CE: ; JPGN-19-452; Total nos of Pages: 5;

JPGN-19-452

SHORT COMMUNICATION

Effect of Nonthermal Processing on Human Milk Bactericidal Activity Against *Escherichia coli*

AQ1 [*†]Olga Barbarska, [*]Kamila Strom, [*]Gabriela Oledzka, [‡§]Calvo Javier, [‡§]Antoni Gaya, [||]María Carmen López-Mendoza, [¶]Malgorzata Rutkowska, [#]Elzbieta Rosiak, and [†]Aleksandra Maria Wesolowska

ABSTRACT

Nonthermal methods are more efficient at preserving various biological properties of human milk, as compared with holder pasteurization (HoP), which is the most common preservation method. This study was performed to assess the effects of nonthermal processing on bactericidal activity against *Escherichia coli* in human milk. Milk samples obtained from the Regional Human Milk Bank in Warsaw at Holy Family Hospital were processed by HoP, irradiated with ultraviolet-C (UV-C) for 5, 10, and 15 minutes (6720 J/L each minute), subjected to 2 variations of high-pressure processing (HPP): 450 MPa for 15 minutes and 200 MPa for 10 minutes + 400 MPa for 10 min, with a 10-minutes break. The samples were then evaluated by a bactericidal assay (raw untreated human milk was used as a control). The bactericidal capacity after HoP was preserved in 12.1% of samples, showing a significant reduction in bactericidal properties compared with in raw milk ($P < 0.05$). The differences between samples preserved by nonthermal methods and raw milk were not significant ($P > 0.05$). Nonthermal methods of human milk treatment better preserve the bactericidal capacity compared with holder pasteurisation. Those alternative technologies to HoP can be proposed after further investigation for milk processing for Human Milk Banks facilities.

Key Words: high-pressure processing, holder pasteurization, human milk bank, pascalization, ultraviolet-C irradiation

(*JPGN* 2020;70: 00–00)

What Is Known

- Holder pasteurization causes significant losses of some milk components, such as immunoglobulins, hormones, enzymes, and cytokines.
- High-pressure processing and ultraviolet-C treatment reduce microbial contamination while preserving many bioactive components.

What Is New

- Nonthermal methods, such as high-pressure processing and ultraviolet-C irradiation, preserve human milk bactericidal activity against *Escherichia coli* better than holder pasteurization.
- Ultraviolet-C treatment retains bactericidal properties against *Escherichia coli* in human milk at a dose of 6720 J/L.

Holder pasteurization (HoP), also known as the low-temperature long-time pasteurization, is commonly used in human milk banks. During this process, milk is incubated in a water bath at 62.5 °C for 30 minutes and then cooled rapidly to a temperature below 4 °C. Holder pasteurization ensures microbiological safety; however, heating of human milk using this method causes great losses in some bioactive components, such as immunoglobulins, hormones, enzymes, and cytokines (1,2). Thus, alternative methods are needed to preserve human milk. Promising results have been obtained using nonthermal methods, such as ultraviolet-C (UV-C) radiation or high-pressure processing (HPP, pascalization). Both methods effectively reduce microbial contamination by at least 5-$\log_{10}$ (2–4) while preserving higher levels of many bioactive components compared with holder pasteurization (5–8).

Various components, such as immunoglobulins, lactoferrin, and lysozyme present in raw untreated human milk synergistically function to exert bactericidal properties (9). The various preservation methods available have different effects on the contents of bioactive components compared in milk (10).

Although nonthermal preservation methods and HoP show similar effectiveness in destroying pathogens, safety issues after pasteurization must be considered. HoP alters the bactericidal properties of milk, and some human milk banks have reported cases of pasteurized milk contamination despite the absence of contamination before processing (11). Picaud and Buffin (12) found that it is necessary to discard pasteurized milk containing any level of pathogens, as these pathogens can greatly multiply during storage.

Received June 13, 2019; accepted February 6, 2020.

