99 (88; 99)	87 (80; 87)	57 (47; 57)
Lactoferrin	84 (66; 105)	85 (69; 100)	13 (10; 13)
Lipase	93 (84; 93)	95 (81; 93)	41 (27; 41)
sIgA	83 (28; 117)	75 (39; 112)	82 (56; 151)

HPP – high-pressure processing; HoP – Holder pasteurisation; sIgA – secretory immunoglobulin A

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in the milk bank, and we sought the gentlest method. We followed the protocol of Demazeau et al. (2018) and Fekraoui et al. (2021). The use of higher pressures with a shorter holding time might be effective in inactivating microbes, including spores, but this method is costly and may not be as gentle on nutritional values. Although many studies have explored high-pressure treatment methods and their effect on nutritional values, most have been conducted on small sample sets. We see the novelty of our work in the fact that we compared 15 batches of breast milk, where we monitored changes in the values of bioactive substances within one donor, using conditions and procedures common to the practice of the milk bank.

Determination of the substances contained in breast milk after the application of high pressure has been addressed by several studies; results have been published in a comprehensive review by Wesolowska et al. (2019). Kontopodi et al. (2022) performed a study of native milk serum protein concentration in HPP milk in comparison with HoP, analysed by the liquid chromatography-mass spectrometry method. They demonstrated that HPP may be considered a suitable alternative to HoP. When compared to the untreated samples, a significant decrease in protein concentration was observed only after HoP ($P < 0.05$).

Dussault et al. (2021) demonstrated that HPP of human milk provides safe milk with less detrimental effects on biochemically active components such as immunoglobulins, lysozyme, lactoferrin, lipase, and cytokines than HoP. Overall, it can be concluded that HPP of HM allows higher preservation of the initial content of bioactive substances than classic HoP.

We demonstrated that the median relative residual lysozyme activity significantly decreased ($P < 0.001$) to 57 (47; 57) % after HoP treatment, which corresponds with the mean residual lysozyme activity (52.3%) reported by Demazeau et al. (2018), while being lower than that reported by Klotz et al. (2017) (65%).

After milk samples were treated with cycled HPP, we did not find any significant change in lysozyme concentrations. The resulting median of biological activity was 99 (88; 99) %. These results are in agreement with Demazeau et al. (2018), who reported residual lysozyme activity greater than 95%. Dussault et al. (2021) reached the same results; they confirmed that there was no significant difference in lysozyme concentration between untreated and HPP using 425 MPa/4 cycles, 6 min hold/4 °C or 37 °C. Mayayo et al. (2016) reported that lysozyme activity was not affected after HPP using 400, 450, or 500 MPa for 30 min. Viazis et al. (2007) reported that HPP using 400 MPa for 30, 60, 90, or 120 min was superior to HoP. Our study also showed that cycled HPP was superior to continuous HPP.

After HoP treatment of milk, there was a significant decrease ($P < 0.001$) in lactoferrin concentration, while treatment with the HPP method led to no statistically significant decrease in lactoferrin concentration. The median lactoferrin content, in our study, after HoP was 13 (10; 15) %, which was lower than the level (approx. 20%) reported by Klotz et al. (2017). The median lactoferrin content after cycled HPP was 84 (66; 105) % in our study and 93, 97% in a study by Demazeau et al. (2018), who used the same high-pressure technology. Dussault et al. (2021), using 425 MPa/4 cycles with 6 min hold at 37 °C, also confirmed the gentleness of the cycled HPP method in preserving lactoferrin activity. Aceti et al. (2020) found that lactoferrin content was well preserved even after HPP using 6 000 bar, 3 min at 19 °C, followed by a 30 s decompression; they also reported that HoP reduced lactoferrin concentrations by 87.5%. These published data confirm our results showing a lower median residual lactoferrin activity of 13 (10;15) % after HoP treatment. Pitino et al. (2022) reported that the concentration of lactoferrin was reduced by only 35% after HoP.

We found a statistically significant decrease ($P < 0.001$) in lipase activity after HoP, where the median retention rate was 41 (27; 41) %, which was lower than the value (47%) reported by Dussault et al. (2021). They also reported no difference in lipase activity in raw milk versus after HPP treatment. This contrasts with our results, in which we found a statistically significant decrease in lipase activity after HPP in cycles ($P = 0.002$) as well as after using continuous HPP ($P = 0.014$). Nevertheless, significantly better results ($P < 0.001$) were achieved using HPP methods compared to HoP. We found no statistically significant difference ($P = 0.240$) between the two HPP methods. The median of residual lipase activity after cycled HPP was 93 (84; 93) %, and after continuous HPP, it was 95 (81; 93) %. Our values are higher than those reported by Demazeau et al. (2018) (retention rates of 80–85 %) using cycled HPP.

