**Impact of time-temperature combinations on the anti-Cytomegalovirus activity and biological components of human milk**

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**Impact:**

- This work questions the standard HoP and open the debate on whether the pasteurization temperature commonly used in milk banks should be lowered to better preserve the biological components of the milk.
- A reduction of HoP temperature at 60 °C determined a significant preservation of anti-HCMV activity and IgA content of donor HM, compared to standard HoP.

- This alternative HoP is highly feasible compared to other substitute pasteurization techniques, since it would employ the same pasteurizer equipment found in most Human Milk Banks.

**ABSTRACT**

**Background:** There is extensive evidence that Holder Pasteurization (HoP) (30 minutes at 62.5°C) has harmful effects on the bioactivities of human milk (HM). We previously demonstrated that lowering HoP temperature is sufficient to inactivate Cytomegalovirus (HCMV). Here, we analyzed the effect of lowering time/temperature on the antiviral activity against HCMV and IgA levels of HM.

**Methods:** 80 HM samples from 5 mothers were pasteurized in a range of temperature (62.5–56°C) and time (40–10 minutes) in a conventional setting of Human Milk Bank. Unpasteurized HM from each mother was used as control. The samples were assayed against HCMV-AD169 strain in cell cultures and IgA levels were determined by ELISA.

**Results:** All HM samples exhibited anti-HCMV activity, to a different extent. An improvement of antiviral activity was observed in samples treated at 60°C, 58°C and 56°C compared to those at 62.5°C, with $ID_{50}$ values near those of unpasteurized milk. Similarly, a better retention in IgA levels was observed reducing the temperature of treatment.

**Conclusions:** We demonstrated that a 2.5°C degree reduction of heat treatment significantly preserved the IgA content and fully restored the anti-HCMV activity of HM, supporting this variant of HoP as a valid alternative to preserve HM bioactivities.

## INTRODUCTION

Human milk (HM) is a synergistic collection of nutrients and biological factors evolutionarily designed to facilitate the transition from the intra-uterine to extra-uterine life[1,2]. While artificial formulas replicate to a greater or lesser extent the nutritional characteristics of breast milk by providing a mix of proteins, lipids, and carbohydrates, they lack the biological factors that provide immunological protection and that favor the maturation of the neonatal digestive and nervous system. These biological components are especially relevant for the premature newborns[3,4]. Consequently, donor human milk (DHM) is prioritized over artificial formula in preterm neonates who cannot receive their mother's own milk[5,6].

The DHM is collected, processed, and stored by the Human Milk Banks (HMBs), who distribute this product with all the sanitary guarantees. To prevent transmission of Cytomegalovirus (HCMV) infection, as well as other bacterial and viral pathogens, HMBs routinely process milk using Holder pasteurization (HoP)[7]. It has been demonstrated that this heat treatment destroys most of the bacterial and viral contamination, including HCMV[8,9]. However, as a collateral effect, there is also a decrease in the activity of several biological factors present in HM. Although it has been shown that HoP only partially affect the macronutrient composition of HM (protein, carbohydrates, and lipids, including polyunsaturated fatty acids)[10,11], there is clear evidence that it determines a total eradication of lipase activity, as well as a substantial decrease in the concentration of various biological components such as IgA, lactoferrin, lysozyme, cytokines, and growth factors. Several reviews have summarized the effect of pasteurization on the composition of HM[12,13].

Due to the deleterious effect of HoP, there is a growing interest in developing a milk-processing method that minimizes the disruption of HM biological components as much as possible[14,15]. Among the alternative methods currently under study, we can mention High Temperature Short Time (HTST) pasteurization, High Pressure Processing (HPP), Ultraviolet

(UV) irradiation, and microwave treatment[16–22]. However, despite interesting results, the main obstacle in their routine use in HMBs is the lack of appropriate and affordable equipment at the HMB scale. Moreover, all results described so far have been obtained using prototypes, except for the HTST.

In this context, we hypothesized that the lowering of the pasteurization temperature and/or time can result in better preservation of the biological components of HM, still maintaining the inactivating effect of pasteurization on microbial contamination. An important advantage of this strategy would be the possibility of reusing the currently existing equipment in the HMBs, simply by modifying the pasteurization settings of temperature and/or time.

With this approach in mind, we have recently shown that pasteurization at 60 ºC for 30 minutes completely eliminates HCMV present in HM, under similar conditions to those used in conventional HMBs[23]. Moreover, there is evidence in the literature that heat treatment under these conditions has the capacity to destroy bacterial and viral (HIV, HTLV-I, Polioviruses, Chikungunya Virus (CHIKV), West Nile Virus (WNV)) contaminations in DHM[9,24,25].

To strengthen this approach, we considered that it was necessary to demonstrate how the decrease in pasteurization temperature results in a protection of the biological components present in breast milk. Although there are published data in this regard, the study has never been carried out under the routine conditions normally used in HMBs. One of the clearest biological functions of milk is to provide protection against infection, especially when the newborn's immune system is not fully developed. Indeed, we have previously described the ability of HM to exert an antiviral effect on HCMV, a pathogenic virus that is easily transmitted through breastfeeding from mother to newborn[26]. Indeed, HCMV is the cause of the most common congenital infection, and is of particular clinical relevance in pre-term infants[27,28]. Although HCMV is shed into HM by almost every seropositive woman, milk-

acquired infection with symptomatic disease is relatively rare, likely due to the protective effects of HM components[29,30]. Therefore, preserving the antiviral activity during HM processing is of great importance. In this work we considered the antiviral activity of HM as a representative model to analyze the effect of milk processing by heat treatment on its biological activities. We analyzed the antiviral activity against HCMV of DHM pasteurized at lower temperatures (56, 58, 60 ºC) than the temperature used in HoP (62.5 ºC), all in the same conditions and with the same equipment that we use in our milk bank.

