



Can high hydrostatic pressure processing be the best way to preserve human milk?



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ABSTRACT

Background: Breastfeeding is one of the most important factors influencing proper child development. When a mother cannot breastfeed, the best alternative, especially for feeding premature infants, is to then use of **human milk (HM)** which has been collected, preserved and stored in **Human Milk Banks (HMB)**.

Scope and approach: In this review, the impact of some stages of the management of HM in HMB on its final biological value and microbiological safety are described. Negative changes in HM components begin during the first stage of frozen storage. In the next stages, further losses occur, which largely depend on the applied method of **microbiota** elimination. Complete inactivation of milk microbiota can be achieved after **pressurization** in the range 500–600 MPa, but similarly for **holder pasteurization (HoP)**, in these conditions unfavorable changes in the milk components take place. At lower pressures, the changes are smaller, but microbiological safety of HM is not achieved.

Key findings and conclusions: Replacing HoP with pressurization does not seem to be justified so far, not only because of the small differences in the retention of valuable HM ingredients, but also because of the high costs associated with the inclusion of the device for generating pressure in the HMB environment. A new solution may be the use of high-pressure milk storage at subzero temperatures, but this hypothesis must be verified.

1. Introduction

The World Health Organization (WHO) recommends breastfeeding exclusively for the first six months and, if possible, continuing until the second year of the child life (Gartner et al., 2005). This recommendation results from the unusual properties of human milk (HM). The composition of HM is perfectly adapted in the quality and quantity of compounds needed for the development of children at every stage of their growth (Ballard & Morrow, 2013). It contains many bioactive substances and immune factors that modulate the immune response and affect the proper growth of intestinal microbiota. Breastfeeding is the best way to feed infants as it is one of the main factors responsible for the proper development of children and maintaining their good health into adulthood. In addition, in the case of premature babies, breastfeeding enables the proper maturation of underdeveloped digestive and immune systems and significantly reduces the risk of certain diseases, such as necrotizing enterocolitis or retinopathy of prematurity, etc. (Meinzen-Derr et al., 2009; Zhou, Shukla, John, & Chen, 2015).

When a mother cannot breastfeed, the best alternative, especially for feeding premature infants, is to then use HM which has been collected in Human Milk Banks (HMB) (WHO, 2011). The procedure for handling with milk in HMB includes the following stages: frozen

storage, pasteurization and re-freezing. During the first stage of frozen storage, inactivation of immune cells such as leukocytes as well as other unfavorable changes, takes place. Although the microbiota of HM is an important component positively influencing the development of the infant, some risk of transferring dangerous diseases as a result of secondary contamination. Therefore, in most HMB, the milk is preserving by heating at 62.5 °C for 30 min (Low Temperature Long Time pasteurization – LTLT pasteurization) (Weaver et al., 2019). Such treatment leads to a significant reduction in the nutritional and biological value of the HM (Picaud & Buffin, 2017). For this reason, new methods for preserving HM are searched for, which will ensure microbiological safety while maintaining the properties of the milk as much as possible.

Such multi-stage milk handling requires great hygienic care so that no secondary microbial contamination occurs at any stage, especially by the spore-forming *Bacillus cereus* that can survive the preservation processes. This work presents a critical review of the literature on the effects of the particular stages of milk management in HMB on its final quality with a special emphasis on the advantages and disadvantages of the high hydrostatic pressure (HHP) technique as a method of preserving HM. HHP has been successfully used in the food industry not only as a non-thermal method of food preservation, but also as a method for creating food products with new functional and sensory properties.

