Raw milk preservation by hyperbaric storage: Effect on microbial counts, protein structure and technological functionality

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Abstract:	The possibility to apply hyperbaric storage (HS) at room temperature (20 °C) as a sustainable approach for preservation of raw skim milk was studied. Samples were stored at 200 and 150 MPa for up to 6 days. Optimal pressure for milk HS was found to be 150 MPa, since no clotting was detected for up to 6 days. 150 MPa-HS caused the irreversible inactivation of inoculated Escherichia coli (5.13 ± 0.33 logCFU mL -1) and Staphylococcus aureus (5.66 ± 0.93 logCFU mL -1) within 2 and 6 days, respectively. Inactivation of total and faecal coliforms (3.0 log reductions) below the detection limit was achieved after just 2 days, whereas lactic acid bacteria and coagulase-positive Staphylococci were inactivated after 6 days. Pressurized storage also caused an increase in proteose peptones and the release of submicelles from casein micelles. Micelles progressively aggregated with pressure-unfolded β-Lactoglobulin. These phenomena led to milk presenting up to 4-fold better foaming capacity, probably due to β-Lactoglobulin unfolding or higher proteose peptones content. This work demonstrated the capability of HS to guarantee milk preservation during storage, and brought attention on the opportunity to consider the technology for milk pasteurization and functionality improvement.
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Dear Editor,

We send to your attention the research article " **Raw milk preservation by hyperbaric storage: effect on microbial counts, protein structure and functionality** " by Federico Basso, Michela Maifreni, Nadia Innocente, Lara Manzocco and Maria Cristina Nicoli. All the authors have read and approved the manuscript.

Hyperbaric storage was investigated as a sustainable emerging technology for non-thermal preservation of milk. To the best of our knowledge, the efficacy of the technology in inactivating native microbial counts and inoculated pathogens has never been evaluated before. Only one paper has been published so far, reporting the effects of the technology on the profile of microbial metabolites in milk. In addition, no information is available on the effect of hyperbaric storage on protein structure and functionality.

In this paper, the application of hyperbaric storage was investigated to assess the effects of the technology on raw skim milk microbiological quality (*i.e.*, counts of naturally present total bacteria, lactic acid bacteria, coagulase-positive *Staphylococci*, faecal coliforms and total coliforms), safety (*i.e.* counts of inoculated *Staphylococcus aureus* and *Escherichia coli*), protein stability (*i.e.*, casein micelles size and whey protein content), colour, appearance and technological functionality (*i.e.* foaming properties). Results demonstrate the potentiality of hyperbaric storage for milk non-thermal pasteurization, defined as the achievement of at least 5 log reductions of the inoculated pathogens. Data also show the capability of the technology to improve milk protein functionality, as indicated by a remarkable enhancement in milk foaming without affecting colour and appearance.

We feel confident that the paper could provide a significant contribution to the understanding of the effects of one of the fastest growing technologies in the field of non-thermal food processing. We hope this article could satisfy the requirements of Food Research International, so that you might consider it for publication in this Journal.

Best regards,

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1 HS (150 MPa; 6 days) reduces by 5 log units *E. coli* and *S. aureus* in raw skim milk

2 HS-induced microbial inactivation is irreversible (for up to 12 days at 4 °C)

3 Casein micelles serve as local aggregation points for HS-unfolded β-Lactoglobulin

4 HS activates milk proteases leading to an increase in proteose-peptones

5 Foaming capacity of HS-treated milk increases up to 4 times

Raw milk preservation by hyperbaric storage: effect on microbial counts, protein structure and technological functionality

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ABSTRACT

The possibility to apply hyperbaric storage (HS) at room temperature (20 °C) as a sustainable approach for preservation of raw skim milk was studied. Samples were stored at 200 and 150 MPa for up to 6 days. Optimal pressure for milk HS was found to be 150 MPa, since no clotting was detected for up to 6 days. 150 MPa-HS caused the irreversible inactivation of inoculated *Escherichia coli* (5.13 ± 0.33 logCFU mL^{-1}) and *Staphylococcus aureus* (5.66 ± 0.93 logCFU mL^{-1}) within 2 and 6 days, respectively. Inactivation of total and faecal coliforms (3.0 log reductions) below the detection limit was achieved after just 2 days, whereas lactic acid bacteria and coagulase-positive *Staphylococci* were inactivated after 6 days. Pressurized storage also caused an increase in proteose peptones and the release of submicelles from casein micelles. Micelles progressively aggregated with pressure-unfolded β-Lactoglobulin. These phenomena led to milk presenting up to 4-fold better foaming capacity, probably due to β-Lactoglobulin unfolding or higher proteose peptones content.