## METHODS

*Milk samples collection and heat treatment*

This project was revised and approved by the Ethics Committee of Balearic Islands (CEIC IB; IB 5024/22 PI). Milk samples from five mothers, who were previously donors at our Human Milk Bank, were obtained from the Biobank of the Balearic Islands Health Research Institute (IdISBa). In details, milk samples from each mother were collected in a time frame between 2 and 15 days. All the milk collected during that period of time was pooled together and aliquoted in 100-mL bottles. All experiments were performed using the pooled milk from each donor. HM in 100-mL bottles was processed with a water bath (Water bath Memmert WPE45, Schwabach, Germany), that it is the same equipment used to pasteurize the donated human milk in our Human Milk Bank. Specifically, HM aliquots were pasteurized at 56, 58, 60 or 62.5 ºC, for 10, 20, 30, 40 minutes respectively. For each mother, one bottle was left untreated (UP, unpasteurized control sample). After treatment, the bottles were rapidly immersed in an ice bath until the temperature decreased below 10 °C. The temperature inside the bottles was monitored and registered with a probe (Flexible thermistor probe, PB-5006-0M5, Tinytag, West Sussex, England). Finally, the milk from the bottles was aliquoted and kept at -30 ºC for subsequent analyses.

*Cell lines and viruses*

Human foreskin fibroblast (HFF-1, ATCC® SCRC-1041) were used for HCMV-AD169 production and for antiviral assays. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated foetal bovine serum (Sigma Aldrich) and 1% antibiotic solution (Zell Shield, Minerva Biolabs, Germany), in humidified 5% $CO_2$ atmosphere at 37 °C.

HCMV strain AD169 (ATCC® VR-538) was propagated on HFF-1 cells. When a clear cytopathic effect developed, supernatants were collected and clarified by centrifugation. Viral

stocks were stored at -80 °C. Viral titers were determined by focus assay on HFF-1 cells in 96-well plates, as described elsewhere[31], and expressed as foci forming units per mL (FFU/mL).

*Cell viability assays*

Cell viability was assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay, as previously described[32]. The 50% and 90% cytotoxic dilutions ($CD_{50}$ and $CD_{90}$) and 95% confidence intervals (CIs) were determined.

*HCMV inhibition assays*

Viral inhibition assays were performed on HFF-1 cells to determine the antiviral activity of milk samples unpasteurized and treated with various time-temperature combinations. HFF-1 were seeded in 96-well plates at a density of $5x10^3$ cell/well. The following day, 2 mL of milk sample were centrifuged at 10,000 g for 60 min at 4 °C, and the fat-free aqueous phase was collected with a 10-mL syringe in a 1.5-mL microtube. Then, the aqueous fractions were serially diluted in DMEM 2% FBS and incubated with HCMV-AD169 at MOI 0.02 (multiplicity of infection, FFU/cell) for 1 hr at 37 °C. Then the mixtures were added to HFF-1 cells for 2 hours. After removing the inocula, the cells were washed 5 times with culture medium and overlaid with 1.2% methylcellulose 2% FBS DMEM medium. After a 5-day-incubation HCMV-AD169 infected cells were visualised by immunocytochemistry. Results were reported as percentage of stained HCMV-infected cells in comparison to controls. The inhibitory dilutions of the milk samples reducing the viral infectivity by 50% (inhibitory dilution-50, $ID_{50}$) and 95% CIs were determined. All the experiments were performed in triplicate.

*IgA quantification*

Total IgA levels were measured in treated and untreated milk samples by using the Human IgA ELISA Kit of Invitrogen (Thermo Fisher Scientific, Waltham, MA), following manufacturer instructions. The test was performed on the serum fraction of milk obtained after centrifugation for 5 minutes at 10,000 g to remove fat. 20 µL of a 1:2000 diluted sample were used, and each sample was tested in duplicate in 3 independent experiments. The IgA level data are expressed as a percentage of loss of IgA levels compared to unpasteurized milk, which was taken as a reference of 100%.

*Data analysis*

All analyses were performed using the software GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). The $CD_{50}$ values and $ID_{50}$ values for inhibition curves were calculated by regression analysis by fitting a variable slope-sigmoidal dose–response curve. The Student's T-test was used to compare $\%ID_{50}$ or %IgA level of heat-treated samples with unpasteurized sample (UP). Significance was reported for p value $<0.05$ (*), $<0.01$ (**) and $<0.001$ (***).

## RESULTS

The study group included 5 healthy donor mothers. The main clinical characteristics of the study group are reported in Table 1. In brief, one had a premature delivery (32 weeks of gestation, 2150 grams of new-born weight) and the rest were born at term (between 38 and 41 weeks of gestation with new-borns weights between 3050 and 3550 grams). Milk samples were collected starting from day 5, 11, 43, 50, 64 after birth, respectively, , with the intention of having milk with different amounts of immunoglobulin A. Four out of five mothers were HCMV-IgG negative.

Human milk (HM) samples were collected and treated at different time-temperature combinations, as described in the Methods section. The pasteurization procedure involved the use of a conventional HoP method with standard equipment according to the international Guidelines of HMBs. Thus, 16 HM heat-treated samples and an unpasteurized (UP) milk sample as control from each mother were obtained and processed for further investigations of anti-HCMV activity and IgA content.

