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Processing methods of donor human milk evaluated by a blood plasma clotting assay

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ABSTRACT

Donor human milk is the first alternative for preterm infants when mother's own milk is not available. Most available human milk banking guidelines recommend classical holder pasteurization to ensure safety by eliminating potential infectious microorganisms. Processing by heat treatment, however, negatively affects functionality and availability of bioactive components naturally present in human milk. Here we compared the effect of five different processing methods on the ability of human milk to induce blood plasma clotting, which was recently described as a bioactive function present in human milk. From thirty lactating women, milk samples were collected, and all milk samples were subjected to holder pasteurization (30 min at 62.5 °C), high-temperature-short-time pasteurization (15 s at 72 °C), high-pressure processing (5 min at 500 MPa), ultraviolet-C irradiation (4863 J/L), or thermo-ultrasonication (6 min at 60 W, at 40 °C). All methods significantly reduced the ability of milk to trigger blood plasma clotting compared to untreated milk, but ultraviolet-C irradiation and high-pressure processing were best at preserving this activity. Taken together, measuring the ability of milk to induce blood plasma clotting may offer a new tool to monitor the effect of human milk processing.

1. Introduction

Mother's own milk is considered the best diet for almost all newborns. Especially preterm infants may benefit from human milk as it is for example related to less intestinal complications such as necrotizing enterocolitis when compared to preterm infant formula feeding (Corpeleijn et al., 2012). The reason human milk contributes to the reduced risk of (intestinal) inflammation is the presence of bioactive substances in human milk (Gila-Diaz et al., 2019). However, sometimes mother's own milk is insufficient or unavailable to preterm newborn infants. The next best choice for these infants is donor human milk (Arslanoglu et al., 2013). Although milk donors are screened for health status, consumption of donated raw milk can still impose a serious health risk to preterm infants due to potential transmission of pathogens, mainly bacteria, cytomegalovirus and human immunodeficiency virus.

Considering these biosafety concerns, almost all human donor milk banks employ holder pasteurization (HoP) before the milk is provided to preterm infants (Weaver et al., 2019). Although HoP effectively eliminates pathogens, it also destroys or reduces (the functionality of) bioactive components present in milk. Therefore, at present there is a quest for alternative milk processing methods that are less harmful to bioactive components (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013a; Dussault et al., 2021).

Recently, we demonstrated that human milk, but not bovine, induces blood plasma clotting, although its precise biological role is uncertain. The ability of milk to induce blood plasma clotting may contribute to the

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relative lower incidence of necrotizing enterocolitis in preterm infants fed own mothers milk when compared to those who are fed cow's milk based preterm formula (Corpeleijn et al., 2012). The ability of human milk to induce blood plasma clotting is due to the presence of cell-derived particles called extracellular vesicles (EVs) (Hu et al., 2020). EVs are small in size, about 50 nm to 1 μm, and are released by all cells into their environment. EVs are enclosed by a phospholipid membrane, and are thought to play various roles in homeostasis, protection, and intercellular communication, the latter by transporting for example genetic information between cells (Yáñez-Mó et al., 2015). The protein that triggers coagulation, a transmembrane protein called tissue factor (TF), is abundantly present in human milk, where it is exclusively present in the phospholipid membrane of EVs (Hu et al., 2020).

Also other human body fluids such as saliva contain TF-exposing EVs that induce blood plasma clotting (Berckmans, Sturk, van Tienen, Schaap, & Nieuwland, 2011). Although it is unknown why such body fluids contain TF-exposing EVs, their presence in saliva has been associated with the reflex of licking a wound, i.e. to promote haemostasis and wound healing, thereby reducing the risk of developing infection. To which extent the EVs in milk may also promote haemostasis, e.g. during nipple skin damage or upon gastrointestinal damage in young infants, is yet unknown. Recently, we observed that TF-exposing EVs from normal human saliva and milk expose not only TF but the complex of TF and its ligand, coagulation factor VII (data not shown), which supports our hypothesis that TF-exposing EVs in milk may support haemostasis. The fact that already in the 1930-ies paediatricians demonstrated that nose bleeds of haemophilia patients could be effectively treated by gauzes soaked in human milk, directly proves that the ability of human milk to trigger blood clotting is indeed sufficient and effective in promoting haemostasis (Glanzmann, 1934; Solé, 1935).

The aim of the study was to determine the effect of five different milk processing methods compared to untreated milk on the ability of human milk to induce blood plasma clotting.