This work demonstrated the capability of HS to guarantee milk preservation during storage, and brought attention on the opportunity to consider the technology for milk pasteurization and functionality improvement.

Keywords: Hyperbaric storage, raw milk, microbial inactivation, non-thermal pasteurization, protein interaction, foaming properties.

1 Introduction

Hyperbaric storage (HS) is an innovative food technology based on hydrostatic pressurization of food inside steel vessels (Fernandes et al., 2014; Santos et al., 2020). Despite conceptually similar to high pressure processing (HPP), which is performed at 400 – 800 MPa for up to 30 min (Aganovic et al., 2020), HS is carried out at moderate pressure (P < 250 MPa) for days, weeks or even months. While HPP is used to achieve cold pasteurization or to assist sterilization, HS performed at room temperature has attracted substantial interest as an alternative to refrigeration for perishable food. The application of the technology is highly sustainable since the maintenance of pressurized conditions can be guaranteed by the sealing of the pressurized vessels solely, accounting for an extremely low energetic cost (Bermejo-Prada et al., 2017). HS has thus been

Abbreviations: HS, Hyperbaric storage; HPP, High pressure processing; UHT, Ultra-high-temperature sterilized milk; BHI, Brain heart infusion broth; MRD, Maximum recovery diluent; PCA, Plate count agar; SC+, Coagulase-positive *Staphylococci*; BP, Baird Parker agar; MRS, Man Rogosa Sharp agar; DLS, Dynamic light scattering; RP-HPLC, Reverse-phase high performance liquid chromatography; FC, faecal coliforms; TC, total coliforms.

proposed as a sustainable alternative to refrigeration for perishable foods, including meat, fish, cheese, fruit juices, seafood and egg white (Basso, et al., 2021; Duarte et al., 2015; Fidalgo et al., 2018; Freitas et al., 2016; Otero et al., 2019; Otero & Pérez-Mateos, 2021; Santos et al., 2019). In these matrices, HS has been demonstrated to prevent microbial growth and to induce significant inactivation of hygiene indices (*e.g.* total bacteria count, yeasts and molds, lactic acid bacteria) and inoculated pathogens, with minimal effects on sensory properties. In particular, application of 100 MPa to watermelon juice reduced the count of total aerobic mesophiles and inoculated *Escherichia coli* and *Listeria innocua* by about 3 log cycles (Pinto et al., 2017). Results of peculiar interest were obtained in the case of fruit juices inoculated with heat- and pressure-resistant sporogenic microorganisms (*i.e.*, *Alicyclobacillus acidoterrestris* and *Bacillus subtilis*) (Pinto et al., 2018, 2019). In this case, HS at 50-100 MPa at room temperature allowed to achieve about 5 log reductions of total endospore count.

It could be inferred that HS can be used to decontaminate foods while storing them. Such possibility could be of upmost value in the case of fresh milk, which is conventionally obtained by pasteurization of raw milk (*i.e.*, thermal preservation), and subsequent storage under refrigerated conditions (4 °C) (Vasavada, 1988). As well known, despite guaranteeing microbiological safety, this approach is associated not only to milk thermal damage upon pasteurization (Syed et al., 2021), but also to high environmental impacts of heat treatment and cold storage (James & James, 2010; Swain et al., 2005; Syed et al., 2021).