We found an insignificant decrease in sIgA values after HoP ($P = 0.053$), cycled HPP ($P = 0.053$), and continuous HPP ($P = 0.060$). Although extreme outlier values are visible in the comparison shown in Figure 5, each of them represents only a single value within the corresponding data set. Moreover, the comparison was based on median sIgA levels, which are

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not influenced by such outliers. Therefore, we believe that these data remain sufficiently informative. These findings agree with those of Dussault et al. (2021), who also reported no significant difference in sIgA between untreated milk and HoP-treated milk. Contador et al. (2013) reported that HPP at 400 MPa for 3 or 6 min retained the same sIgA levels as untreated milk, while higher pressure (600 MPa) preserved only 40–50 % of the initial content. Permanyer et al. (2010) also reported that HPP (using 400 MPa, 5 min at 12 °C) was gentle and resulted in 100% retention of sIgA, while HoP left only 72% of the initial sIgA content. Mayayo et al. (2016) reported even lower levels of IgA preservation (43%) after HoP. Aceti et al. (2020) also reported a significant reduction in sIgA content after HoP but also after very high-pressure HPP (6 000 bar, 3 min at 19 °C). Demazeau et al. (2018) reported lower retention rates compared to our results (HoP 46.3 vs. 82%, cycled HPP 64 vs. 83%).

Reference values of lysozyme and lactoferrin are virtually unknown and dependent on the length of lactation and the absolute amount of protein in expressed HM. It is very difficult to establish reference values for analytes in human milk because its composition is very individual and dependent on many factors (mother's diet, environment, period of lactation, timing of lactation, etc.). The composition of the milk produced by a particular mother undergoes very dynamic changes that accurately reflect the current needs of the breastfed child. However, studies have dealt with bioactive component levels in breast milk. The concentration of sIgA in human milk has been reported to range from 0.22 to 83.7 mg·mL^{-1}; the highest levels were determined in colostrum (Mickleson and Moriarty 1982; Gan et al. 2019; Lis-Kuberka et al. 2021). The typical concentration of lysozyme is about 200–400 μg·mL^{-1}. Lönnerdal et al. (2017) determined lysozyme content in breast milk in the range 0.28 1.1 mg·mL^{-1} (age 0–90 days) and lactoferrin content in the range 1.44–5.05 mg·mL^{-1} (age 0–360 days). Czosnykowska-Łukacka et al. (2019) reported that the mean value of lactoferrin concentration was lowest at 1–12 months of lactation (3.39 ± 1.43 g·L^{-1}), significantly increasing at 13 18 months (5.55 ± 4.00 g·L^{-1}; $P < 0.006$) and remaining at comparable levels at 19–24 months versus after 24 months (5.02 ± 2.97 and 4.90 ± 3.18 g·L^{-1}). Montagne et al. (2001) determined that concentrations of lactoferrin vary between 2.0 and 5 g·L^{-1}.

Studies have sought to determine reference values for bioactive components in the serum of healthy newborns. Bayram et al. (2019) determined mean values for IgA content (mg·dL^{-1}): 953 ± 262.19 (age 0–30 days), 429.5 ± 145.59 (age 1–3 months), and 482.43 ± 236.80 (age 4–6 months). As mentioned above, it is very difficult to establish reference values for analytes in breast milk due to interindividual variability between donors. For this reason, we monitored changes in the content of substances within 1 donor (each sample of untreated milk was divided into four aliquots), and we present the residual percentage after the treatment methods. We found that cycled HPP did not lead to a statistically significant reduction in the tested bioactive substances relative to the amounts present in raw, untreated milk.

CONCLUSION

Better results were achieved when using cycled HPP (350 MPa/5 min/38 °C) in terms of preserving the initial content of the bioactive components sIgA, lactoferrin, and lysozyme, as well as lipase activity. This method, therefore, appears promising for processing breast milk. However, before introducing this method into routine clinical practice, further validation studies must be carried out to determine the content of other nutritional substances and, last but not least, the microbial safety of the product, and the results must be implemented into a hazard critical control point system to ensure the safety of the recipient.

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