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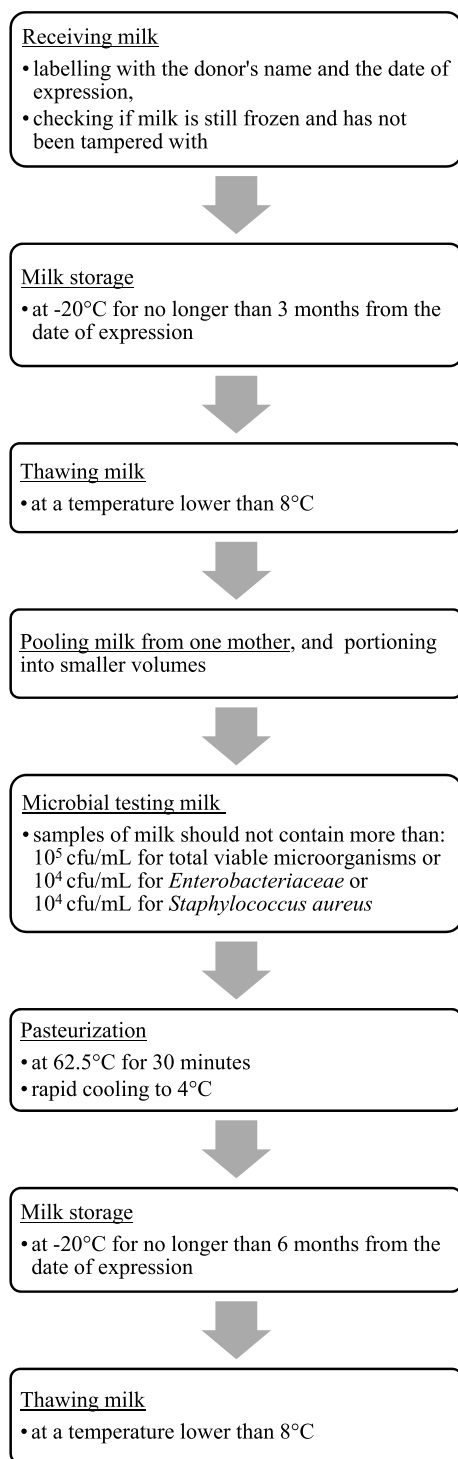


Fig. 1. The procedure applied for handling with milk in the HMB.

Interesting possibilities for preserving not only food, but also biological material are offered by the application of high pressures at subzero temperatures. The effect of high pressure on water phase transitions can be used not only to inactivate microorganisms, but also to quickly freeze material (pressure-assisted-freezing), for rapid thawing (pressure-assisted-thawing) or to keep the material unfrozen at temperatures down to -22°C . Under pressure conditions that do not damage cellular structures below 30 MPa, the use of this technique is not excluded for storing biological material, even including unaltered organs ((Kalichevsky, Knorr, & Lillford, 1995) (Takahashi et al., 2001)).

1.1. Procedures for handling milk in HMB

The stages of handling milk in HMB after being taken from a donor are shown in Fig. 1. Mothers, who have passed the verification process and been accepted as a donor, deliver milk to the bank in chilled or frozen form. Each portion of milk is precisely marked with the first name and surname of the mother and checked for damage to the packaging. If the sample is delivered in a frozen form, it is also controlled whether it has been partially defrosted. The milk is then placed in the freezer at -20°C and it can stay there for up to three months from the date of expression. During these three months, when the need arises, milk is thawed at a temperature lower than 8°C , portions from one mother are pooled and the microbiological purity of the milk is checked. If the milk satisfies the microbiological criteria, it undergoes LTLT pasteurization (so-called holder pasteurization – HoP) followed by rapid cooling down to 4°C . From the pasteurized batch of material, samples are taken to determine the effectiveness of the pasteurization, and the remaining milk is frozen again and stored at -20°C for up to a maximum of six months from the date of milk expression. When it is a needed, the post-pasteurization process milk is thawed at a temperature lower than 8°C and delivered to the neonatology department. Such regular routine handling methods ensure to a large extent, microbiological safety, but can induce changes in the valuable nutrients and biological active substances contained in HM. Additionally, sometimes there are problems with the contamination of the milk by spore-forming pathogenic *Bacillus cereus*, which may survive all the treatments that the milk is subjected to and threaten the beneficiaries of HMB (Lewin, Delage, Bernier, & Germain, 2019). Milk from milk banks is one of the first sources to be suspected in cases of *B. cereus* infection in premature infants fed pasteurized breast milk, as is frequently reported and borne out (Rigourd et al., 2018).