Milk pressurization has been proven to be particularly challenging, due to the high sensitivity of milk proteins to hyperbaric conditions (Huppertz, Fox, et al., 2006). In particular, casein micelles disintegration has been often observed under pressure due to solubilization of colloidal calcium phosphate, resulting in milk clotting (Anema et al., 2005; Huppertz et al., 2002; Huppertz, Kelly, et al., 2006; Kiełczewska et al., 2020; Needs, Capellas, et al., 2000). Nevertheless, this effect was not detected when pressure was applied in the HS range (Huppertz et al., 2004). Although circumstantial, this evidence suggests that HS might be applied to milk without inducing clotting phenomena. Nevertheless, the effects of prolonged pressurizations (*e.g.*, days/weeks) on raw milk and, in particular, on casein micelles structure, are unknown.

Based on these considerations, the aim of the present study was to investigate the possibility of HS to be applied as sustainable milk preservation treatment, and to evaluate the potentiality of the technology as a non-thermal pasteurization approach. To this aim, the effects of HS on milk physical stability, colour, microbiological quality and safety, and functional properties were evaluated in raw skim milk. The research was organized in different consequential steps: milk was initially stored at different pressures (150, 200 MPa) and analyzed for absence of clotting (dynamic light scattering) and colour changes (tristimulus colorimetry). Following, the attention was focused on the HS treatment performed at pressure showing no clotting for up to 6 days, taken as average shelf life of fresh pasteurized milk under refrigerated conditions (Palmeri et al., 2019). The capacity of HS to achieve milk preservation was evaluated based on its capability to control the naturally occurring milk microflora (*i.e.*, total bacteria, lactic acid bacteria, coagulase-positive *Staphylococci*, faecal coliforms and total coliforms) and to reduce the microbial load of inoculated *E. coli* and *S. aureus*. Finally, milk attitude to be processed into stable foams was assessed, and foaming performance was related to protein profile changes.

2 Materials and Methods

2.1 Samples preparation

Ultra-high-temperature sterilized (UHT) and raw skim milk were obtained at a local food retailer and a local milk processing plant, respectively. Approximately 100 mL aliquots of milk were poured in polyethylene/ethylene vinyl alcohol/polypropylene pouches (15 × 30 cm; 80 μm thickness, water vapor permeability < 1 g · m^{-2} · 24 h^{-1}; Niederwieser Group S.p.A., Campogalliano, Italy), which were heat-sealed with headspace not exceeding 5% of samples volume (Orved, VM-16, Musile di Piave, Italy).

Milk samples for microbiological analyses were prepared separately. For the inoculum, bacteria suspensions containing *Escherichia coli* 8048 and *Staphylococcus aureus* 226 were prepared from the bacterial culture collection of the Department of Agricultural, Food, Animal and Environmental Sciences of the University of Udine (Italy). Strains were maintained at -80 °C in Brain Heart Infusion broth (BHI, Oxoid, Milan, Italy) with 30% sterile glycerol as cryoprotectant until use. From stock cultures, the strains were plated on BHI culture media, and incubated at 37 °C for 24 h. The inoculations were carried out by suspending plated pure cultures of each microorganism in 5 mL of BHI at 37 °C for 24 h. Subsequently, the cells were collected by centrifugation at 14,170 × *g* for 10 min at 4 °C (Beckman, Avanti TM J-25, Palo Alto, CA, USA) and washed three times with Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy). The final pellet was suspended in MRD. An aliquot of the bacteria suspension was added to approximately 50 mL UHT milk or raw milk to obtain a final concentration of 10^5 - 10^6 CFU mL^{-1}.

2.2 Hyperbaric storage

A HS working unit assembled by Comer Srl (Bologna, Italy) was used. It consisted of a water-tight steel vessel (Hystat, Slaithwaite, Huddersfield, UK) pressurized by a Haskel International high pressure pump (Burbank, CA, USA). The pressure-mediating fluid was an aqueous solution containing 0.2% (w/w) potassium sorbate and 0.2% (w/w) sodium benzoate (Carlo Erba Reagents Srl, Milan, Italy) to prevent mold growth in the fluid reservoir. Packaged samples were introduced in the vessel and pressurized at 200 and 150 MPa at room temperature (20 ± 2 °C). Control samples were stored under refrigerated conditions (4 °C, 0.1 MPa). At increasing time during storage for up to 6 days, samples were removed from the HS vessel or from the refrigerator, and analyzed.