*Effect of different time-temperature combinations on the anti-HCMV activity of HM.*

As described in the Methods section, HM samples were clarified by centrifugation to obtain the aqueous fraction, which was used for the following assays. Previous works showed that the aqueous fraction of HM was the preferable biological matrix for cell-based assays, as it had a lower impact on cell viability than the whole milk sample[26,33]. Therefore, preliminary experiments were conducted to determine the effect on cell viability of the aqueous fraction dilutions of HM samples. Almost all undiluted samples (1:1 dilution) showed a toxic effect on HFF-1 cells, the cell line selected for the antiviral assays. On the contrary, as reported in Figure 1, the 1:2 dilution of HM samples did not affect cell viability, with a few exceptions, which exhibited a ≥90% cell viability at the 1:4 dilution. Based on these results, the antiviral

activity of HM samples was evaluated at non-cytotoxic dilutions for each sample, in order to exclude the possibility that the observed antiviral effect was due to cellular toxicity.

To assess the impact of different time-temperature combinations on the intrinsic anti-HCMV activity of the aqueous fraction of HM, in vitro antiviral assays were performed by treating the virus before and during cell infection with serial dilutions of UP or heat-treated HM samples, as described in Figure 2A. An immunostaining procedure was used to detect HCMV-infected cells, generating a dose-response curve for each tested HM sample. The antiviral activity was expressed as $ID_{50}$ value, i.e., the dilution of HM sample able to inhibit 50% of viral infectivity. This approach is validated and widely accepted[34–36].

The $ID_{50}$ values for all tested HM samples are reported in Table S1. As we demonstrated in a previous work[26], UP samples were endowed with intrinsic anti-HCMV activity, albeit with a significant inter-individual variability ($ID_{50}$ from 0.0180 to 0.0957). Moreover, all heat-treated samples exhibited some degree of anti-HCMV activity, with $ID_{50}$ values ranging from 0.0143 to >0.5. Figure 2B shows the mean % changes of $ID_{50}$ values from the whole study population for all time-temperature combinations tested, compared to the UP samples. The antiviral activity of HM exhibited a time- and temperature-dependent reduction following treatment, to different extents considering the specific time-temperature combination. Treatment at 62.5 °C was associated to the greatest reduction in antiviral activity at all times tested, as compared to the other tested temperatures. In particular, the 10-minutes treatment at 62.5 °C caused a mean reduction of 131% of antiviral activity compared to the UP, and this loss of activity at 62.5 °C was more pronounced as the treatment time increased, reaching up to a mean 260% reduction at 40 minutes. On the other hand, the antiviral activity was better preserved for all other time-temperature combinations. Indeed, the anti-HCMV activity of 60, 58, 56 °C samples was reduced less than 50% for all tested times, compared to the UP. Of note, only the 40-minutes treatment at 60 °C exhibited a >50% loss of activity (i.e., 79%).

This global trend was maintained considering individually each mother, although with some interesting differences (Figures 2C-G). In particular, HM samples from mother B exhibited the greatest loss of antiviral activity when treated at 62.5 °C for all times tested (reduction >400% compared to UP), with $ID_{50}$ values >0.5 (Figure 2D). Interestingly, mother B's UP sample exerted the weakest anti-HCMV activity of all tested UP samples ($ID_{50}$ of 0.0957). As shown in Figure 2F, HM samples from mother D followed a similar trend to that of mother B, with a great reduction of antiviral activity when treated at 62.5 °C. However, treatments at lower temperatures (60, 58, 56 °C) of HM samples from mothers B and D resulted in a significant improvement of antiviral activity, compared to 62.5 °C treatments, with $ID_{50}$ increases lower than 64% (except for a 267.8% increase at 60 °C for 40 minutes for mother B). On the contrary, HM samples from mothers A and E showed only a slight reduction in the antiviral activity of HM following heat-treatments (Figures 2C and 2G). Specifically, an $ID_{50}$ increase minor than 100% was observed for all time-temperature combinations, compared to the UP, except for the 30- and 40-minutes treatments at 62.5 °C that determined an important loss of antiviral activity (>100%). As for mother C, HM samples exhibited the minor loss of anti-HCMV activity following heat treatment at all time-temperature combination tested (<50% compared to UP) (Figure 2E). Of note, the UP sample from mother C also showed the highest antiviral activity compared to UPs from other mothers ($ID_{50}$ of 0.018).

Altogether, these results indicated that treatments at lower temperatures and/or shorter times than those of standard HoP are able to preserve more effectively the intrinsic anti-HCMV activity of human milk.

*Effect of different time-temperature combinations on the IgA content of HM.*

Another important characteristic of the human milk, which has been proven to be negatively affected by pasteurization processes, is the content of immunoglobulins[17]. Thus, we investigated how lowering times and/or temperatures of the pasteurization process could

impact on the immunoglobulin content of HM. Specifically, we assessed the IgA content, since IgA is the major immunoglobulin class present in human milk.

To this end, the different HM samples treated as described in the Methods were centrifuged in order to avoid the interference of the fat component, and the skimmed milk was used to test for IgA concentration by using a commercial ELISA test. Considering that in the literature IgA concentrations in HM show a great inter-study variability up to 50-fold[37], we consider that the IgA content of our UP samples ranging from 142.51 to 299.96 μg/mL are similar to those reported in literature.