It is believed that during the whole milk handling cycle, the pasteurization process is of key importance for the quality of this milk. In many works, it has also been shown that the storage of raw milk has already caused the first changes in its temperature-sensitive ingredients (Hanna et al., 2004; Marinković et al., 2015). Most of the studies have focused on the bacteriological, nutritional and immunological effects of storage.

1.2. The effect of frozen storage on the ingredients of raw HM

Some authors report a minimal effect on HM by freezing and found no significant changes in the macronutrients – protein and lactose – and biologically important components: some vitamins, lactoferrin, or IgA and IgG after freezing for three months. However, a decrease in antioxidant activity with frozen storage at the currently recommended temperature was also observed. Many milk components change with storage, including immune cells, which are completely damaged by the ice that forms during freezing. Storage also reduces the activity of the antioxidant enzymes, lysozyme and protease (Table 1). Otherwise, it is difficult to separate the effects of freezing from thawing. The latter process is associated with lipolysis, emulsification and protein denaturation in milk. Freezing has been noted to break the emulsion between milk fat globules and the aqueous fraction (Keenan & Patton, 1995). Recently, Orbach, Mandel, Mangel, Marom, and Lubetzky (2019) demonstrated that storage of milk up to 24 weeks at -80°C caused less loss of fat and energy than storage at -20°C .

1.3. Effect of pasteurization methods on some components of HM

The most common method for the inactivation of milk microbiota is heating at 62.5°C for 30 min. The effect of HoP on milk's components has been widely described in the literature. This method leads to the loss of some biologically active components and the cells that are crucial for the defense against infections, such as immunoglobulins, leukocytes, lymphocytes, secretory immunoglobulin A (sIgA),



Table 1
Effect of frozen storage on raw HM quality.

Milk component	Time of storage at –20 °C	Impact	References
Macronutrients:			
Protein:	3 month	NC	Friend et al., 1983
Lactose	3 month	NC	Friend et al., 1983
Fat	3 month	↓	Friend et al., 1983
	6 month	↓	Orbach et al. (2019)
Vitamins			
Biotin	3 month	NC	Friend et al., 1983
Niacin	3 month	NC	Friend et al., 1983
Panthenic acid	3 month	NC	Friend et al., 1983
Ascorbic acid	3 month	↓	
a-Tocopherol	4 month	NC	Moffat et al., 1987
Enzymes			
Lipase	3 month	↑	Friend et al., 1983
Lysozyme	3 month	↓	Friend et al., 1983
Protease	3 month	↓	Friend et al., 1983
Immunological factors			
IgA	3 month	NC	Evans et al. (1978)
IgG	3 month	NC	Evans et al. (1978)
Lactoferrin	3 month	NC	Evans et al. (1978)
α-Antytrypsin	3 month	↓	Evans et al. (1978)
Cells	3 month	↓	
Antioxidant activity	1 week	↓	Hanna et al. (2004)
Activity superoxide dismutase (SOD)	1 moth	↓	Marincović et al., 2015
Glutathione peroxidase (GPx)	1 month	↓	Marincović et al., 2015
Glutathione reductase	1 month	NC	Marincović et al., 2015
Microbiota	3 month	NC	own results (unpublished data)

NC - no change, ↓- reduction in content or activity, ↑ - increase in content or activity.

Table 2
Effect of HoP and pressurization on HM properties and its microbial quality.

Component of milk	Relative content/activity [%] after			
	Holder pasteurization	Pressurization at		
		200–400 MPa	500–600 MPa	193 MPa, –20 °C
SOD	97	–	–	157
CAT	43	–	–	81
GPx	52	–	–	79
Leukocytes	4	17	6	–
sIgA	50	–	–	65
IgA	36–50	90	50	–
Lactoferrin	53	81	50	86
Lysozyme	84	100	100	100
Vitamin C	80	99	96	93
Antioxidant activity	93	–	–	100
Tocopheroles	99	94	97	–
Aldehydes	300	180	1580	–
Ketones	197	195	375	–
Aliphatic hydrocarbons	135	193	158	–
Microbiota	Not detected in 1 mL	Reducing about 2 log cycles	Not detected in 1 mL	Reducing about 2 log cycles

immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), lactoferrin, lysozyme and cytokines (Picaud & Buffin, 2017). Therefore, new methods for preserving HM are searched for, which will ensure microbiological safety, but maintain the properties of milk as much as possible. The high pressure technique is, in this context, the most promising method. Recently, many works have appeared on this topic. The following section presents a critical review of the literature

regarding the effect of HoP and HHP on selected components of human milk. These data have been also summarized in Table 2.

