Effect of Alternative Pasteurization Techniques on Human Milk's Bioactive Proteins

*Arianna Aceti, †Laura Cavallarin, *Silvia Martini, ‡Marzia Giribaldi, *Francesca Vitali, §Simone Ambretti, ||Vittorio Zambrini, and *Luigi Corvaglia

ABSTRACT

Objectives: Human milk (HM) feeding leads to improved outcome for preterm infants. When mother's milk is unavailable, pasteurized donor HM (DHM) is the recommended alternative over formula. The Holder pasteurization (HoP) method is universally performed in HM banks; however, it is known to impair several functional HM components. The aim of this study was to compare the efficacy of HoP with 2 innovative processing methods (high-temperature short-time [HTST] pasteurization and high-pressure processing [HPP]) in preserving some bioactive HM protein components.

Methods: HM samples from donors of the Bologna HM bank were collected and divided into 4 subsamples: 1 was kept raw, and each of the others was processed using a different technique (HoP, HTST, and HPP at 600 MPa for 3 minutes). Total protein content, secretory immunoglobulin A (sIgA), and lactoferrin contents were compared.

Results: Both HM lactoferrin and sIgA content were negatively affected, but to a different extent, by each method: sIgA was preserved by HTST, with only HPP leading to a significant reduction (−38.8%); lactoferrin content was strongly reduced by HoP (−87.5%) and HTST (-83.5%), and preserved by HPP. Variations in protein profile were seen for all processing methods, being more relevant for HoP, followed by HTST and, finally, by HPP. All the 3 methods lowered the untreated HM microbial counts to undetectable levels, in accordance with national guidelines.

Conclusions: Both HTST and HPP better preserved the original HM protein profile, compared to HoP. They, however, affected differently some bioactive HM components involved in immune response and antibacterial activity.

Key Words: donor human milk, human milk pasteurization, lactoferrin, protein profile, secretory IgAs

(*JPGN* 2020;70: 508–512)

What Is Known

- Donor human milk represents the best alternative to own mother's milk for preterm infants.
- To comply with microbiological safety standards, donor human milk is usually pasteurized in human milk banks using the Holder pasteurization method, which is known to impair several donor human milk bioactive properties.
- Pasteurization methods alternative to Holder pasteurization such as high-temperature short-time and high-pressure processing are currently under investigation.

What Is New

- High-temperature short-time and high-pressure processing reduced microbiological counts to undetectable level.
- Secretory immunoglobulin A is better preserved by high-temperature short-time; lactoferrin content is strongly reduced by both Holder pasteurization and high-temperature short-time and preserved by high-pressure processing.
- The highest variations in protein profile are seen after Holder pasteurization.

Human milk (HM) represents the optimal feeding for preterm infants, especially for those born with a very low (<1500 g) birth weight (1). Mother's own milk is uniquely tailored for each newborn, both in its nutritional composition and in the non-nutritive bioactive factors that promote survival and healthy development

Received August 3, 2019; accepted December 12, 2019.

From the *Department of Medical and Surgical Sciences, Neonatal Intensive Care Unit, AOU Bologna, University of Bologna, Bologna, the †Institute of Sciences of Food Production, National Research Council, Grugliasco, Turin, the ‡Council for Agricultural Research and Economics (CREA), Research Centre for Engineering and Agro-Food Processing, Torino, the §Operative Unit of Clinical Microbiology, AOU Bologna, and the ||Department of Quality, Innovation, Safety, Environment, Granarolo S.p.A., Bologna, Italy.

Address correspondence and reprint requests to Marzia Giribaldi, PhD, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Centro di Ricerca in Ingegneria e Trasformazioni Agroalimentari c/o Area della Ricerca, Strada delle Cacce 73-10135, Torino, Italy (e-mail: marzia.giribaldi@crea.gov.it).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (*www.jpgn.org*).