From the *Department of Medical Biology, the †Laboratory of Human Milk and Lactation Research at Regional Human Milk Bank in Holy Family Hospital, Faculty of Health Sciences, Department of Medical Biology, Medical University of Warsaw, Warsaw, Poland, the ‡Banc de Llet Materna, Fundació Banc de Sang i Teixits de les Illes Balears, the §Terapia Celular e Ingeniería Tisular, Institut d'Investigació Sanitària Illes Balears, Palma de Mallorca, the ||Department of Food Science and Technology, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain, the ¶High Pressure Physics, Polish Academy of Science, and the #Warsaw University of Life Sciences - SGGW, Institute of AQ2 Human Nutrition Sciences, Warsaw, Poland.

Address correspondence and reprint requests to Aleksandra Maria AQ3 Wesolowska, PhD, Litewska 14/16 00-575, Warsaw, Poland (e-mail: aleksandra.wesolowska@wum.edu.pl).

Funding source: Sandander Universitates project - development of innovative scientific research in cooperation with Banc de Llet Materna, Fundació Banc de Sang i Teixits de les Illes Balears, Palma de Mallorca, Spain.

The authors report no conflicts of interest.

DOI: 10.1097/MPG.0000000000002673

CE: ; JPGN-19-452; Total nos of Pages: 5;

JPGN-19-452

This study was conducted to assess the effect of nonthermal processing on human milk bactericidal activity against *Escherichia coli.*

METHODS

Samples of approximately 130 mL of human milk obtained from Regional Human Milk Bank in Warsaw at Holy Family Hospital were collected from 6 women. The average nutritional values, expressed in g/100 mL, were as follows: fat 3.71 ± 0.66; total protein 1.03 ± 0.16; crude protein 0.80 ± 0.13; carbohydrates 7.25 ± 0.20; and total solids 12.18 ± 0.73. The average energy value was 67.83 ± 6.40 kcal/100 mL. The Bioethics Committee of Warsaw Medical University approved the protocol for this noninterventional study (admission number AKBE/59/15). Milk was collected at the women's homes using an electric pump or through manual methods. Samples were collected from several milk expression sessions. The samples were delivered to the human milk bank (HMB) within 24 h of collection. Before transport, the samples were stored in a domestic refrigerator and then transported to the HMB in a cooling bag.

Each sample was divided into 5 aliquots: raw milk; HoP; HPP 450 MPa (HPP 450); HPP 200 + 400 MPa (HPP 200 + 400); and UV-C treatment. The UV-C aliquot was exposed to irradiation for 15 minutes, with samples collected every 5 minutes. The maximum time between sample extraction and the microbiological and bactericidal assay was 48 hours. During this time, the samples were subjected to HoP, HPP, and UV-C treatment. Before and after the processes, the samples were stored at 4 °C.

A 50-mL sample was treated by HoP with an automatic Human Milk Pasteurizer S90 Eco (Sterifeed, Medicare Colgate, Ltd., Devon, England) under the recommended conditions of 62.5°C for 30 minutes, followed by rapid cooling to a temperature below 4 °C.

HPP of 10-mL samples was performed in 2 manners: 450 MPa for 15 minutes; 200 MPa for 10 minutes, 10-minutes break, 400 MPa for 10 minutes. The samples were exposed to high-pressure treatment at the Institute of High Pressure Physics, Polish Academy of Sciences, using a U 4000/65 apparatus (designed by Unipress Equipment, Institute of High Pressure Physics, Poland). The maximum pressure achievable by the apparatus is 600 MPa; the treatment chamber volume is 0.95 L. A mixture of distilled water and polypropylene glycol (1 : 1) was used as the pressure-transmitting fluid. The working temperature of the apparatus ranged from −10 °C to +80 °C. A pressure of up to 600 MPa was generated in 15–25 seconds, and the release time was 1 to 4 seconds.