2. Methods

2.1. Sample collection

Lactating mothers were recruited in Amsterdam University Medical Centers, Amsterdam, The Netherlands. Written informed consent was obtained from all participants. The study protocol was approved by the local Medical Ethical Committee. Milk was collected by complete expression of all milk in one breast, and milk samples were collected in disposable bisphenol A-free bottles by using a breast pump. The milk was stored at −20 °C. If the milk was collected at home, samples were stored at −20 °C in their home freezer, then transported on dry ice to the hospital.

2.2. Processing methods

To thaw the frozen milk, the bottle was put in a refrigerator overnight at 4 °C. Milk samples were split into 6 aliquots after which one baseline sample was stored untreated and the other aliquots underwent one out of five different processing methods: HoP (30 min at 62.5 °C), high-temperature-short-time pasteurization (HTST; 15 s at 72 °C), high-pressure processing (HPP; 5 min at 500 MPa), ultraviolet-C irradiation (UVC; 4863 J/L), or thermo-ultrasonication (TUS; 6 min at 60 W, 40 °C). The choice of milk processing methods was based on a review (Peila et al., 2017), and the specific conditions for HPP, HTST, UVC irradiation and TUS were derived from studies performed at the Wageningen University (Liu et al., 2020; Mank et al., 2021).

For HoP, 30 mL milk from each donor was poured into 50 mL sterile Greiner tubes. The tubes were placed in a shaking water bath at 150 rotations per minute (rpm), where they were heated for 30 min at 62.5 °C. Subsequently all tubes were placed in an ice bath for 15 min for cooling to <4 °C. The temperature was continuously monitored using a temperature data logger RS PRO 1384 (RS Components B.V., The Netherlands).

For HTST, a laboratory scale pasteurizer was built. The system includes a peristaltic pump (Watson Marlow 505S), two water baths representing the heating and the holding section, and an ice bath representing the cooling section. Milk samples of 10 mL from each donor were heated for 15 s at 72 °C, after which they were cooled to <10 °C.

For HPP, milk samples of 10 mL from each donor were poured into sterile pouches and subjected to 500 MPa for 5 min using a pilot scale high pressure pasteurizer (Resato, the Netherlands). This HPP pasteurizer used water as the pressure transmission medium.

For UVC irradiation, we used an experimental set up that was based on a previously published approach (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013b). Milk samples of 60 mL from each donor were poured into sterile glass beakers of 150 mL. A UVC germicidal lamp (PL-S 5 W, UVC radiation 1.1 W, Philips, The Netherlands) was used as the source of UVC irradiation and was positioned diagonally in the beaker to ensure a vortical flow. The samples were stirred on a magnetic stirring plate at 500 rpm, with a 4 × 20 mm stirring rod (IKA RH basic 2, Germany) during the whole process. The approximate UVC dose was 4863 J/L.

For TUS, a sonifier (Branson Digital Sonifier® 450, 50–60 Hz) that was fitted with a sound enclosure (Branson Emerson Technologies, GmbH & Co) and a microtip probe was used. The sonifier was then connected to a circulating water bath and milk samples of 20 mL from each donor were transferred into a glass beaker surrounded by circulating water of 40 °C. All experiments were performed at an amplitude of 58%, which produced an output of 60 W, as displayed by the instrument. The sonifier was set on a pulse-pause mode with a continuous pulse of 59.9 s and a pause of 20 s for a treatment time of 6 min. After treatment, the samples were cooled in an ice-water bath to 4 °C. A data logger (RS PRO 1384) was used to monitor the temperature at all times.

After all treatments, the samples were stored at −20 °C until analysis.

2.3. Blood collection and blood plasma preparation

To prepare a pool of normal human blood plasma, citrate-anticoagulated blood was collected from about 400 healthy subjects with informed consent by venepuncture through a 21-gauge needle using Vacutainer tubes (BD, Mississauga, Canada). The first 2 mL of blood was discarded to exclude contamination from venepuncture. The collected blood was centrifuged for 5 min at 4190 *g* at 18 °C to separate blood cells from the blood plasma. Subsequently, blood plasma was collected and transferred into a new tube, which was centrifuged for 15 min at 3000 *g* at 15 °C to remove most of the remaining blood platelets. After collection of the platelet-depleted blood plasma, the blood plasma was pooled, mixed, and stored as 500 μL aliquots at −80 °C until use.