The work presented in the article was supported by the Italian Ministry of Education, University and Research Agrifood National Cluster in the frame of the project ''PROS.IT- Promotion of consumer's health: nutritional implementation of Italian agroindustrial products.''

L.C. and M.G. are the inventors of a pending patent on the HTST pasteurizer for human milk used in the paper (Patent no. EP 15176792.8-1358). The remaining authors report no conflict of interest.

DOI: 10.1097/MPG.0000000000002598

(2). When mother's own milk is unavailable or insufficient, the use of donor HM (DHM) is recommended (3): recent studies have shown that DHM feeding is associated with a reduction of neonatal morbidities including necrotizing enterocolitis (4,5). Furthermore, it has been documented that the availability of DHM has a paradoxical beneficial effect in increasing the rates of breastfeeding among mothers who deliver prematurely (4,6). To ensure its quality and safety, DHM must be provided by an HM bank (HMB) (3): specific recommendations for the preparation, pasteurization, and distribution of DHM exist in many countries worldwide (7–12). As for the process of pasteurization, the Holder pasteurization (HoP) method is universally recommended by all HMBs, because, at present, it is the only method for which validated devices are commercially available, and for which an extensive amount of evidence on safety and efficacy exists (8,13–15). It is, however, well known that HoP, which is a thermal process performed on bottled DHM at 62.5°C for 30 minutes, followed by fast cooling to a temperature of 4°C, impairs several functional HM components, including immunoglobulins (Igs), lactoferrin, lipases, as well as other enzymatic activities, some cytokines, and some vitamins (8,13–15). For this reason, further research is currently being aimed at testing pasteurization methods alternative to HoP, which would be capable of retaining the largest variety of HM bioactive properties to the highest extent, without affecting DHM microbiological safety (8,13–16).

Several alternative processing methods are being investigated in recent years: these include thermal processes, such as high-temperature short-time pasteurization (HTST), and nonthermal methods, such as high-pressure processing (HPP) and ultraviolet irradiation, and mixed techniques, such as (thermo-)ultrasonic processing (8,13,15–17). HTST and HPP are considered as the most promising alternatives to HoP for DHM (16,17), being the most widely studied at present. The main limitation to their applicability as routine pasteurization techniques in HMBs is the lack of specific instrumentation, validated in relevant HMB environment. This technical gap is being progressively filled, because specific devices to be operated with small milk volumes have been recently developed for the use in HMBs (18,19).

The aim of the present study is to contribute to the innovation in the field by directly comparing the efficacy of the 2 most promising techniques, HTST and HPP, to standard HoP. To this aim, prototyped pasteurization equipment and commercial devices are used. Changes in the protein profile of DHM, and specific HM bioactive components (lactoferrin and secretory IgA content [sIgA]), are addressed.

METHODS

Ethics

The study protocol was approved by the Ethical Committee of Sant'Orsola-Malpighi Hospital, Bologna, Italy (study 165/2015/U/Tess). HM samples were collected from donors of the Bologna HMB, after signing an informed written consent.

Collection of Human Milk Samples

HM samples were collected, approximately 3 months after delivery, from 5 HM donors following the requirements of the Bologna HMB. Mothers were asked to express milk by a breast pump after carefully washing their hands and breast. HM was collected using sterile, single-use breast pump kits into sterile polypropylene bottles. HM samples were stored at −20°C at the HMB until processed, and then handled following the routine protocol used for DHM (11): they were removed from the freezer several hours before the analyses and processed only when completely thawed.