Figure 3A shows the mean % changes of IgA content from the whole study population for all time-temperature combinations tested, compared to the UP samples. In line with the results of the antiviral activity, we observed that IgA content of heat-treated HM samples was significantly reduced after treatment, to different extents considering the specific time-temperature combination. Interestingly, as opposed to the clear time-dependent reduction in antiviral activity, the reduction in IgA content of HM samples was not significantly affected by the increasing treatment time. On the other hand, lowering temperature determined a better preservation of IgA content. Specifically, the greatest loss of IgA content was assessed for treatment at 62.5 °C for all times tested (mean 46%). As the treatment temperature decreased, the IgA content was gradually preserved compared to the UP, up to an approximate mean 70% retention of IgA at 56 and 58 °C.

As reported in Figures 3B-F, we observed the same trend of IgA content reduction at increasing temperature-treatments for the individual mothers, with interesting inter-individual differences. HM samples from mothers A and B exhibited the greatest reduction in IgA levels following treatment (Figures 3B and 3C). In particular, 62.5 and 60 °C treatments determined a reduction higher than 50% at all times tested, whereas a mean 47% loss of IgA content was observed for 58 and 56 °C. Of note, 62.5 °C treated sample from mother A exhibited the

greatest loss of IgA content compared to UP (mean 66%); however, it is important to point out the UP sample from mother A also presented the lowest content of IgA of all mothers (142.51 μg/mL). For mothers C, D, and E, all the time-temperature combinations never caused a decrease in IgA content greater than 50% compared to UP. Specifically, as shown in Figure 3E, HM samples from mother D showed a temperature-dependent loss of IgA content in the range of approximately 45-30%. Instead, mothers C and E exhibited the minor loss of IgA following treatment compared to the other mothers, with a mean reduction of 35% at 62.5 °C, a less pronounced reduction at 60 °C (around 20%) and a mild reduction at 58 °C and 56 °C (approximately 0-15%) (Figures 3D and 3F).

*Effect of 30-minutes treatment at different temperatures on the biological properties of HM.*

Since the Holder Pasteurization consists of a 30-minutes treatment at 62.5 °C, we compared the variation of anti-HCMV activity and IgA content of HM following a 30-minutes pasteurization at different temperatures for the global population of the study **(**Figure 4).

As shown in Figures 4A, the anti-HCMV activity of HM was significantly reduced at a 62.5 °C treatment ($p < 0.05$), whereas reducing the temperature of only 2.5 °C degrees (i.e., 60 °C) preserved the antiviral activity of HM similarly to that of UP milk. HM samples treated at lower temperatures (58, 56 °C) exhibited an improvement of antiviral activity comparable to that of 60 °C. Accordingly, Figure 4C shows an increment of viral infectivity, expressed as brown-stained infected cells, for 62.5 °C -treated sample, compared to the unpasteurized and heat-treated samples at 60, 58, 56 °C. Our results confirmed that the antiviral activity of HM is negatively affected by the pasteurization temperature.

In regard to the IgA content of 30-minutes heat-treated HM, we observed a temperature-dependent effect (Figure 4B). At 62.5 °C, we observed the greatest reduction of IgA compared to UP ($p < 0.001$), whereas with the lowering of temperature treatment, the IgA content was increasingly retained.

## DISCUSSION

It is widely accepted that pasteurization of DHM, while essential to avoid the risk of infectious disease transmission, has harmful effects on various features of the bioactivity of HM[12,13,38]. In the present paper, we investigated how the reduction of time and/or temperature of the HoP can positively affect the retention of important biological features of HM, such as its intrinsic antiviral activity and the levels of immunoglobulins, resulting in a more rich and complete food for the new-born infant. Although the negative effect of pasteurization on HM components has been previously described in the literature, to the best of our knowledge, this is the first detailed study on different time-temperature combination treatment effect on milk biological properties performed in a conventional setting of HMBs, with standard HoP procedures. In particular, we examined the anti-HCMV potential and IgA content of the breast milk from 5 donor mothers, after heat treatment at standard HoP conditions (30 minutes at 62.5 °C) or at various combinations of time-temperature (40 – 10 minutes; 62.5 – 56 °C).

The intrinsic antiviral activity of HM against human viruses causing disease in new-borns and children has been explored in previous works[34,36]. In particular, the anti-HCMV potential has been well established for both the HM itself and some specific components[31,39]. Accordingly, in the present work, all tested unpasteurized human milk samples were endowed with net anti-HCMV activity, with $ID_{50}$ values in the range of 0.0180-0.0957, comparable to those reported in the literature[26]. These significant inter-individual differences were expected, as various studies reported a high variability of milk composition between individuals and a consequent variability of antiviral potential and other bioactivities of HM[40]. Although the investigated mothers in this study are seronegative for HCMV-IgGs (except for mother A, whose serostatus was not assessable), their HM still exerted anti-HCMV activity, clearly indicating that additional immune or non-immune factors also contribute to the intrinsic anti-HCMV

activity of HM, other than specific immunoglobulins against the virus. Therefore, this observation stimulates further studies to identify yet unknown antiviral factors and combinatorial antiviral mechanisms of the human milk[41].