2.4. Blood plasma clotting assay

To monitor the effect of processing methods on the ability of milk to trigger blood plasma clotting, blood plasma was thawed at 37 °C, followed by centrifugation at 18,900 *g* at 4 °C for one hour to remove residual cell fragments and debris as described earlier (Hu et al., 2020). A schematic overview of the blood plasma clotting assay is shown in Fig. 1. Human milk samples were thawed at 37 °C and then immediately placed on melting ice until use. In preliminary experiments, we made milk titration curves (0.04% to 20% (vol/vol)) in blood plasma to find an optimal milk concentration to trigger clotting (Fig. 2), and we arbitrarily chose 5% milk (vol/vol) as the optimal concentration. To obtain a final concentration of milk of approximately 5% (vol/vol), milk (50 μL) was diluted in 150 μL saline and gently mixed to ensure homogeneity. From this four-fold diluted milk sample, 20 μL was added to 70 μL blood plasma in a flat bottom 96 wells plate and mixed. In this mixture the milk provides the EV-bound TF, the initiator of clotting, and the blood plasma provides the coagulation factors. Because calcium ions are

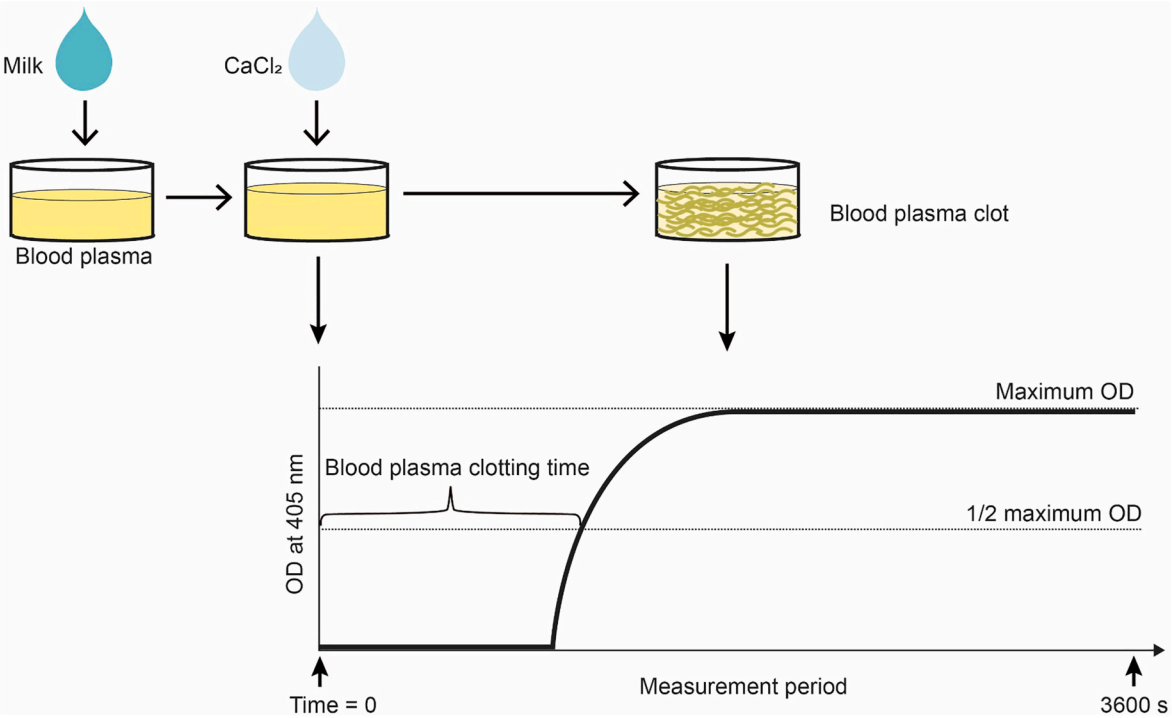

Fig. 1. Principle of the blood plasma clotting assay.
Human milk is added to blood plasma, mixed, and pre-heated at 37 °C. To initiate blood plasma clotting, $CaCl_2$ is added ($t = 0$). Clot formation is recorded by monitoring the optical density (OD) of blood plasma for one hour at 37 °C. When the blood plasma clotting starts, the OD increases. The clotting time is automatically recorded as the ½ maximum OD.

required to allow binding of coagulation factors to the EV membrane, and because calcium ions are chelated by the anticoagulant citrate to inhibit coagulation, $CaCl_2$ was added (15 μL $CaCl_2$ (100 mmol/L stock)) to allow blood plasma clot formation. After mixing, clotting was monitored for one hour at 37 °C by measuring the optical density at λ = 405 nm using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA).