Human Milk Processing

HM pools (400 mL each) were obtained from individual donors by collecting milk during few consecutive days. Each milk pool was divided into 4 subsamples (100 mL each): 1 sample was not treated, 1 sample was pasteurized by HoP, 1 by HTST, and the last by HPP. Each subsample was analyzed for selected indicators of protein and microbial quality. HoP was performed at the Bologna HMB, following the standard pasteurization procedure for DHM which is used in our neonatal intensive care unit. Specifically, HoP was performed by a standard HM Holder pasteurizer (S90 TES, Medicare Colgate LTD, Cullompton, UK). DHM was pasteurized using a temperature of 62.5°C for 30 minutes (tolerance ± 0.5°C), and then cooled to 4°C in 60 minutes (tolerance ± 0.5°C). HPP was performed at HPP Italia, Traversetolo, Italy, using an industrial AV-30 device produced by Avure Technologies, Inc (Middletown, OH); HPP was performed by applying a 6000 bar pressure for 3 minutes to bottled HM by using water at 4°C. Temperature increases to 19°C during compression. Decompression time is about 30 seconds. HTST pasteurization was performed as previously described (19), using a patented proprietary device (Patent number: EP 15176792.8-1358), which is a benchtop device consisting in a system of tubular heat exchangers for heating and for cooling. The temperature was monitored along the pasteurization steps by specific digital probes (tolerance ± 0.5°C).

Human Milk Analyses

All analyses were performed separately on each individual milk sample from different donor mothers. Processed (HoP, HPP, and HTST) and untreated HM were assayed for total protein, sIgAs and lactoferrin content, and for protein profile. sIgAs were measured on 1:10,000 diluted samples, using an ELISA kit (BioVendor, Brno, Czech Republic) and following the manufacturer's instructions, in triplicate. Lactoferrin quantity was determined in triplicate on 1:20,000 diluted samples (1:40,000 for untreated HM), using an ELISA kit (BioVendor) and following the manufacturer's instructions. Total protein content (TPC) was determined on samples skimmed by centrifugation at 2000 *g* at 4°C for 30 minutes, using 2DQuant kit (GE Healthcare Italia, Milan, Italy), in duplicate, following the manufacturer's instructions. The protein profile (in nonreducing conditions, 5 μg of proteins) was visualized by monodimensional electrophoresis on a 10-well 12% Nu-PAGE precast gel (Thermo Fisher Scientific, Waltham, MA) with MES (Thermo Fisher Scientific) as running buffer, on a Novex Mini-cell (Thermo Fisher Scientific) at 200 V. The gels were stained with Blue Coomassie Colloidal stain, and band intensity was quantified by densitometry following the protocol already described in a previous study (20).

Microbiological safety analyses were performed following the Italian guidelines for HMBs (11), on both untreated and processed HM samples, to verify the compliance of each processing technique to the HMB requirements. Each HM sample was assessed for total bacterial load (Plate Count Agar, Kima Meus, Piove di Sacco, Italy), presence of enterobacteriaceae (Herellea Agar, Kima Meus) and *Staphylococcus aureus* (Mannitol Salt Agar-Kima Meus). Bacterial counts were reported as Colony Forming Units (CFU)/mL. In order to comply with the standards required by HMB guidelines (11), in raw HM total viable bacteria count must be $<10^5$ CFU/mL, and both Enterobacteriaceae and *S aureus* $<10^4$ CFU/mL; after pasteurization, no bacteria should be detected.

TABLE 1. Median (interquartile range) in total protein, lactoferrin, and secretory IgA content (g/L) of 5 human milk samples before and after different pasteurization techniques: Holder (HoP), high pressure processing, and high-temperature short-time pasteurization

	HM	HoP	HPP	HTST	$P < 0.05$
Total proteins	7.2 (7.1–9.6)	7.6 (7.6–10.4)	7.5 (6.7–9.2)	7.4 (6.5–10)	ns
Lactoferrin	0.82 (0.68–1.38)*	0.13 (0.09–0.15)†	0.62 (0.52–0.77)*	0.19 (0.11–0.19)†	0.002
Secretory IgAs	2.3 (2.3–2.8)*	1.7 (1.3–1.9)*,†	1.4 (1.3–1.8)†	1.8 (1.7–2.1)*,†	0.030

P values for significant analysis of variance comparisons are reported in the last column (ns = not significant).
HM = human milk; HoP = Holder pasteurization; HPP = high-pressure processing; HTST = high-temperature-short-time; IgA = immunoglobulin A.
*,† Different classes of homogeneity according to Tukey (secretory IgAs) and Steel-Dwass (lactoferrin) post-hoc test.