2.3 Image acquisition

Images were acquired using an image acquisition cabinet (Immagini & Computer, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milano, Italy). The digital camera was placed on an adjustable stand positioned at 45 cm from a cardboard base covered with white paper where 15 mL glass vials containing the milk samples were placed. Lighting was provided by 4100W frosted photographic floodlights, positioned to minimize shadow and glare

2.4 Colour

A tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head was used to determine milk colour. The instrument was standardized against a white tile before analysis. Samples were poured into Petri dishes, positioned on top of the standardization tile and analyzed. Colour was expressed in L*, a* and b* scale parameters.

2.5 Microbiological analyses

Decimal dilutions of milk samples were prepared in MRD (Oxoid, Milan, Italy) and plated in specific culture media according to the microorganisms analyzed. Total bacterial count was enumerated on Plate Count Agar (PCA, Oxoid, Milan, Italy) and the plates were incubated at 30

± 1 °C for 48-72 h; *S. aureus* and coagulase-positive *Staphylococci* (SC+) were plated and counted on Baird Parker agar (BP, Oxoid, Milan, Italy) after incubation at 37 ± 1 °C for 24-36h; *E. coli*, and fecal and total coliforms were determined on ColiID (bioMerieux, Grassina, Italia) and the plates were incubated at 37 ± 1 °C for 24h; lactic acid bacteria (LAB) were enumerated on Man Rogosa Sharp agar (MRS, Oxoid, Milan, Italy) after incubation at 30 ± 1 °C for 48h. The results were expressed as the decimal logarithm of colony forming units per milliliter of milk (logCFU mL^{-1}); the detection of limit (L.o.D.) was 0 logCFU mL^{-1} for *E. coli* and coliforms, and 1 logCFU mL^{-1} and *S. aureus*, coagulase-positive *Staphylococci*, TBC, LAB, respectively.

2.6 Casein micelles size

Casein micelles size was determined by dynamic light scattering (DLS) analysis adapting the method from Segat et al. (2015). Milk samples were diluted 1:100 (v/v) with MilliQ water and inserted into 1 cm optical pathway cuvettes. Particle size was determined at 20 °C by using a dynamic light scattering system (NanoSizer 3000, Malvern Instruments, Malvern, UK) equipped with a Peltier temperature control system. The refractive index was set at 1.333 and the viscosity was approximated to that of pure water at 20 °C. The occurrence of milk clotting was identified in correspondence of the formation of aggregates with size higher than 5 μm.

2.7 Whey protein profile

Whey was obtained from milk samples by isoelectric precipitation (pH 4.6) of casein by addition of HCl 1 M. Whey samples were frozen and kept at – 18 °C until analysis. Thawed samples were diluted 1:5 (v/v) with MilliQ water and subjected to reverse-phase high performance liquid chromatography (RP-HPLC) as previously described by De Noni et al. (2007). The RP-HPLC apparatus was a 230 Pro Star (Varian Inc, Palo Alto, CA, USA), equipped with a 7725i injector (Rheodyne, Cotati, CA, USA) and a PLRP-S column (4.6 mm i.d. × 150 mm, 5 mm, 300 Å from Polymer Laboratories, Shropshire, UK) kept at 40 °C. The detector was a Varian 330 Pro Star UV-Vis spectrophotometer set at 205 nm. Samples were eluted by applying a gradient of solvents: A (0.1% (v/v) trifluoroacetic acid in MilliQ water); B (0.1% (v/v) trifluoroacetic acid in acetonitrile; Sigma Aldrich, Milan, Italy). Eluting solvents were filtered through 0.45 μm cutoff HV DURAPORE® membrane filters (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Cork, Ireland). The elution gradient, as solvent B proportion (v/v), was as follows: 0-8 min, 25-35%; 8-10 min, 35-36%; 10-17 min, 36-38%; 17-23 min, 38-45%; 23-23.5 min, 45-100%; 23.5-25 min, 100-25%. The flow rate was 1.0 mL min^{-1}. Peak assignment was performed according to Innocente et al. (2011). β-Lactoglobulin (β-Lg) was quantified by using a calibration curve obtained from standard solutions (Sigma Aldrich. Milan, Italy) in the 0-2 g L^{-1} concentration range (R^2_{adj} = 0.9843).