Milk samples were treated by UV-C as described previously (2). First, 50 mL of milk was deposited into a graduated cylinder, and then a germicidal UV-C lamp of 26 cm in length with 8 W of output power (LIT-06; Instrumentación Científico Técnica S.L., La Rioja, Spain) was introduced vertically. Because of the size of the glass cylinder, only 70% of the lamp was immersed in the milk (18.2 cm). The rest of the lamp was covered with aluminium foil. During UV-C irradiation, the milk was agitated with a magnetic stirrer to create a low-velocity vortex flow. The milk was treated for 15 minutes, and 5-mL samples were collected every 5 minutes. The milk samples were treated with a radiation dose of 6720 J/L every minute ($60\ s \times 8\ W \times 0.7$ (70% immersion of the lamp)/$0.05\ L = 6720$ J/L). The dosage remained the same during all processes, independently of the volume reduction because of the concurrent reduction in the lamp surface in contact with the milk.

Raw milk samples were subjected to microbiological analysis using the traditional plate method according to ISO standards (TVC, norm: PN-EN ISO 4833:2004–1:2013–12) and *E. coli* (norm: PN-ISO 16649–2-:2004). Samples were seeded directly or diluted depending on the expected level of contamination. All analyses were performed in duplicate into 2 parallel Petri dishes.

E coli NCTC 9111 (serotype O111: K58 (B4): H-) was obtained from Coleccion Española de Cultivos Tipo (CECT, Burjassot, Valencia). The bactericidal assay was performed as described previously (12). Bacteria were cultured on nutrient agar overnight, suspended in peptone water, and adjusted to an absorbance to 3×10^8 colony-forming units/mL. From this dilution, 0.2 mL was used to inoculate 0.8 mL of the milk samples. All samples were cultured at 37 °C for 2 h. Control samples were prepared by mixing 0.2 mL of bacteria solution with 0.8 mL of brain heart infusion broth (Biocorp, Warsaw, Poland).

The degree of bacteriolysis was calculated as the difference between *E coli* counts in the control and milk samples, expressed as a percentage of the control sample counts.

$$\%\,\text{reduction} = \frac{\text{N0} - \text{Nf}}{\text{N0}} \times 100$$

Where N0 is control and Nf is the study sample.

The results of the bactericidal assay are presented as percentages compared with the control sample counts (percentage reduction in *E coli* growth). The statistical significance of the differences between raw milk and samples subjected to each treatment method was assessed using the Mann-Whitney *U* test; when $P < 0.05$, the difference was considered as significant. Data were analysed using STATISTICA version 13.1 software (Stat Soft, Inc., Tulsa, OK).

RESULTS

The total viable count (TVC) was determined in all raw milk samples (4.21 ± 0.72 $\log_{10}$ colony-forming units/mL). No *E coli* was detected in any of the samples.

The bactericidal capacity against *E coli* was 46.6% in raw milk. In samples treated by HPP and UV-C, *E coli* growth was reduced by 29.6% to 50.3% and 57.6% to 62.6%, respectively. The differences between these samples and raw milk were not significant ($P > 0.05$). The bactericidal activity against *E coli* of samples after HoP was 12.1%, revealing a significant reduction in bactericidal properties compared with those in raw milk samples ($P < 0.05$) (Table 1).

TABLE 1. Effect of nonthermal processing on human milk bactericidal activity against *Escherichia coli*

	Reduction of *E coli* growth (%) Mean ± SD*	Mann-Whitney *U* test in relation to fresh milk	
		Z	*P*
Raw milk	46.60 ± 42.14	–	–
Holder pasteurization	12.12 ± 19.07	2.985	0.003
HPP 450 MPa	29.60 ± 52.35	−0.965	0.335
HPP 200 + 400 MPa	50.34 ± 39.62	−0.127	0.899
UV-C, 5 min	57.55 ± 37.02	−0.847	0.397
UV-C, 10 min	62.57 ± 40.48	−1.270	0.204
UV-C, 15 min	57.59 ± 41.57	−0.921	0.357

E coli = Escherichia coli.
*Difference between *E coli* counts in control (brain heart infusion broth) and n milk samples, expressed as a percentage compared with control sample counts.