Statistical Analysis

TPC, lactoferrin, and secretory IgA contents were reported as median values and interquartile ranges. In order to evaluate difference among untreated HM and the 3 different processing methods, 1-way analysis of variance with Tukey post-hoc comparison was used for TPC and sIgA, and Kruskal-Wallis with Steel-Dwass post-hoc comparison for lactoferrin. A P value <0.05 was considered as statistically significant.

RESULTS

Protein Fraction Analysis

Results of protein fraction analyses are summarized in Table 1. TPC of defatted HM was quantified before and after each processing method. TPC of the 5 analyzed HM samples before processed varied between 5 and 12.1 g/L (median: 7.2 g/L); the total amount of HM proteins was not affected significantly by any processing method.

Lactoferrin content of untreated HM varied between 0.54 and 1.59 g/L (median: 0.82 g/L) and was affected negatively by all the 3 processing methods. A high reduction in HM lactoferrin content was observed following both thermal pasteurization methods, with HoP having the greatest effect (−87.5% after HoP and −83.5% after HTST). The difference between the 2 thermal methods was not significant. On the contrary, HPP was found to preserve HM lactoferrin content. Secretory IgA content of untreated HM varied between 2.1 and 3 g/L (median: 2.3 g/L) and was negatively affected by all processing methods, but to a different extent. In particular, only HPP led to a significant reduction of sIgA content, with an almost double reduction if compared to HTST (0.9 g/L with respect to 0.5 g/L).

In Figure 1, the protein profile of the 5 pooled HM subsamples in nonreducing conditions is reported; for 1 sample, the reducing protein profile is also displayed. For all the HM samples, the reducing conditions were not suitable to visualize any difference in protein band abundance. When nonreducing conditions were used, variations on specific HM protein band abundance were seen. Moreover, these changes were common to all individuals, despite minor differences in the baseline HM profile. Variations seemed more relevant for HoP, followed by HTST and, finally, by HPP.

In Figure 2, 1 individual protein profile was magnified to highlight the bands accounting for visible changes. The identity of the proteins contained in these bands was assessed by comparison with previous publications by our group (19–21): protein aggregates (mainly lactoferrin) were increased by all processing methods,