1.4. Secretory immunoglobulin A

sIgA binds to the mucosal epithelium and inhibits bacterial or viral adherence to mucosal cells and the penetration of allergens. The sIgA content in colostrum (milk secreted up to five days postpartum) amounts to 7 g/L and constitutes more than 80–90% of all antibody content. The other immunoglobulins: IgG – protects against bacterial and viral infections, and IgM – acts as an anti-infective agent of humoral immunity. Their contents in colostrum amount to 0.1 and 2.5 g/L, respectively. The concentration of immunoglobulins significantly declines with time of milk maturity and reaches a plateau in mature milk (four to six weeks postpartum). However, they are still an important immunological factor (Palmeira & Carneiro-Sampaio, 2016). HHP, similarly to HoP, affects the composition of milk, especially the immune components. In these conditions, the leukocyte, IgA, IgM and IgG content decreases drastically in milk (Contador, Delgado-Adámez, Delgado, Cava, & Ramírez, 2013; Sousa, Delgadillo, & Saraiva, 2014). The changes described by different research groups regarding the quantity of sIgA in HM after HoP are variable. According to Lima, Wagner-Gillespie, Perrin, and Fogleman (2017), the amount of sIgA in HM after the HoP lowered only slightly but Chang et al. (2013) and Czank, Prime, Hartmann, Simmer, and Hartmann (2009) showed that the concentration of this protein decreased by about 26 and 30%, respectively. An even greater loss of IgA content, by 40%, after HoP, was reported by Contador et al. (2013) and 64% by Braga and Palhares (2007). The concentration of sIgA after pressurization at 193 MPa at –20 °C decreased by 35% (Malinowska-Pańczyk, Martysiak-Żurowska, Puta, & Kuznierewicz, 2017). The data on the impact of HHP on sIgA are slight, but some works describe the effect of HHP on IgA in HM. According to Contador et al. (2013), pressurization at an ambient temperature led to a decrease in the IgA content by 10% at 400 MPa and at a higher pressure, 600 MPa, by 50%. Using an elevated temperature in the pressure process caused a drastic reduction in content of this immunoglobulin. At a pressure of 300 MPa at 50 °C, the level of IgA is already lowered to 48% of the initial concentration. Increasing the pressure and temperature of the process caused even greater losses. At a pressure of 600 MPa at 80 °C, the IgA concentration in HM was less than 1 mg/L (from the initial quantity 1–1.6 g/L in mature milk) (Delgado et al., 2013). On the other hand, treatment of colostrum at 200 and 400 MPa at 8 °C allowed for the complete preservation of IgA, but a pressure of 600 MPa at the same temperature resulted in a 20% decrease in the content of this immunoglobulin (Sousa et al., 2014).

1.5. Lactoferrin (LF)

LF is a glycoprotein that has many important functions in the body, including affecting the proper course of the bone mineralization process (Kowalska, Gruczyńska, & Bryś, 2015). It induces growth of the bacteria responsible for the proper development of the gastrointestinal flora, e.g. *Bifidobacterium* sp. LF is characterized by the broadest spectrum of antimicrobial activity among all proteins in milk and plays a key role in the immune response of the newborn – it stimulates the body to produce the immunoglobulins involved in the defense against the penetration of pathogens and the development of infection. For this purpose, it also activates T and B lymphocytes (Lis, Orczyk-Pawilowicz, & Kątnik-Prastowska, 2013). The LF content in raw milk amounted to about 1.9 g/L (Malinowska-Pańczyk et al., 2017; Nagasawa, Kiyosawa, Kuwahara, Fukuwatari, & Suzuki, 1972). HoP caused drastic, around 60%, LF losses in HM (Chang et al., 2013). The use of high pressure at subzero temperature caused a significantly smaller loss of LF. After pressurization at 193 MPa, about 86% LF remained in tested milk (Malinowska-Pańczyk et al., 2017). Similarly, Mayayo et al. (2014) showed that a pressure of 350 MPa at 20 °C only slightly contributed to

the denaturation of LF. The increase in pressure enhanced the level of denaturation of this protein. Pressurization at 500 and 600 MPa for 10 min caused a reduction of lactoferrin by 30 and 45%, respectively (Mayayo et al., 2014).