The effect of HM pasteurization on the intrinsic antiviral activity has been poorly investigated so far. Previous studies conducted by our group showed that HoP significantly reduced the anti-HCMV activity of HM[26,36], while a recent work from Kothari *et al.* reported on the partial preservation of anti-HCMV activity of HoP-processed milk[42]. However, the impact of different time-temperature combinations of the HoP procedure has never been studied in regard to the intrinsic anti-HCMV activity of HM. Here, we demonstrated that all heat treatments at various combinations of time-temperature (40 – 10 minutes; 62.5 – 56 °C) preserved the anti-HCMV activity, although to different extents. Generally, significant retention of antiviral activity was observed in samples treated at 60, 58, and 56 °C, with an $ID_{50}$ increase minor than 50% compared to UP milk. By contrast, heat treatment at 62.5 °C determined a more significant loss of anti-HCMV activity, with $ID_{50}$ increases higher than 100% compared to UP milk. Moreover, we showed a time-dependent loss of antiviral activity, for all tested temperatures. The fact that all heat-treated HM samples were active in inhibiting HCMV infectivity to a variable extent suggests that some physical or biochemical properties of human milk which are minimally affected by heating treatment must contribute to the overall anti-HCMV activity. For example, a recent study from Francese *et al*. reported that the anti-HCMV activity of an abundant component of human milk, i.e., glycosaminoglycans, was maintained after a HoP-like heat treatment[31].

Similar to the inter-individual variability of anti-HCMV activity for UP milk samples, we also observed variability among mothers in the preservation of activity after heat-treatment. Interestingly, the degree of retention of antiviral activity after heat-treatment appeared to be correlated to the activity of unpasteurized milk. Specifically, the weakest antiviral activity

was associated to the highest loss of activity, as evidenced for mother B, and, similarly, the strongest anti-HCMV activity of UP HM, observed for mother C, was correlated to the minor loss of activity after heat-treatment. Of note, HM from mother C was the sample collected earliest, which our group previously demonstrated to possess the highest antiviral potency compared to milk collected later[26].

Similarly, we assessed the retention of total IgA in HM after pasteurization. The effects of thermal treatment on the IgA content of HM are well described, as IgA is the most abundant class of immunoglobulins present in this biofluid and plays an important contribution in the biological and nutritional role of human milk[24,38,43]. Here, we observed that HoP treatment for 30 minutes at 62.5 °C caused a mean decrease in IgA content of 46% (±7.5%), in accordance with the literature that reports an IgA reduction in the range of 30-50%[44–46]. After treatment at different times-temperatures (40 – 10 minutes; 62.5 – 56 °C), IgA levels were significantly reduced at all combinations tested, compared to UP milk. In particular, a temperature-dependent loss of IgA was evidenced, with a mean reduction of 28% (±11.1%), 30% (±9.0%), 38% (±10.1%) at 56 °C, 58 °C and 60 °C respectively. Interestingly, the degree of IgA retention following heat treatment was not significantly affected by the treatment time. This finding was in line with the work of Czank *et al*., which demonstrated that temperature, and not holding time, is critical for the retention of various biological factors of HM, including IgA, lactoferrin and lysozyme[24]. However, those data were obtained using a pasteurizer-like model for experimental use, whereas our study was conducted treating HM under conventional conditions and settings of HMBs.

While HoP still represents the “gold-standard” and most commonly adopted technique in HMBs to inactivate pathogens in DHM, it determines an important loss of HM bioactivity. Therefore, many attempts were made to identify new alternative feasible treatment options, that would improve the balance between inactivation of pathogens and preservation of

biological components of HM. Various techniques have been proposed as substitutes for HoP. Among them, HTST pasteurization emerged as a valuable alternative pasteurization, being able to increase the retention of the antiviral activity and some bioactive and nutritional components of HM compared to HoP[36,47,48]. However, Klotz *et al*. recently observed that HTST is less effective than HoP in reducing bacterial load[49]. Other studies focused on the optimization of the HoP procedure by modifying its parameters, in order to improve the bioactivity of pasteurized HM, while still inactivating breast milk-transmitted pathogens. For example, Capriati *et al*. applied a modified HoP procedure (peak treatment temperature of 72.5 °C, immediately followed by a cooling process), which resulted in a better retention of triglycerides compared to standard HoP[50]. Moreover, a study on the effect of different time-temperature combinations showed that a 30-minutes treatment at 57 °C was sufficient to inactivate by 99.9% the yield of all bacterial species tested (i.e., E. coli, S. epidermidis, E. cloacae, B. cereus, and S. aureus)[24]. However, there's still need for an in-depth characterization of the susceptibility of other potential milk contaminating bacteria to heat treatment with reduced temperature. It's widely recognized that Bacillus cereus possesses the ability to resist HoP due to its sporulating ability, and therefore still represents the primary cause of discarded pasteurized DHM[51]. Our variant of pasteurization would not overcome the limit of traditional HoP. However, recently, Jandová et al. showed that the risk of B. cereus growth during thawing and warming of contaminated DHM was almost eliminated if the milk was used within 1 h after warming[52]. Furthermore, recent studies demonstrated the relatively small risk of B. cereus infection following the ingestion of pasteurized DHM[53], and that airborne or direct or indirect contact are the main sources of most, if not all, cases of severe B. cereus neonatal infections[54].

Concerning breastfeeding transmitted viruses, there is clear evidence that a 60 °C treatment is sufficient for the total inactivation of common viral pathogens found in HM[8,9]. In particular,

the Human Immunodeficiency Virus (HIV) and the Human T-cell Leukemia Virus (HTLV), two of the most dangerous viral pathogens transmitted through breast milk, were fully inactivated by thermal treatment at 55-60 °C[55,56]. Moreover, thermal treatment at 55 °C was shown to almost completely inactivate high-risk and low-risk Human Papillomaviruses (HPV)[57]. In regards to the most common breastfeeding-transmitted virus, i.e., HCMV, our group recently demonstrated that pasteurization at 60 °C for 30 minutes was effective in inactivating it, using a procedure that closely resembles common HoP practice in HMBs[23].