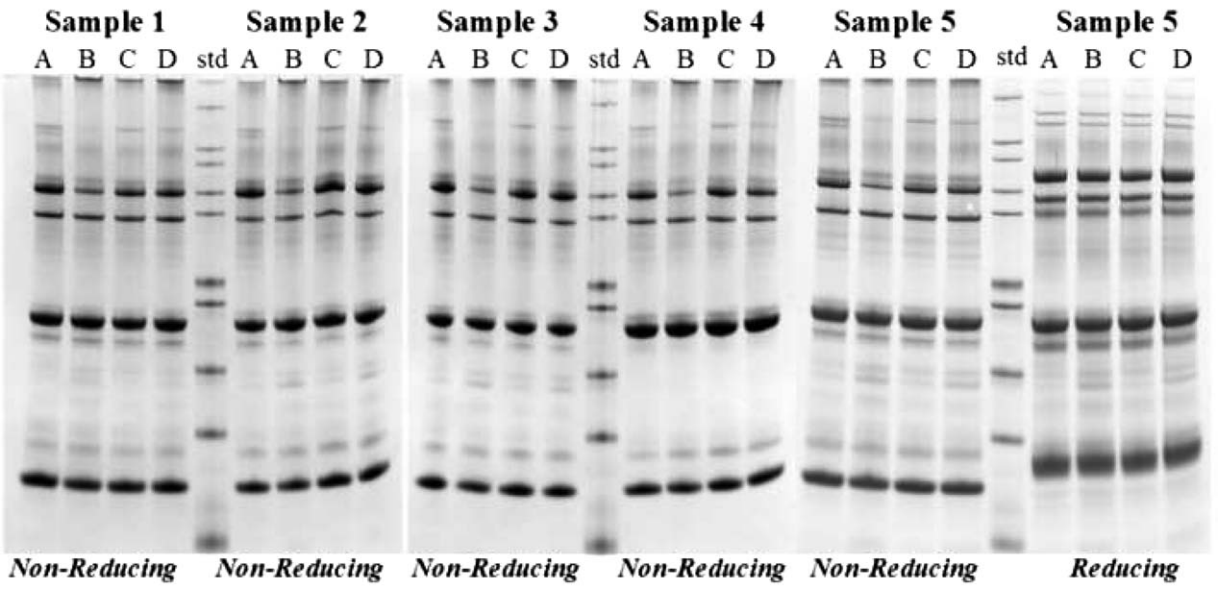

FIGURE 1. NuPAGE total protein profile of human milk (HM) after different pasteurization processes. Colloidal Coomassie brilliant blue stained. Each image (sample) is representative of 1 pooled HM subsample. Std: mass markers Mark12 (Thermo Fisher Scientific). A: Unpasteurized HM; B: holder pasteurized HM; C: high pressure processed HM; D: high-temperature short-time pasteurized HM. Sample 5 protein profile is represented in both absence (nonreducing) and presence (reducing) of dithiothreitol.

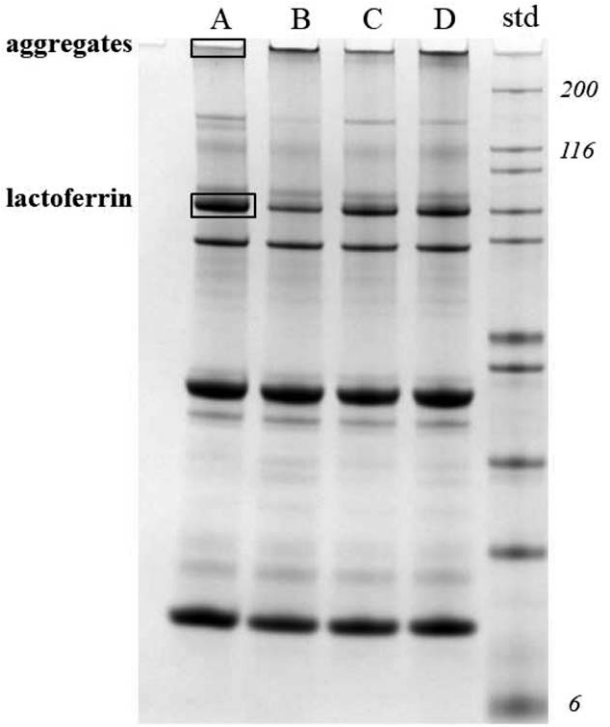

FIGURE 2. NuPAGE total protein profile of human milk (HM) after different pasteurization methods. Colloidal Coomassie brilliant blue stained. Std: mass markers (kDa) Mark12 (Thermo Fisher Scientific). A: unpasteurized HM; B: holder pasteurized HM; C: high pressure processed HM; D: high-temperature short-time pasteurized HM.

although to a different extent (+16% for HPP, >40% for both thermal pasteurization—Supplementary Fig. 1, Supplemental Digital Content, *http://links.lww.com/MPG/B760*). As a consequence, native lactoferrin band was decreased compared to raw HM, but the variation was significant only for HoP (−35%—Supplementary Fig. 1, Supplemental Digital Content, *http://links.lww.com/MPG/B760*).

Microbiological Analyses

Each processing method proved to comply with safety standards required by HMB guidelines. Specifically, total bacterial count in all the raw HM samples was $<10^5$ CFU/mL and Enterobacteriaceae and *S aureus* count were lower than 10^4 CFU/mL. Bacterial count was undetectable in all the processed HM samples, treated by HoP, HTST, or HPP.