1.6. Lysozyme

In our work, we estimated that the activity of lysozyme in mature HM is about 28,000 U/mL (Malinowska-Pańczyk et al., 2017). Sousa et al. (2014) showed that the activity of lysozyme in colostrum was about 18,000 U/mL, whereas Lima et al. (2017) found that the lysozyme activity in raw HM was 8000 U/mL. Lysozyme is an enzyme which is stable in an acidic environment. A neutral pH increases its susceptibility to inactivation at elevated temperatures, hence it should be inactivated during heating of HM. Unfortunately, the data regarding changes in the content/activity of lysozyme after HoP are ambiguous. Many authors have shown that after heating at 62.5 °C for 30 min the amount of lysozyme decreases drastically. Koenig, de Albuquerque Diniz, Correia Barbosa, and Costa Vaz (2005) showed that under these conditions the lysozyme content decreased by about 75%. However, Evans, Ryley, Neale, Dodge, and Lewarne (1978) showed a reduction of only 23% while Czank et al. (2009) by about 53%. A significant loss of lysozyme activity after HoP, as much as 50 and 44%, was demonstrated by Lima et al. (2017) and Sousa et al. (2014), respectively, while Viazis, Farkas, and Allen (2007) showed only a 21% decline, and Malinowska-Pańczyk et al. (2017) only a 16% reduction and these changes were not statistically significant. No changes in lysozyme activity were also reported by Mayayo et al. (2016) after heating of HM at 65 °C for 30 min. A pressure of 193 MPa did not change the activity of lysozyme (Malinowska-Pańczyk et al., 2017). Also, Sousa et al. (2014) showed that pressurization at 200, 400 and 600 MPa for 2.5–30 min did not change the activity of this enzyme, while Mayayo et al. (2016) noted an increase in its activity after pressure treatment in a range from 300 to 650 MPa at room temperature for 30 min.

1.7. Antioxidant enzymes

SOD, GPx and CAT are an important group of antioxidant enzymes present in HM. The content or activity of these enzymes in HM is several times higher than in cow's milk and their role is the scavenging and elimination of free radicals in infants (Zivković et al., 2015). SOD catalyzes the dismutation of superoxide anion and prevents the formation of other reactive oxygen species and their derivatives. Additionally, it affects the number of neutrophils – natural body defense cells in milk. The product of SOD is hydrogen peroxide, which is converted to water by CAT or GPx. The function of the latter enzymes in HM is not yet fully known. The maximal activity of SOD, CAT and GPx amounted to 1.27 U/mL, 19.15 nmol min⁻¹ mL⁻¹ and 10.18 nmol min⁻¹ mL⁻¹, respectively (Martysiak-Żurowska, Puta, & Kielbratowska, 2019). Similar results have been described by other authors (Savić, Vojinović, Zvezdanović, Cosić, & Savić, 2005; Silvestre et al., 2008; Yuksel, Yigit, Cinar, Atmaca, & Onaran, 2015). Marinković et al. (2015) showed that activity of SOD drastically decreased, by about 50%, after HoP. However, it was noted that the activity of this enzyme did not change after heating at 62.5 °C for 30 min (Martysiak-Żurowska et al., 2019). A high pressure of 193 MPa at subzero temperature caused an increase in the activity of this enzyme by 57% (Malinowska-Pańczyk et al., 2017). The reasons for the increase in the activity of this enzyme after pressurization may be twofold. Firstly, it is a well-known phenomenon that the direction of change in enzyme activity depends on the pressure value (Mozhaev, Lange, Kudryashova, & Balny, 1996). The second, more likely, may be due to the release of this protein from neutrophils (leukocytes). As demonstrated by Delgado et al. (2013) and Contador et al. (2013), these cells are almost completely destroyed by pressure 300–900 MPa and the HoP conditions. Probably, this protein denatures under high pressure, but the amount of enzyme released from milk cells

compensates for these losses and even increases its activity. When using high temperature, the enzyme is also likely to be released from neutrophils, which compensates for the denaturation of this protein under these conditions, so that the activity does not change.