2.8 Foaming properties

Two different foaming methods, based on mechanical agitation or on steam injection, were used. For the mechanical-based method, the procedures applied by Kamath et al. (2008) and Ho et al. (2019) were adapted. In particular, 25 mL milk aliquots were poured into 100 mL beaker, equilibrated at 20 °C for 1 h, heated to 50 ± 3 °C in a microwave oven (Panasonic Ne-1643, 1600 W, applied for 8 s) and foamed using a commercially available mechanical milk frother for 15 s. For the steam-based method, 90 mL of milk was poured into 250 mL beakers and the foam was generated using a steam injection system purposedly built to simulate catering steam frothers. Steam was injected in the samples for 5 s, so that milk reached a temperature of 70 ± 5 °C. For both methods, the height of the milk surface (h_i) from the bottom of the beaker was measured with

a Metrica monobloc precision venier caliper (Metrica S.p.A., San Donato M.se, MI, Italy). Foam height was measured after (h_0) and 15 min (h_{15}) and the foaming capacity and foam stability were expressed as follows:

$$Foaming\ capacity\ (\%) = h_0/h_i \cdot 100 \quad \text{(Eq.1)}$$

$$Foam\ stability\ (\%) = h_{15}/h_0 \cdot 100 \quad \text{(Eq.2)}$$

2.9 Data analysis

Microbiological analyses were performed in single on samples from two independent experiments and are reported as mean ± standard deviation. Data of particle size, colour and foaming properties were obtained by at least triplicate measurements. These data are reported as mean ± standard deviation and were subjected to one-way analysis of variance (ANOVA) and Tukey's Honest Significant Differences test ($p < 0.05$) using R v. 3.6.1 for Windows (The R foundation for statistical computing). RP-HPLC data were obtained in duplicate and reported as mean ± standard deviation.

3 Results and discussion

3.1 Identification of pressure conditions for milk hyperbaric storage

Preliminary trials were performed to identify the maximum pressure level that could be applied to milk without leading to significant changes in its physical stability within the typical shelf life of refrigerated pasteurized milk (*i.e.* up to 6 days) (Palmeri et al., 2019). To this aim, samples were stored at 150 and 200 MPa until milk clotting was detected by DLS in correspondence of large aggregates (Table 1).

Milk showed the presence of a monodispersed (Polydispersity index = 0.09 ± 0.04) particle family with 169 nm size, representing casein micelles (De Kruif, 1999). Under HS at 200 MPa, two distinct phenomena were observed (Table 1): a progressive increase in casein micelles size and the appearance of a novel family of smaller particles (about 50 nm). The latter became evident after 30 min-HS, and can be associated to sub-micellar particles, which occurred as a consequence of pressure-induced micelle fragmentation and reassociation (Gebhardt et al., 2006). After 1.5 h of HS, casein micelles aggregated to form large particles exceeding 5 μm in size, indicating the onset of clotting. When HS was performed at 150 MPa, the increase in casein micelle size and their fragmentation occurred at a much slower rate. In particular, sub-micellar particles became detectable only after 2 days (Table 1). As casein better tolerated less intensive HS, milk clotting was detected only after 6 days. It is worth noting that, when milk clotted, casein micelles were significantly larger (370 nm) if milk was stored at 150 MPa rather than at 200 MPa (250 nm). This indicates that milk clotting was not the result of micelle enlargement solely. In fact, many Authors reported that pressure-induced clotting primarily occurs due to aggregation of sub-micellar particles, whereas an increased micelle size is mainly attributable to interactions between micelles and pressure-unfolded whey proteins (Anema et al., 2005; Huppertz et al., 2004; Huppertz & De Kruif, 2007; Needs, Capellas et al., 2000; Needs, Stenning, et al., 2000). Independently on storage conditions, no changes in luminosity were observed in all samples (data not shown), indicating that casein micelle modifications (Table 1) did not affect the optical properties of milk colloidal system. The effects of pressurized storage on milk appearance were also evaluated by assessing color parameters a* (redness) and b* (yellowness) (Figure 1).