2.5. Statistics

The blood plasma clotting time, as a measure for the presence TF, the initiator of coagulation, was analyzed using a Wilcoxon signed rank test (SPSS version 26.0 software, SPSS Inc., Chicago, IL). A probability value (P) of less than 0.05 was considered to be statistically significant. All data are shown as median and interquartile range, unless indicated otherwise.

3. Results

3.1. Baseline of donors

Milk samples were collected from 30 healthy women at median 4 months (range 2 weeks - 13 months) postpartum.

3.2. Effect of processing methods on the ability of human milk to induce blood plasma clotting

In preliminary experiments, we diluted unprocessed human milk from one donor to measure its ability to trigger blood plasma clotting. From Fig. 2A it is clear that the ability of milk to trigger blood plasma clotting is concentration dependent. For example, addition of 20% (5-fold dilution) milk (vol/vol) resulted in almost immediate blood plasma clotting, whereas at a final dilution of 0.2% (500-fold dilution) the blood plasma clotting was hardly recordable within one hour. Therefore, we tested 1% and 5% final concentrations of milk in blood plasma (vol/vol) for all processing methods studied. As shown in Figures 2B (1%) and 2C (5%), at a final concentration of 5%, the blood plasma clotting time of all processing methods was within the detection range, and therefore this concentration was chosen to study the clotting ability of untreated and processed milk samples from 30 donors.

Fig. 3 shows that after addition of 5% (vol/vol) untreated human milk ($n = 30$) the blood plasma clotting time was 68 s (median; interquartile range 34–99). Compared to untreated milk, treatment of milk samples with HoP increased the blood plasma clotting time to 1259 s (686–3156; $P < 0.001$), whereas TUS and HTST increased the blood plasma clotting time to 1256 s (512–3276) and 809 s (564–1189), respectively (both $P < 0.001$). Treatment of milk with HPP modestly affected the ability of human milk to induce blood plasma clotting, with a blood plasma clotting time of 239 s (133–315; $P < 0.001$). UVC, however, preserved the ability of human milk to induce blood plasma clotting best from all tested processing methods with a blood plasma clotting time of 92 s (44–150), although this is still significantly prolonged compared to untreated milk ($P = 0.033$). Also, UVC preserved clotting activity significantly better than HPP ($P < 0.001$). Supplementary Table 1 shows the blood plasma clotting times of all individual donors.

4. Discussion

In the present study, we found that non-thermal methods preserve the blood plasma clotting ability of human milk better than thermal methods. Recently, we showed that human milk induces blood plasma clotting, and this milk activity is sensitive to HoP (Hu et al., 2020). In the present study, we confirm these findings. In addition, HTST, a processing method that reduces thermal damage of bioactive components in human milk, also impairs the ability of human milk to induce blood plasma clotting, indicating that heating-based processing methods are not optimal to preserve this bioactive function (Escuder-Vieco et al.,