On the other hand, CAT and GPx activity decreases after pressure of 193 MPa at –20 °C by about 20%, and to a greater extent (50%) after thermal pasteurization (Malinowska-Pańczyk et al., 2017). These enzymes are also found in neutrophils and the destruction of these cells during the heating and pressurization probably leads to the release of these proteins and then to denaturation. The loss of activity after pressurization at 193 MPa is probably caused by the greater susceptibility of these enzymes to inactivation under these conditions (Malinowska-Pańczyk et al., 2017). The effect of high pressure at an ambient temperature has not yet been determined.

1.8. Volatile profile

The composition of the volatile flavor compounds of HM provides information about the global changes that occur after processing and the adverse reaction that appear, e.g. lipid oxidation. Contador, Delgado, García-Parra, Garrido, and Ramírez (2015) showed that the both HoP and HHP affect the volatile profile of HM. The effect of pressurization depended on the level of pressure and the processing time. The volatile profile of raw HM and pressurized at 400 MPa for 3 min practically did not differ. The greatest changes occurred after application of 600 MPa for 6 min. HoP and pressurization (600 MPa, 6 min) led to a decrease in the content of alkanes and cycloalkanes. In the case of terpenes, only HoP increased their concentration, with the level of D-limonene, especially, higher by about 70% compared to raw milk. During heating, a new compound α -pinene was also created. It belongs to the bicyclic monoterpenoids and does not normally occur in the volatile profile of raw milk.

Heating did not cause changes in the concentration of carboxylic acids, but pressurization (600 MPa, 6 min) led to an increase in their content. In these conditions (HoP and 600 MPa, 6 min), formation of volatile compounds such as aldehydes, furans and pyrans, which are formed as a result of increased lipid oxidation and the degradation of sugars and amino acids in the Maillard reaction, takes place (Contador et al., 2015). These changes cause the formation of a negative odor when processing HM. In particular, 5-(hydroxymethyl)-2-furancarboxaldehyde is formed during thermal and pressure processing. The presence of this compound is associated with poor food quality and an unhealthy effect in the consumer. The largest amount of this compound was detected in the volatile profile of HM treated by 600 MPa for 6 min so it is not recommended to use these conditions for milk preservation (Contador et al., 2015).

1.9. HM microbiota

HoP is an effective method of milk microbiota inactivation, but its effectiveness depends on the initial level of microbial contamination. To obtain the complete effect of pasteurization, the total number of bacteria in the milk should not be greater than 10⁵ cfu/mL (Malinowska-Pańczyk et al., 2019). In the case of HHP, Windyga et al. (2015) showed that the number of HM microbiota reduced by about 2.0, 0.5 and 2.5 log cycles after pressurization at 300 MPa at 4, 20 and 50 °C, respectively. Partial inactivation of HM microbiota was also achieved after pressurization at 193 MPa and –20 °C. The total bacterial count, the number of lactic acid bacteria (LAB) and coagulase-positive staphylococci was reduced after treatment at 193 MPa by about 2.6, 2.3 and 2.1 log cycles, respectively (Malinowska-Pańczyk et al., 2017).