While no significant changes in redness and yellowness were detected in refrigerated milk, a slight but progressive increase in these parameters was detected upon milk HS (Figure 1). Although not visually perceivable to the naked eye, this minor colour change could be attributed to pressure-triggered non-enzymatic browning. Reportedly, the early condensation steps of the Maillard reaction can be favoured by pressure since, in some cases, they can be characterized by a negative activation volume (Hill et al., 1996; Isaacs & Coulson, 1996).
Based on these results, milk physical stability could be guaranteed for up to 6 days by storing it at pressure as high as 150 MPa. The latter was thus deemed as the optimal pressure level for milk HS, and further experiments were conducted by applying these conditions.

3.2 Effect of hyperbaric storage on milk hygiene and microbiological safety

Since milk is not an inherently sterile matrix, microbiological analyses were firstly performed to assess the effect of HS on the naturally occurring microflora. To this aim, total bacteria count (TBC), lactic acid bacteria (LAB), coagulase-positive *Staphylococci* (SC+), fecal coliforms (FC) and total coliforms (TC) microbiological quality indexes were considered. The latter were followed during pressurized storage for up to 6 days, using refrigerated milk as reference (Table 2).
In fresh raw milk, the value of all the considered indexes was relatively high, ranging from about 2 to circa 4 logCFU mL^{-1}. The detection of FC and SC+ indicated the potential occurrence of dangerous microorganisms, such as *E. coli* and *S. aureus*. During refrigerated storage for up to 6 days, all the microbial indexes progressively increased with the only exception of SC+, which remained relatively stable. In particular, TBC and FC grew by more than 2 logCFU mL^{-1} after 6 days, whereas LAB and TC increased by less than 1 log unit. These results are in agreement with the well-known weak bacteriostatic capacity of refrigeration in raw milk (Griffiths et al., 1987), potentially allowing the development of pathogens. On the contrary, HS at 150 MPa caused the reduction of all microorganisms below the detection limit. In particular, FC and TC were inactivated within 1 and 2 days, respectively. Differently, the gram-positive species comprising SC+ and LAB better withstood pressurized conditions and, similarly to TBC, were reduced below the detection limit only after 6 days-HS.
Based on these results, the efficacy of HS as a potential approach for milk pasteurization was evaluated. To this aim, counts of milk spiked with *E. coli* and *S. aureus* (5-6 logCFU mL^{-1}) pressurized at 150 MPa for up to 6 days were compared to those of analogous samples submitted to refrigeration. Possible interferences provided by the presence of native milk bacteria (Table 2) were made negligible by firstly performing the challenge test using UHT skim milk. The results are shown in Table 3.
The application of refrigerated conditions did not affect the load of the inoculated microorganisms, which remained unchanged during the 6 days-storage. Oppositely, HS progressively reduced both *E. coli* and *S. aureus* loads below the detection limit. The complete inactivation of *S. aureus* required the application of 150 MPa for 6 days, whereas *E. coli* was undetectable in milk samples after just 2 days. It is likely that the remarkably higher resistance of gram-positive bacteria to pressure, which is due to their thick peptidoglycan cell wall layer, allowed *S. aureus* to better withstand HS conditions as compared to *E. coli* (Wuytack et al., 2002). Similar results were previously observed during HS of egg white inoculated with *Salmonella enterica* and *S. aureus* as well as of watermelon juice spiked with *E. coli* and *L. innocua* (Basso, et al., 2021; Pinto et al., 2017).

With the aim of validating the encouraging results obtained with UHT milk, the challenge test was repeated on raw skim milk. In this case, the presence of native milk microorganisms was evaluated by performing TBC counts concomitantly to *E. coli* and *S. aureus* ones. The results are reported in Table 4.

Similar to what observed for UHT skim milk, the application of refrigeration did not induce any variation in the counts of inoculated *E. coli* and *S. aureus* while increased TBC by roughly 1 log unit. On the other hand, milk TBC counts decreased during HS, showing a reduction that ranged from about 3 to 5 logCFU mL^{-1}. Moreover, pressurized storage promoted 5 log units-inactivation of both *E. coli* and *S. aureus*, with high similarity with the inactivation efficacy observed in UHT milk (Table 3). It is noteworthy that a 5-log reduction has been suggested as a reasonable criterion by different Authors to assess the potential of non-thermal technologies for milk pasteurization (Alberini et al., 2015; Matak et al., 2005; Mussa & Ramaswamy, 1997; Ruiz-Espinosa et al., 2013; Stratakos et al., 2019). Data shown in Tables 3 and 4 clearly evidence that such a criterion can be reached by storing milk at 150 MPa for 6 days. This result suggests the potentiality of HS for non-thermal pasteurization of milk.