In this context, current practice (62.5 °C for 30 min) may be considered excessive for pasteurizing donor human milk in order to inactivate the most common breastfeeding pathogens. Herein, we demonstrated that a reduction of HoP temperature at 60 °C determined a significant preservation of anti-HCMV activity and IgA content of donor HM, compared to standard HoP. In particular, reducing the temperature of pasteurization of only 2.5 °C degrees preserved the antiviral activity of HM similarly to that of unpasteurized milk. Thus, our work, along with previous literature, further supports this variant of HoP as a valid alternative to better preserve bioactivity of HM and simultaneously ensure microbiological safety. Moreover, this alternative HoP is highly feasible compared to other substitute pasteurization techniques, since it would employ the same pasteurizer equipment found in most HMBs. Despite its promising effect on the preservation of HM bioactivities, further studies are necessary to confirm the inactivation of other clinical strains of common breast milk-transmitted viruses and bacteria, before introducing a 2.5 °C reduction in the temperature setting of HM pasteurization.

**Study limitations**

This study was carried out on human milk samples from 5 mothers that were collected at different times after delivery (from 5 to 64 days). Considering the limited number of samples, we cannot rule out that this heterogeneity has prevented to obtain more consistent results. Moreover, the sample size meant that we were not able to evaluate the impact of the lactational stage on antiviral activity of pasteurized milk. Indeed, it would be interesting to assess whether the effect of pasteurization at different times/temperatures on antiviral activity observed in this study was conserved among colostrum, transitional, and mature milk.

## DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

**REFERENCES**


1. Thome, U. H. & Boquien, C.-Y. Human Milk: An Ideal Food for Nutrition of Preterm Newborn DOHAD (DEVELOPMENTAL ORIGIN OF HEALTH AND ADULT DISEASES) CONTEXT AND NUTRITIONAL PROGRAMMING. *Front. Pediatr. | www.frontiersin.org* **6**, 295 (2018).
2. Lönnerdal, B. Bioactive Proteins in Human Milk—Potential Benefits for Preterm Infants. *Clin. Perinatol.* **44**, 179–191 (2017).
3. Abrams, S. A., Landers, S., Noble, L. M. & Poindexter, B. B. Donor human milk for the high- risk infant: Preparation, safety, and usage options in the United States. *Pediatrics* **139**, (2017).
4. Li, Y. *et al.* Pasteurization Procedures for Donor Human Milk Affect Body Growth, Intestinal Structure, and Resistance against Bacterial Infections in Preterm Pigs. *J. Nutr.* **147**, 1121–1130 (2017).
5. Quigley, M. & McGuire, W. Formula versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst. Rev.* **2014**, (2014).
6. BREASTFEEDING, S. O. *et al.* Breastfeeding and the Use of Human Milk. *Pediatrics* **129**, e827–e841 (2012).
7. Weaver, G. *et al.* Recommendations for the establishment and operation of Human Milk Banks in Europe: A consensus statement from the European Milk Bank Association (EMBA). *Front. Pediatr.* **7**, 53 (2019).
8. Gayà, A. & Calvo, J. Improving Pasteurization to Preserve the Biological Components of Donated Human Milk. *Front. Pediatr.* **6**, 288 (2018).
9. Pitino, M. A., O'connor, D. L., McGeer, A. J. & Unger, S. The impact of thermal pasteurization on viral load and detectable live viruses in human milk and other matrices: A rapid review. *Appl. Physiol. Nutr. Metab.* **46**, 10–26 (2021).

10. Paulaviciene, I. J. *et al.* The Effect of Prolonged Freezing and Holder Pasteurization on the Macronutrient and Bioactive Protein Compositions of Human Milk. *https://home.liebertpub.com/bfm* **15**, 583–588 (2020).

11. García-Lara, N. R. *et al.* Effect of holder pasteurization and frozen storage on macronutrients and energy content of breast milk. *J. Pediatr. Gastroenterol. Nutr.* **57**, 377–382 (2013).

12. Peila, C. *et al.* The Effect of Holder Pasteurization on Nutrients and Biologically-Active Components in Donor Human Milk: A Review. *Nutr. 2016, Vol. 8, Page 477* **8**, 477 (2016).

13. Picaud, J. C. & Buffin, R. Human Milk—Treatment and Quality of Banked Human Milk. *Clin. Perinatol.* **44**, 95–119 (2017).

14. Moro, G. E. *et al.* Processing of donor human milk: Update and recommendations from the European Milk Bank Association (EMBA). *Front. Pediatr.* **7**, 49 (2019).

15. Aceti, A. *et al.* Effect of Alternative Pasteurization Techniques on Human Milk's Bioactive Proteins. *J. Pediatr. Gastroenterol. Nutr.* **70**, 508–512 (2020).

16. Giribaldi, M. *et al.* A Preliminary Assessment of HTST Processing on Donkey Milk. *Vet. Sci. 2017, Vol. 4, Page 50* **4**, 50 (2017).

17. Escuder-Vieco, D., Espinosa-Martos, I., Rodríguez, J. M., Fernández, L. & Pallás-Alonso, C. R. Effect of HTST and Holder Pasteurization on the Concentration of Immunoglobulins, Growth Factors, and Hormones in Donor Human Milk. *Front. Immunol.* **9**, 2222 (2018).

18. Wesolowska, A. *et al.* New achievements in high-pressure processing to preserve human milk bioactivity. *Front. Pediatr.* **6**, 323 (2018).