The microbiota of HM were not detected in milk samples only after pressurization at 500 MPa regardless of the process temperature (Pitino et al., 2019; Windyga et al., 2015). Complete reduction of HM microbiota can also be achieved after pressurization at 600 MPa or after two-

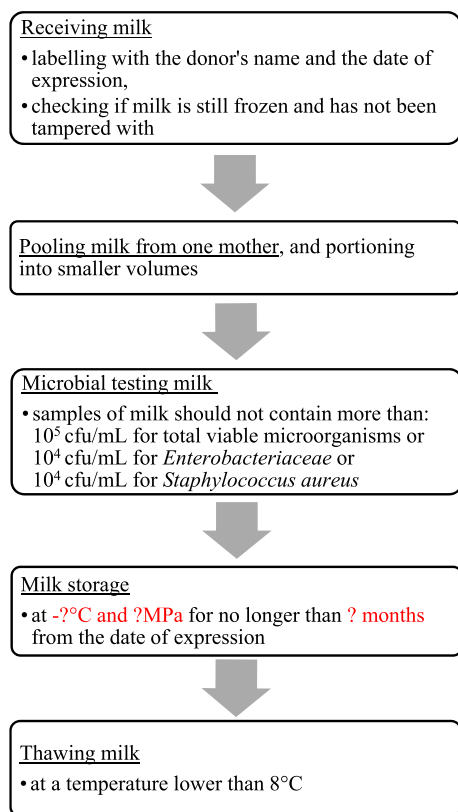


Fig. 2. Hypothetical scheme for handling with HM in HMB.

stage pressurization (100 MPa + 600 MPa; 200 MPa + 400 MPa; 200 MPa + 600 MPa) (Wesolowska et al., 2018). Demazeau et al. (2018) showed that total inactivation, not only the vegetative bacterial cells of *S. aureus*, but also the spores of *B. cereus* intentionally inoculated into HM (populations $\sim 10^6$ cfu/mL), was reached after four cycles of pressurization at 350 MPa at 38 °C.

1.10. Future trends

The search for methods of HM preservation, as an alternative to HoP, is the focus of many research groups around the world. The literature data presented above, shows that unfavorable changes in HM components take place during pressurization. The magnitude of the changes depends on the range of pressures used. At pressures up to 400 MPa, the changes are inconsiderable but do not allow the achievement of the expected reduction of microorganisms. Higher pressures lead to complete inactivation of HM microbiota, but also cause degradation of important biologically active ingredients in HM. It is also unknown how HHP will affect viruses that can also be transmitted through human milk. The introduction of this technique into HMB also has other limitations, e.g. high investment and operating costs and the continuing lack of a device of the appropriate size and weight that will properly and safely work in the HMB environment (Moro et al., 2019).

A new approach to the preservation, and also the handling, of milk in HMB may be the use of moderate pressures, up to 200 MPa at subzero temperatures. According to Bridgman (1912), high pressure reduces the freezing point of water to -22 °C at 207.5 MPa. Therefore, above this temperature, a sample placed in a sealed vessel in an unfrozen state is affected by the pressure. Our preliminary studies indicate that during storage of milk under high pressure at 60 MPa and -5 °C, the microbiota of HM is not detected after 14 days, nor at 156 MPa and -15 °C after 24 h. Under these conditions, the lysozyme and antioxidant activity of HM and LF content are not changed after 30 days of storage.

Before using HM stored under high-pressure low-temperature conditions, comprehensive testing will be needed to determine the optimal storage conditions. The advantage of storage HM under high pressure is the relatively inexpensive apparatus (Malinowska-Pańczyk, Kołodziejaska, Murawska, & Wołosewicz, 2009) that can be easily re-scaled to larger volumes. The use of such conditions may also create new opportunities allowing the simplification of the procedure for handling with milk from eight to five stages (Fig. 2) and reducing the risk of secondary contamination by *Bacillus cereus*.

A new way of preserving HM may also be to use high pressure homogenization (HPH) but currently there is no data on the effects of such treatment on the microbiota and components of HM. The use of HPH for preserving cow's milk indicates that this alternative method allows obtaining a product with a shelf life similar to high pasteurized milk (90 °C, 15 s) in terms of its microbiological and physicochemical properties (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007). HPH is also a promising method for processing pharmaceuticals (Yadav & Kale, 2019). The effect of HPH on the biological activity of HM will depend on many factors, e.g. homogenizer construction, the type of pressure pump or piston pumps, the geometry and design of valves, etc. According to Yadav and Kale (2019), in the near future the HPH will have a broader impact at both industrial and research levels, therefore it can be expected that research results will soon appear in the literature regarding the effect of HPH on the nutritional and biological properties of HM.

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