To evaluate the capability of HS to extend the shelf life of milk after depressurization, inoculated and pressurized raw skim milk was further stored under refrigerated conditions for 12 days. During this period, *E. coli* and *S. aureus* remained undetectable, and TBC values did not change (data not shown). This result demonstrates the irreversibility of HS-induced microbial inactivation and highlights the capability of the technology of extending milk microbiological stability for several days after decompression.

3.3 Effect of hyperbaric storage on whey proteins

To better investigate the effect of hyperbaric storage on milk proteins, the attention was focused on the role of whey proteins in micelle enlargement (Table 1). Whey was thus recovered from differently stored milk and subjected to RP-HPLC. In accordance with De Noni et al. (2007), chromatograms indicated the presence of the full whey protein spectrum in fresh milk (Figure 2). In particular, α-lactalbumin (α-La), bovine serum albumin (BSA) and β-lactoglobulin (β-Lg) were eluted at about 20, 22 and 25 min, respectively. Moreover, the presence of proteose-peptones was clearly indicated by the occurrence of a broad, irregular peak at 13 min (Innocente et al., 2011). During refrigerated storage, milk whey proteins content did not change (chromatograms not shown), indicating optimal maintenance of their structure. Contrarily, a significant loss of β-Lg was observed in the samples stored at 150 MPa (Figure 2). Quantitative analysis showed that β-Lg concentration decreased from 2.38 ± 0.28 (fresh sample) to 0.44 ± 0.10 and 0.11 ± 0.08 g L^{-1} after 1 and 6 days of hyperbaric storage, respectively. These results are probably due to extensive pressure-induced unfolding of β-Lg, which is highly pressure-sensitive (Huppertz et al., 2004; Huppertz, Fox, et al., 2006) and prone to interact with κ-casein molecules in relatively stable complexes (Cho & Singh, 2003). It can be thus inferred that milk whey was deprived of β-Lg since it separated along with casein. In other words, casein micelles would locally support aggregation of pressure-unfolded β-Lg molecules, which would have accumulated onto their surfaces, thus leading to the observed increase in milk casein size (Table 1) (Patel & Huppertz, 2014; Scollard et al., 2000). This hypothesis was further confirmed by statistical analysis, which revealed strong negative correlation (r = - 0.838) between β-Lg concentration and casein micelles size. HS also induced a progressive increase in proteose-peptones content (Figure 2), suggesting that casein hydrolysis by native milk proteases (*e.g.*, plasmin) was favoured by HS (Garcia et al., 2017).

According to García-Risco et al. (2003), this phenomenon resulted from pressure-induced modification of casein structure, which made them prone to proteolytic enzymes.

3.4 Effect of hyperbaric storage on milk foaming properties

The observed effects of HS on milk proteins indicate the possibility to employ pressurized storage to improve the technological performance of milk. For instance, due to their exceptional surface activity, unfolded β-Lg and proteose-peptones formed by HS could be of peculiar interest for milk foaming.

To assess whether protein structural changes induced by hyperbaric storage could steer the attitude of milk to be further processed into foams, differently stored milk samples were analyzed for foaming properties by using two alternative methods (Table 5). The first one was based on mechanical agitation and moderate heating. According to the literature, besides being representative of milk foaming processes carried out at domestic level (Silva et al., 2008), this procedure allows to accurately evaluate foaming performances. Subsequently, a steam injection-based method was also applied, which can be considered the gold standard for foamed milk preparations (*i.e.*, *cappuccino*, *macchiato*, and *latte*) in the catering sector (Silva et al., 2008).