19. Kontopodi, E. *et al.* Effects of High-Pressure Processing, UV-C Irradiation and Thermoultrasonication on Donor Human Milk Safety and Quality. *Front. Pediatr.* **10**,

305 (2022).

20. Christen, L., Lai, C. T., Hartmann, B., Hartmann, P. E. & Geddes, D. T. Ultraviolet-C Irradiation: A Novel Pasteurization Method for Donor Human Milk. *PLoS One* **8**, e68120 (2013).

21. Leite, J. A. S. *et al.* The effect of microwave-assisted heating on bioactive and immunological compounds in donor human milk. *LWT* **161**, 113306 (2022).

22. Martysiak-Żurowska, D., Malinowska-Pańczyk, E., Orzołek, M., Kusznierewicz, B. & Kiełbratowska, B. Effect of microwave and convection heating on selected nutrients of human milk. *Food Chem.* **369**, 130958 (2022).

23. Gayà, A. *et al.* Analysis of Thermal Sensitivity of Human Cytomegalovirus Assayed in the Conventional Conditions of a Human Milk Bank. *Front. Pediatr.* **9**, (2021).

24. Czank, C., Prime, D. K., Hartmann, B., Simmer, K. & Hartmann, P. E. Retention of the Immunological Proteins of Pasteurized Human Milk in Relation to Pasteurizer Design and Practice. *Pediatr. Res. 2009 664* **66**, 374–379 (2009).

25. Leydold, S. M. *et al.* Chikungunya virus and the safety of plasma products. *Transfusion* **52**, 2122–2130 (2012).

26. Donalisio, M. *et al.* Anti-cytomegalovirus activity in human milk and colostrum from mothers of preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **67**, 654–659 (2018).

27. Kabani, N., & Ross, S. A. Congenital Cytomegalovirus Infection. *J. Infect. Dis.* **221**, S9–S14 (2020).

28. Manicklal, S., Emery, V. C., Lazzarotto, T., Boppana, S. B., & Gupta, R. K. The "Silent" Global Burden of Congenital Cytomegalovirus. Clin. Microbiol. Rev. 26, 86–102 (2013).

29. Hamprecht, K., & Goelz, R. Postnatal Cytomegalovirus Infection Through Human Milk in Preterm Infants: Transmission, Clinical Presentation, and Prevention. *Clin.*

*Perinatol.* **44**, 121–130 (2017).

30. Trombetta, C. M., Viviani, S., Montomoli, E., & Marchi, S. Seroprevalence of antibodies to cytomegalovirus in pregnant women in the Apulia region (Italy). *J. Prev. Med. Hyg.* **62**, E372–E376 (2021).

31. Francese, R. *et al.* Human milk glycosaminoglycans inhibit cytomegalovirus and respiratory syncytial virus infectivity by impairing cell binding. *Pediatr. Res.* 1–8 (2022).

32. Cagno, V. *et al.* In vitro evaluation of the antiviral properties of Shilajit and investigation of its mechanisms of action. *J. Ethnopharmacol.* **166**, 129–134 (2015).

33. Civra, A. *et al.* Human Colostrum and Derived Extracellular Vesicles Prevent Infection by Human Rotavirus and Respiratory Syncytial Virus in Vitro. *J. Hum. Lact.* **37**, 122–134 (2021).

34. Francese, R. *et al.* Anti-Zika virus and anti-Usutu virus activity of human milk and its components. *PLoS Negl. Trop. Dis.* **14**, 1–24 (2020).

35. Conzelmann, C. *et al.* Storage-Dependent Generation of Potent Anti-ZIKV Activity in Human Breast Milk. *Viruses 2019, Vol. 11, Page 591* **11**, 591 (2019).

36. Donalisio, M. *et al.* High temperature-short time pasteurization has a lower impact on the antiviral properties of human milk than holder pasteurization. *Front. Pediatr.* **6**, 304 (2018).

37. Rio-Aige, K. *et al.* The Breast Milk Immunoglobulinome. *Nutr. 2021, Vol. 13, Page 1810* **13**, 1810 (2021).

38. Young, B. E., Murphy, K., Borman, L. L., Heinrich, R. & Krebs, N. F. Milk Bank Pooling Practices Impact Concentrations and Variability of Bioactive Components of Donor Human Milk. *Front. Nutr.* **7**, 1–6 (2020).

39. Donalisio, M. *et al.* Extracellular Vesicles in Human Preterm Colostrum Inhibit

Infection by Human Cytomegalovirus In Vitro. *Microorg. 2020, Vol. 8, Page 1087* **8**, 1087 (2020).

40. Gidrewicz, D. A. & Fenton, T. R. A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. *BMC Pediatr.* **14**, (2014).
41. Wedekind, S. I. S. & Shenker, N. S. Antiviral properties of human milk. *Microorganisms* **9**, (2021).
42. Kothari, A. *et al.* Preservation of Anti-cytomegalovirus Activity in Human Milk Following High-Pressure Processing Compared to Holder Pasteurization. *Front. Nutr.* **9**, 1–7 (2022).
43. Dussault, N. *et al.* Comparison of the Effect of Holder Pasteurization and High-Pressure Processing on Human Milk Bacterial Load and Bioactive Factors Preservation. *J. Pediatr. Gastroenterol. Nutr.* **72**, 756–762 (2021).
44. Müller, K. *et al.* Holder Pasteurization: Comparison of Water-Bath and Dry-Tempering Devices. *Front. Pediatr.* **10**, 1122 (2022).
45. Irazusta, A. *et al.* High-pressure homogenization and high hydrostatic pressure processing of human milk: Preservation of immunological components for human milk banks. *J. Dairy Sci.* **103**, 5978–5991 (2020).
46. Adhisivam, B. *et al.* Effect of Holder pasteurization on macronutrients and immunoglobulin profile of pooled donor human milk. *https://doi.org/10.1080/14767058.2018.1455089* **32**, 3016–3019 (2018).
47. Escuder-Vieco, D. *et al.* High-Temperature Short-Time and Holder Pasteurization of Donor Milk: Impact on Milk Composition. *Life 2021, Vol. 11, Page 114* **11**, 114 (2021).
48. Kontopodi, E. *et al.* High-Temperature Short-Time Preserves Human Milk's Bioactive Proteins and Their Function Better Than Pasteurization Techniques With Long

Processing Times. *Front. Pediatr.* **9**, (2022).