DISCUSSION

The availability of DHM provided by HMBs has been recently included among the milestones for enteral nutrition of preterm infants developed in the last century (22). Despite a huge number of research efforts, current treatment of DHM is, however, still unsatisfactory in terms of retention of those bioactive components which are believed to mediate the beneficial clinical effects of HM in preterm infants, such as the reduction of necrotizing enterocolitis and the improvement of long-term neurocognitive outcome (23,24). For this reason, current research is directed toward the identification of novel pasteurization methods which would be capable to preserve HM bioactive components without affecting microbiological safety. To date, a number of articles on the effects of innovative processing techniques for DHM pasteurization have been published and the results of these articles have been summarized in recent reviews (13–16,25). Evidences on the impact of innovative technologies are currently being evaluated by several research groups (18,19,26–30). The assessment of new pasteurization technologies is hampered by the increase in parameters to be monitored, others than time and temperature, especially for HPP, which is being tested in a high variety of pressure settings (26,27). In an effort to elucidate the relative advantages of the most promising innovative technologies over the traditional HoP method, we designed a study to compare the effects of HoP, as performed in HMBs, versus HTST, performed with a patented proprietary device validated for treating HM, and continuous HPP on individual HM samples. Most previous reports on the issue (28–30) were conducted by simulating the processing treatments on small HM volumes or even on single protein fractions, but no report to date used real HMB conditions for processing whole individual HM samples to compare the 3 methods. In the tested conditions, none of the processing methods affected the total amount of proteins, thus confirming previous observations (13–15).

On the contrary, some differences were found in the quantity of specific proteins, as assessed by ELISA and by protein electrophoresis: HTST was found to better preserve the original content of sIgAs, in comparison to HPP, to a higher extent in comparison to the standard processing method. These results are in contrast with previous report (29), which found a double rate of degradation of IgAs after HoP and HTST, as simulated on very low amounts (40 μL) of skimmed HM. It is to mention that one of the reasons for this discrepancy is that, in our study, HTST is performed by a prototyped continuous flow device, rather than in batch. Through this technique, the damage caused by heat is minimized by forming a thin layer of milk flowing in a continuous tube system, requiring very short time to reach the operating temperature, and, equally relevant, minimizing times for cooling down the milk, which is not the case in batch processes. As reported previously (19), the whole heating-pasteurizing-cooling time for the prototype required about 1.5 minutes, instead of typical cycle times of >1.5 hours for batch processes (15). The rate of retention of sIgAs in our study for HoP and HPP was similar to that reported by Permanyer et al (30). We can speculate that some sort of aggregation, or complexing, of sIgAs is induced by pressure, causing a similar reduction to that caused by more extended thermal treatments, such as HoP. Because HoP was found to decrease significantly more that HTST the antiviral activity of HM, which is highly correlated to sIgAs content (26), possible impact of HPP reduction of sIgAs content on antiviral activity should be carefully evaluated in future research.

An opposite trend was found, in the tested conditions, for another important antibacterial factor, lactoferrin. Lactoferrin content was strongly reduced by both thermal pasteurization techniques, as measured by ELISA tests, whereas it was better preserved by HPP. Recently, Wesolowska et al (27) found that HPP at 600 MPa for 10 minutes allowed retaining 55% of the original lactoferrin content. In the current study, HPP at same pressure for 3 minutes was enough to bring microbial growth to undetectable level, thus retaining almost 65% of the original lactoferrin content in raw HM. The tendency of native HM lactoferrin to be denaturated following processing was investigated (28), and the kinetics of HM lactoferrin denaturation as a consequence of HPP reported in that study are in accordance to our present data. Nevertheless, efficacy of HPP for 3 minutes at 600 MPa in guaranteeing microbial safety should be better confirmed in future by inoculation and validation studies (31), such as those performed for the HTST prototype used in the present study (19). Very recently, a validation study was published for raw bovine milk (32), indicating that, in a 400 to

600 MPa range, only 600 MPa pressure for 3 minutes was able to provide a satisfactory reduction (above 4log CFU), for dangerous pathogens such as *Escherichia coli*, *Salmonella* spp, and *Listeria monocytogenes*, comparable to that required by legislation before and after pasteurization of acceptable DHM samples.