DISCUSSION

The bacteriostatic effect of human milk depends on many factors, including bioactive components with bactericidal properties, such as lysozyme, lactoferrin, and lactoperoxidase. HoP, a low-temperature long-time treatment, is the most commonly used thermal method for eliminating microorganisms from human milk (62.5 °C for 30 min). This approach significantly reduces lactoperoxidase (50%–88%), lactoferrin (39%–100%), and lysozyme (33%–76%) (10).

Only 1 study has directly assessed the bactericidal properties of human milk after pasteurization (Silvestre et al). It was observed 52.27% reduced growth of *E coli* in the milk after HoP and 36.69% after high-temperature short-time treatment (75 °C for 15 s) compared with untreated milk, which showed a value of 70.10% (13). Growth of *E coli* is among the many factors decreased by thermal pasteurization of human milk. Alternative techniques to improve the pasteurization process are currently on demand. One of the most promising and extensively studied are nonthermal techniques.

The effectiveness of HPP was demonstrated previously by Viasis et al who demonstrated the antimicrobiological efficacy of 400 MPa HPP, which reduced the levels of *Staphylococcus aureus*, *Streptococcus agalactiae*, *Listeria monocytogenes*, and *E. coli* by at least 6-log10 within 2 to 30 minutes (4). Furthermore, 30-minutes treatment with 400 MPa resulted in no significant differences in lysozyme activity, and 85.6% of IgA was retained. In contrast, retention after HoP was 60.5% for lysozyme and 51.2% for IgA (14). High-pressure values more than 600 MPa have negative effects, such as inducing modifications in bioactive components or altering the organoleptic properties of the product. Therefore, to ensure high microbial safety and inactivation of spores, such as those of *Bacillus cereus* and the vegetative form of bacteria, repeated pressure pulses applied by Meyer (15) may be the best solution.

Recently, Wesolowska et al performed HPP and found that 200 MPa, 10 minutes; interval 10 minutes; 400 MPa were the most optimal conditions for preserving the bioactive properties of human milk. No significant differences were observed between the contents of lactoferrin, insulin, and human growth factor in samples treated with this method and raw milk. A slight reduction in IgG of 17.76% (reduction for HoP 49.04) and leptin of 86.12% were observed (8).

Despite the growing number of reports demonstrating the effectiveness of treating human milk by HPP, no studies have examined the bactericidal capacity of this method. In this study, both methods, 200 MPa for 10 minutes at intervals of 10 minutes followed by 400 MPa and 450 MPa for 15 minutes, did not negatively affect bactericidal activity against *E coli* in human milk.

UV-C irradiation is also a promising method for treating milk but the appropriate conditions of preservation and approaches for precisely calculating the dosage have not been determined. Additionally, previous studies used various methods, making it difficult to compare the results (3,6,16).

Christen et al demonstrated the relationship between the total solids content in human milk and dose of UV-C irradiation required to reduce microbial contamination. To obtain a 5-log10 reduction in bactericidal contamination in milk with a total solids content of 107 g/L, treatment at 289 ± 17 J/L was necessary; for milk with a total solids content of 146 g/L, the required dose was as high as 945 ± 164 J/L (3). A dose of 4863 J/L caused a 5-log10 bacterial reduction in all samples. Additionally, this dosage did not significantly alter the bile salt-stimulated activity or alkaline phosphatase activity (3). In the second study, greater retention of sIgA, lactoferrin, and lysozyme was observed compared with those obtained using the holder method (89 ± 4% vs 49 ± 3%, 87 ± 11% vs 9 ± 4%, 75 ± 9% vs 41 ± 14%, respectively; raw milk as control) (6).

Another study investigating UV-C irradiation as a new method of human milk preservation involved illuminating the milk placed on a metal sample plate for different time periods and from different distances. The irradiation dose was measured using a Gigahertz-Optik xX911 meter (Türkenfeld, Germany) with a UV-3718 detector. The study showed that a dose of 64 mJ/cm^2 UV-C was effective for inactivating cytomegalovirus (16).