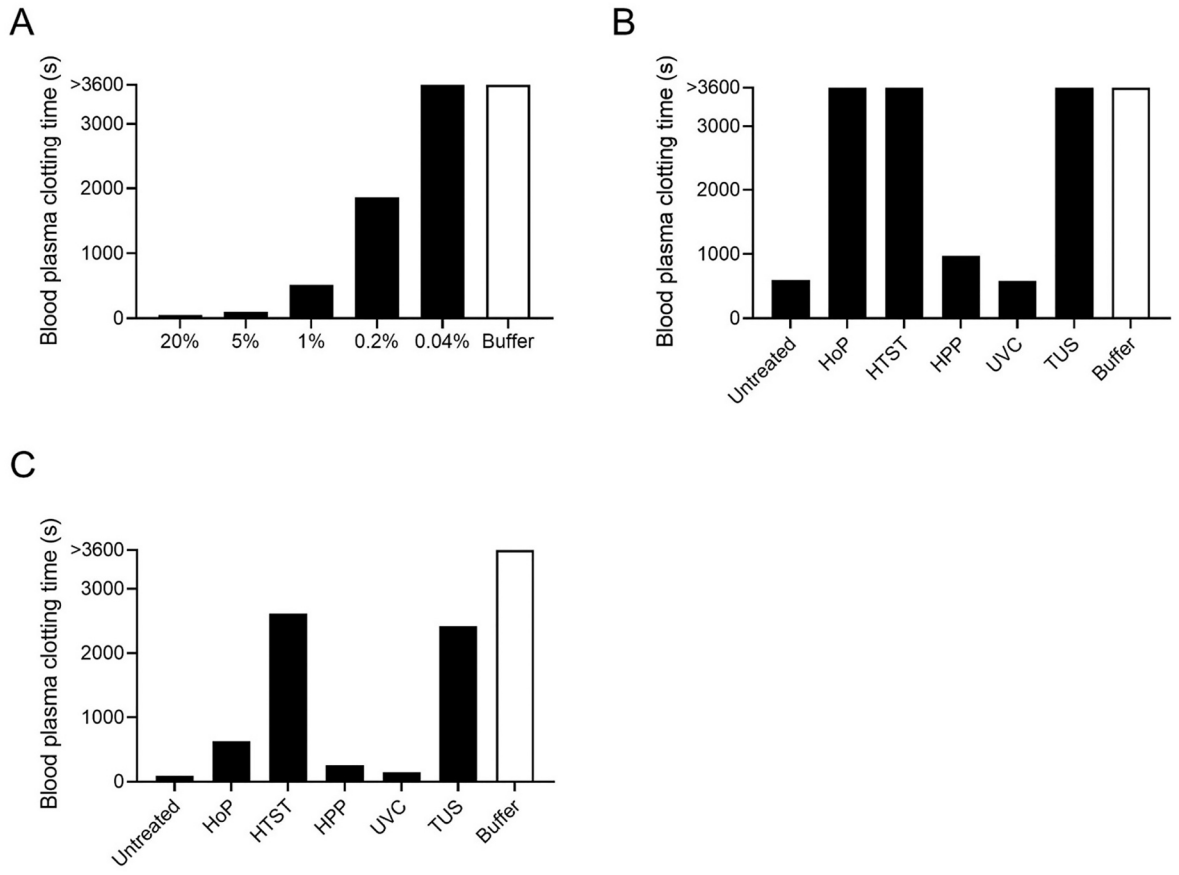

Fig. 2. Finding the optimal concentration of human milk in a blood plasma clotting assay.
Dilutions of human milk from a single donor were added to human plasma to find the optimal dilution (A). Based on the results from (A), the blood plasma clotting times were measured in the presence of 1% (B) or 5% (C) of untreated and processed milk from the same donor (vol/vol). Ssaline was used as negative control. HoP: holder pasteurization; HPP: high-pressure processing; HTST: high-temperature-short-time pasteurization; TUS: thermo-ultrasonication; UVC: ultraviolet-C irradiation.

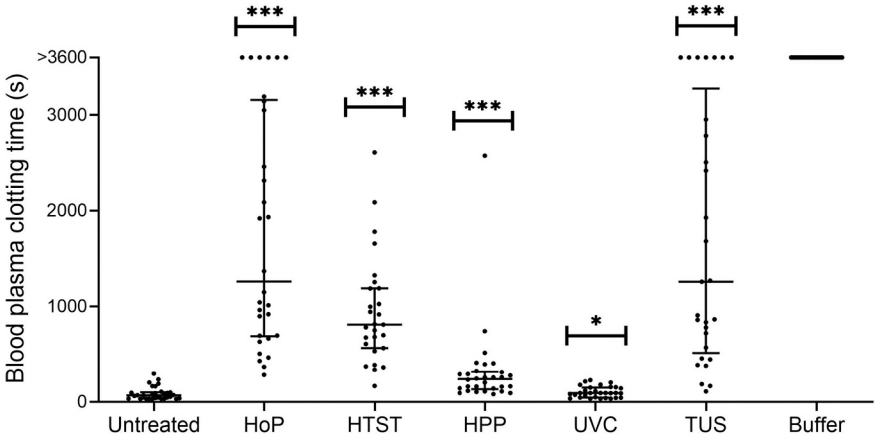

Fig. 3. Effect of milk processing methods on blood plasma clotting.
Human milk was collected from 30 healthy lactating women. The clotting of blood plasma was measured for one hour, i.e. 3600 s (s), in the presence of 5% untreated or processed milk (vol/vol). Buffer (saline) was used as negative control ($n = 30$). HoP: holder pasteurization; HPP: high-pressure processing; HTST: high-temperature-short-time pasteurization; TUS: thermo-ultrasonication; UVC: ultraviolet-C irradiation; $^{*}P < 0.05$; $^{***}P < 0.001$.