When evaluating protein band intensity by protein electrophoresis under reducing conditions, the lactoferrin band intensity was comparable among all the samples, including raw milk. When running the tests in nonreducing conditions, as in other reports (19,20,28), we observed that conformational changes of lactoferrin occurred, driven by the thermal treatments, resulting in an increase of high molecular weight aggregates, causing a decrease of the original lactoferrin band. This phenomenon was significant only in HoP-treated milk. This observation highlights the limits of techniques based on antibody recognition to detect aggregated complexes of lactoferrin, thus limiting the exact quantification of the absolute lactoferrin content in HM treated by thermal methods. The effect of conformational changes of lactoferrin on its bioavailability and bioactivity remains to be assessed.

In conclusion, the results of the present study show that HTST and HPP affect differently some of the bioactive HM components involved in immune response and antibacterial activity. In addition, both methods demonstrate to better preserve the original protein profile of raw milk, compared to standard HoP. Further studies should be aimed at characterizing residual protein bioactivity in HM treated with the 2 different processing methods.

Acknowledgments: The authors would like to thank Michele Morbarigazzi at HPP Italia for performing High Pressure Processing on donor human milk samples and Sara Antoniazzi at the Institute of Sciences of Food Production of the Italian National Research Council for helping in performing protein analyses.

REFERENCES

1. American Academy of Pediatrics. Section on Breastfeeding. Breastfeeding and the use of human milk. *Pediatrics* 2012;129:e827–41.
2. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am* 2013;60:49–74.
3. Committee on Nutrition—Section on Breastfeeding—Committee on Fetus and Newborn. Donor human milk for the high-risk infant: preparation, safety, and usage options in the United States. *Pediatrics* 2017;139:e20163440.
4. Kantorowska A, Wei JC, Cohen RS, et al. Impact of donor milk availability on breast milk use and necrotizing enterocolitis rates. *Pediatrics* 2016;137:e20153123.
5. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *J Pediatr* 2010;156:562.e1–7.e1.
6. Arslanoglu S, Moro GE, Bellù R, et al. Presence of human milk bank is associated with elevated rate of exclusive breastfeeding in VLBW infants. *J Perinat Med* 2013;41:129–31.
7. Agência Nacional de Vigilância Sanitária - Brasil. Banco de Leite Humano: Funcionamento, Prevenção e Controle de Riscos. *http://www.redeblh.fiocruz.br/media/blhanv2008.pdf*. Accessed February 8, 2019.
8. ESPGHAN Committee on NutritionArslanoglu S, Corpeleijn W, Moro G, et al. Donor human milk for preterm infants: current evidence and research directions. *J Pediatr Gastroenterol Nutr* 2013;57:535–42.
9. Hartmann BT, Pang WW, Keil AD, et al. Best practice guidelines for the operation of a donor human milk bank in an Australian NICU. *Early Hum Dev* 2007;83:667–73.
10. Human Milk Banking Association of North America (HMBANA). Guidelines for the establishment and operation of a donor human milk. HMBANA. 4th Edition 2019. *https://www.hmbana.org/our-work/publications.html*. Accessed July 29, 2019.
11. Arslanoglu S, Bertino E, Tonetto P, et al., Italian Association of Human Milk BanksArslanoglu S, Bertino E, et al. Guidelines for the establishment and operation of a donor human milk bank. *J Matern Fetal Neonatal Med* 2010;23:1–20.