Despite the higher treatment dosage, the bactericidal activity against *E coli* obtained in our study was the same as that reported by Christen et al, which showed that bacteria incubated in holder-pasteurized human milk grew significantly faster than those in both untreated and UV-C-irradiated human milk. No significant difference was observed between untreated and UV-C-irradiated samples (6).

In our study, no significant differences were found between raw milk and samples treated by HPP and UV-C. The standard deviations were, however, high (SD = 37.02–52.35), which may be because the devices are not standardized compared with the automatic pasteurizer. The results for HoP were more consistent (SD = 19.07), and only these samples showed significant differences compared with raw milk (Table 1).

In conclusion, nonthermal methods of human milk preservation, such as HPP and UV treatment may better preserve bactericidal activity against *E coli* compared with the HoP method. Other reports have indicated that samples treated by these methods retain significant levels of components important for the bactericidal properties of human milk, such as immunoglobulins and lysozyme.

There were some limitations to this study. The work presents the preliminary results obtained using samples from only 6 women. Additionally, we only detected *E coli*. Further studies are needed to evaluate the bactericidal activity against other microorganisms and contents of bioactive factors responsible for bacteriostatic properties in individual samples. In UV-C, the dose was limited by the equipment used. Although our study showed that under selected conditions, UV-C irradiation does not affect the bactericidal capacity, it is necessary to develop a reproducible treatment method and to determine the optimal irradiation dosage that ensures microbiological purity while maintaining bioactive properties. The significant resistance of bactericidal properties to UV-C observed in this study should be validated in further studies.

REFERENCES

1. 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:pii: E477.
2. Moro GE, Billeaud C, Buffin R, et al. Processing of donor human milk: update and recommendations from the European Milk Bank Association (EMBA). *Front Pediatr* 2019;7:49.
3. Christen L, Lai CT, Hartmann B, et al. Ultraviolet-C irradiation: a novel pasteurization method for donor human milk. *PLoS One* 2013;8:e68120.
4. Viazis S, Farkas B, Jaykus L. Inactivation of bacterial pathogens in human milk by high-pressure processing. *J Food Prot* 2008;71:109–18.
5. Li Y, Nguyen DN, de Waard M, et al. Pasteurization procedures for donor human milk affect body growth, intestinal structure, and resistance against bacterial infections in preterm pigs-3. *J Nutr* 2017;147:1121–30.
6. Christen L, Lai CT, Hartmann B, et al. The effect of UV-C pasteurization on bacteriostatic properties and immunological proteins of donor human milk. *PLoS One* 2013;8:e85867.
7. 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.
8. Wesolowska AM, Sinkiewicz-Darol E, Barbarska O, et al. New achievements in high-pressure processing to preserve human milk bioactivity. *Front Pediatr* 2018;6:323.

9. Hamosh M. Protective function of proteins and lipids in human milk. *Neonatology* 1998;74:163–76.
10. Wesolowska AM, Sinkiewicz-Darol E, Barbarska O, et al. Innovative techniques of processing human milk to preserve key components. *Nutrients* 2019;11:pii: E1169.
11. Dewitte C, Courdent P, Charlet C, et al. Contamination of human milk with aerobic flora: Evaluation of losses for a human milk bank. *Arch Pediatr* 2015;22:461–7.
12. Picaud JC, Buffin R. Human milk-treatment and quality of banked human milk. *Clin Perinatol* 2017;44:95–119.
13. Silvestre D, Ruiz P, Martinez-Costa C, et al. Effect of pasteurization on the bactericidal capacity of human milk. *J Hum Lact* 2008;24:371–6.
14. Viazis S, Farkas BE, Allen JC. Effects of high-pressure processing on immunoglobulin A and lysozyme activity in human milk. *J Hum Lact* 2007;23:253–61.
15. Meyer RS. High pressure sterilization of foods. *Food Technol* 2000;54:67–72.
16. Lloyd ML, Hod N, Jayaraman J, et al. Inactivation of cytomegalovirus in breast milk using ultraviolet-C irradiation: opportunities for a new treatment option in breast milk banking. *PloS One* 2016;11:e0161116.