2018). Also TUS strongly affected the ability of milk to induce clotting. TUS-created cavitation kills microorganisms by mechanical and sonochemical damage, and this method may in the same way also damage EVs, possibly explaining the failure of a subset of milk samples to induce blood plasma clotting (Lim, Benner, & Clark, 2019).

In our present study, three milk processing methods involve a heating step, i.e. HoP, HTST, and TUS. These thermal processing methods are known to reduce the levels of native immune-active proteins and enzymes (Peila et al., 2016), and all three methods reduce the ability of TF to trigger blood plasma clotting. Also HPP may induce irreversible structural changes of proteins and enzymes present in milk (Balasubramaniam, Barbosa-Cánovas, & Lelieveld, 2016), but such changes are not expected to occur at the mild conditions that were applied in the present study.

In contrast, HPP is better at preserving bioactive components as immunoglobulins and growth factors than HoP and HTST (Demazeau et al., 2018), and in the present study we detected a modest effect of HPP on the ability of milk to induce blood plasma clotting. HPP destroys the

tertiary structure of proteins, thereby impairing the microbial internal biochemistry, but HPP only seems to modestly affect the ability of milk to induce blood plasma clotting, which may be due to the fact that we applied relatively mild HPP conditions. UVC is a gentle and safe alternative for human milk processing as this method preserves more bioactive factors than HoP and HTST (Cappozzo, Koutchma, & Barnes, 2015; Li et al., 2017). From our present results, UVC hardly affects the ability of human milk to induce blood plasma clotting, and thus UVC seems best at preserving the structure and function of EV-exposed TF.

From Fig. 3 it is clear that the variation in blood plasma clotting times is larger for thermal processing methods than for HPP and UVC. When sufficient functional TF is added to blood plasma, the blood plasma clotting reaction (which is an exponential reaction) will start immediately. When TF is absent (as in the buffer condition), or when functional TF is present at a low concentration (after heat treatment), the blood plasma will not clot or will clot late. Thus, because the concentration of functional TF is a critical factor in the blood clotting reaction, the observed variation in blood plasma clotting times is less for methods that only modestly affect the functional activity of TF.

Limitations of the present study are that a contribution of TF from non-EV origin can not be excluded because whole milk was stored, and experiments were not performed in duplicate, although in a preliminary experiment the variation was <15% (Supplementary Fig. 1).

In conclusion, we found that UVC and HPP, gentle milk processing methods both known to preserve bioactive components, hardly affect the ability of human milk to trigger blood plasma clotting. In contrast, HoP, HTST, and TUS, processing methods that are not optimal for preserving bioactive components of milk, all three impaired the ability of milk to induce blood plasma clotting.

To which extent measuring blood plasma clotting is an alternative, additional and/or superior bioassay to monitor the effect of donor human milk processing methods, and to which extent the results from this assay are associated with clinical outcome, clearly requires head-to-head comparisons and additional studies. Nevertheless, the blood plasma clotting assay is easy, fast and robust, and may be included to future milk processing studies to investigate its potential clinical relevance. Ultimately, if proven safe, this should lead to the routine implementation of more gentle processing methods of donor human milk. This could then improve its clinical benefits for preterm infants, in case mother's own milk is insufficient.

Author statement

Conceptualization: YH, JT, RN; Methodology: EK, EM, CHPvdA, KH, RMvE, YH; Formal analysis and investigation: YH, EK, EM, CHPvdA, JBvG, KH, RMvE, JT, RN; Data curation: EK, EM; Writing – original draft preparation: YH, EK, EM; Writing – review and editing: YH, EK, EM, CHPvdA, JBvG, KH, RMvE, JT, RN; Visualization: YH; Supervision: JT, CHPvdA, JBvG, RN.

Declaration of Competing Interest

C.H.P.v.d.A. reports participating in scientific advisory boards and giving lectures in educational symposia for Nutricia Early Life Nutrition, Baxter, and Nestlé Nutritional Institute. J.B.v.G. is member of the National Health Council and founder and director of the National Human Donor Milk Bank in the Netherlands.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2022.102938.

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