12. National Institute for Health and Care Excellence. Donor milk banks: service operation. NICE Guideline 2010. Last update July 2018. *https://www.nice.org.uk/guidance/cg93*. Accessed July 29, 2019.
13. Moro GE, Arslanoglu S. Heat treatment of human milk. *J Pediatr Gastroenterol Nutr* 2012;54:165–6.
14. Peila C, Moro GE, Bertino E, et al. The effect of holder pasteurization on nutrients and biologically-active components in donor human milk: a review. *Nutrients* 2016;8:477.
15. Picaud J-C, Buffin R. Human milk—treatment and quality of banked human milk. *Clin Perinatol* 2017;44:95–119.
16. Peila C, Emmerik NE, Giribaldi M, et al. Human milk processing: a systematic review of innovative techniques to ensure the safety and quality of donor milk. *J Pediatr Gastroenterol Nutr* 2017;64:353–61.
17. Sousa SG, Delgadillo I, Saraiva JA. Human milk composition and preservation: evaluation of high-pressure processing as a nonthermal pasteurization technology. *Crit Rev Food Sci Nutr* 2016;56:1043–60.
18. Escuder-Vieco D, Espinosa-Martos I, Rodríguez JM, et al. High-temperature short-time pasteurization system for donor milk in a human milk bank setting. *Front Microbiol* 2018;9:926.
19. Giribaldi M, Coscia A, Peila C, et al. Pasteurization of human milk by a benchtop high-temperature short-time device. *Innov Food Sci Emerg Technol* 2016;36:228–33.
20. Baro C, Giribaldi M, Arslanoglu S, et al. Effect of two pasteurization methods on the protein content of human milk. *Front Biosci* 2011;3:818–29.
21. Giribaldi M, Ortoffi M, Giuffrida MG, et al. Effect of prolonged refrigeration on the protein and microbial profile of human milk. *Int Dairy J* 2013;31:121–6.
22. Van Goudoever JB. Nutrition for preterm infants: 75 years of history. *Ann Nutr Metab* 2018;72:25–31.
23. Patel AL, Kim JH. Human milk and necrotizing enterocolitis. *Semin Pediatr Surg* 2018;27:34–8.
24. Schneider N, Garcia-Rodenas CL. Early nutritional interventions for brain and cognitive development in preterm infants: a review of the literature. *Nutrients* 2017;9:187–92.
25. Wesolowska A, Sinkiewicz-Darol E, Barbarska O, et al. Innovative techniques of processing human milk to preserve key components. *Nutrients* 2019;11:pii: 1169.
26. Donalisio M, Rittà M, Francese R, et al. High temperature-short time pasteurization has a lower impact on the antiviral properties of human milk than holder pasteurization. *Front Pediatr* 2018;6:1–7.
27. Wesolowska A, Sinkiewicz-Darol E, Barbarska O, et al. New achievements in high-pressure processing to preserve human milk bioactivity. *Front Pediatr* 2018;6:323.
28. Mayayo C, Montserrat M, Ramos SJ, et al. Kinetic parameters for high-pressure-induced denaturation of lactoferrin in human milk. *Int Dairy J* 2014;34:246–52.
29. Mayayo C, Montserrat M, Ramos S, et al. Effect of high pressure and heat treatments on IgA immunoreactivity and lysozyme activity in human milk. *Eur Food Res Technol* 2016;242:891–8.
30. Permanyer M, Castellote C, Ramírez-Santana C, et al. Maintenance of breast milk Immunoglobulin A after high-pressure processing. *J Dairy Sci* 2010;93:877–83.
31. Moro GE, Billeaud C, Rachel B, et al. Processing of donor human milk: update and recommendations from the European Milk Bank Association (EMBA). *Front Pediatr* 2019;7:49.
32. Stratakos AC, Inguglia ES, Linton M, et al. Effect of high pressure processing on the safety, shelf life and quality of raw milk. *Innov Food Sci Emerg Technol* 2019;52:325–33.