49. Klotz, D. *et al.* High-Temperature Short-Time Treatment of Human Milk for Bacterial Count Reduction. *Front. Pediatr.* **6**, (2018).
50. Capriati, T. *et al.* A Modified Holder Pasteurization Method for Donor Human Milk: Preliminary Data. *Nutr.* **11**, 1139 (2019).
51. Jandová, M. *et al.* Bacillus cereus as a major cause of discarded pasteurized human banked milk: A single human milk bank experience. *Foods*. **10**, 1–15 (2021).
52. Jandová, M. et al. Quantitative Risk Assessment of Bacillus cereus Growth during the Warming of Thawed Pasteurized Human Banked Milk Using a Predictive Mathematical Model. Foods. 11, 1037 (2022).
53. Lewin, A., Delage, G., Bernier, F. & Germain, M. Banked Human Milk and Quantitative Risk Assessment of Bacillus cereus Infection in Premature Infants: A Simulation Study. *Can. J. Infect. Dis. Med. Microbiol.* **2019**, Article ID 6348281 (2019).
54. Lewin, A. *et al.* Bacillus cereus infection in neonates and the absence of evidence for the role of banked human milk: Case reports and literature review. *Infect. Control. Hosp. Epidemiol.* **40**, 787–93 (2019).
55. Hoque, S. A. *et al.* Transient heating of expressed breast milk up to 65°C inactivates HIV-1 in milk: A simple, rapid, and cost-effective method to prevent postnatal transmission. *J. Med. Virol.* **85**, 187–193 (2013).
56. Kenji YAMATO, H. *et al.* INACTIVATION OF LYMPHOCYTE-TRANSFORMING ACTIVITY OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I BY. *J. Cancer Res.* **77**, 13–15 (1986).
57. Donalisio, M. *et al.* Inactivation of high-risk human papillomaviruses by Holder pasteurization: Implications for donor human milk banking. *J. Perinat. Med.* **42**, 1–8

(2014).

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## AUTHOR CONTRIBUTIONS

M.D., A.G.: substantial contributions to conception and design. I.A., J.C., M.R., S.G. M.L.: acquisition of data. I.A., J.C., M.R.: analysis and interpretation of data. I.A., J.C.: drafting the article. M.D., A.G., D.L.: revising the article critically for important intellectual content. All authors: final approval of the version to be published.

## COMPETING INTERESTS

The authors declare no competing interests.

## CONSENT STATEMENT

The study was revised and approved by the Ethics Committee of Balearic Islands (CEIC IB; IB 5024/22 PI). Each milk donor signed a written consent form for the use of donated milk for research purposes, where mother's and infant's data protection were assured.

**FIGURE LEGENDS**

**Figure 1. Evaluation of the effect of HM samples on cell viability.** Cells were treated with serial dilutions of HM samples unpasteurized (UP) or heat-treated at different time-temperature combinations, under the same experimental conditions as the antiviral assays. The graph reports for each HM sample the first dilution at which ≥90% cell viability was observed, as determined by MTS assay.

**Figure 2. Effect of different time-temperature combination treatments on anti-HCMV activity of HM. A.** Protocol for heat treatment and processing of HM samples, and for HCMV inhibition assays for the evaluation of anti-HCMV activity. **B-G.** The variations in anti-HCMV activity following heat treatment at various time-temperature combinations in comparison to UP are reported as a global analysis (**B**) and for the individual mother A, B, C, D, E (**C-G**). Data are reported as % change in $ID_{50}$ values compared to UP ± confidence intervals 95% for three independent experiments, and as mean % change in $ID_{50}$ values ± SEM for the global analysis of the 5 mothers.

**Figure 3. Effect of different time-temperature combination treatments on IgA content of HM.** The variations in IgA content following heat treatment at various time-temperature combinations in comparison to UP are reported as a global analysis (**A**) and for the individual mother A, B, C, D, E (**B-F**). Data are reported as % of IgA content compared to UP, and as mean % of IgA content compared to UP ± SEM for the global analysis of the 5 mothers.

**Figure 4. Effect of treatment for 30 minutes at 62.5 °C, 60 °C, 58 °C, and 56 °C on the anti-HCMV activity and IgA content of HM samples. A-B.** The variation in anti-HCMV

activity (**A**) and in IgA content (**B**) of HM samples from the global study population (5 mothers) are reported as mean percentages of $ID_{50}$ values and IgA content (± SEM), respectively, in comparison to UP. Heat-treated samples were compared with UP using a Student's T-test. n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **C.** Representative images of HFF-1 cells infected with HCMV-AD169 are reported for the 30-minute assays for the unpasteurized sample (UP), and heat-treated samples (i.e., 62.5, 60, 58, 56 °C). Infected cells and foci are brown by immunostaining. Magnification, 200X.