Refrigeration had almost no effect on milk foaming properties. This might have been due to slight hydrolysis of milk proteins, as a result of the activity of microbial enzymes (Table 2) (Ho et al., 2019). Differently, HS caused a remarkable progressive increase (~ 4-fold after 6 days) in mechanically-induced foaming capacity, without detriment to the foam stability (Table 5). Similar to the mechanical procedure, the steam injection foaming method highlighted a progressive increase in the foaming capacity (about 35% after 6 days) and no changes in the foam stability of pressurized milk (Table 5). These results indicate that the enhancement of milk foaming induced by HS would be relevant for both domestic and catering-related uses, suggesting that preparations based on foamed milk might be attained using lower amounts of milk if the latter was previously subjected to pressurized storage. Data also confirm the hypothesis that unfolding of β-Lg and formation of proteose-peptones during HS improved milk foaming capacity (Figure 2) (Buccioni et al., 2013; Innocente et al., 2011). However, based on their excellent foaming activity, proteose-peptones were reasonably the major driver of these phenomena, as also supported by the strong positive correlation (r=0.9085) between foaming capacity and proteose-peptones RP-HPLC peak area (data not shown).

4 Conclusions

This work demonstrates the efficacy of hyperbaric storage (150 MPa for 6 days) as a preservation treatment for raw milk, and the potentiality of the technology for non-thermal milk pasteurization. HS was actually capable to irreversibly reduce the load of *E. coli* and *S. aureus* by 5 log units with minimal effects on milk physical stability, while significantly boosting foaming capacity. Besides representing an efficacious alternative to milk thermal stabilization, hyperbaric storage might serve as pretreatment in the manufacturing of dairy-based products where milk protein properties are crucial. For instance, the pressure-induced enhancement of milk proteins surface activity could be particularly interesting to improve the rheological properties of ice cream and recombined dairy creams. Moreover, the possibility to integrate β-Lg in the curd could allow to significantly boost yield, nutrient value and functional properties of cheese and fermented milk derivatives.

The application of hyperbaric storage could be easily extended to preserve liquid matrices other than milk, for which non-thermal technologies can be applied for pasteurization. It also shows potential as an alternative to thermal sterilization, whose inactivation capacity might be attained

by applying HS for sufficient time. Nevertheless, for each food matrix, a clear understanding of the kinetics of alterative phenomena at pressurized conditions is needed, since they might be not negligible during prolonged HS.
The implementation of hyperbaric storage in food industries will strictly depend on the availability of working units viable for industrial application, easy to operate, and feasible from an economic perspective.

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Table 1: Size and content of casein micelles and sub-micellar particles in raw skim milk during HS for increasing time at 200 and 150 MPa.

Pressure (MPa)	Time (h)	Micelles size (nm)	Intensity (%)	Sub-micellar particles size (nm)	Intensity (%)	Aggregates size (nm)	Intensity (%)
0	0	169.1 ± 2.6^{g}	100.0 ± 0.0^{a}	-	-	-	-
150	0.5	173.2 ± 2.3^{g}	100.0 ± 0.0^{a}	-	-	-	-
	1	170.9 ± 2.8^{g}	100.0 ± 0.0^{a}	-	-	-	-
	2	167.1 ± 4.6^{g}	100.0 ± 0.0^{a}	-	-	-	-
	3	172.5 ± 5.0^{g}	100.0 ± 0.0^{a}	-	-	-	-
	15	217.1 ± 5.2^{e}	100.0 ± 0.0^{a}	-	-	-	-
	18	223.1 ± 4.1^{e}	100.0 ± 0.0^{a}	-	-	-	-
	24	237.0 ± 4.0^{d}	100.0 ± 0.0^{a}	-	-	-	-
	48	275.8 ± 7.9^{b}	96.7 ± 3.1^{a}	52.1 ± 8.0^{a}	6.5 ± 1.4^{ab}	-	-
	120	377.9 ± 11.0^{a}	96.5 ± 3.3^{a}	51.1 ± 2.8^{a}	5.9 ± 0.9^{ab}	-	-
	144	371.1 ± 8.1^{a}	99.8 ± 0.5^{a}	-	-	5280.0 ± 396.0^{a}	2.0 ± 0.8^{a}
200	0.17	175.4 ± 3.1^{fg}	100.0 ± 0.0^{a}	-	-	-	-
	0.33	186.8 ± 3.2^{f}	100.0 ± 0.0^{a}	-	-	-	-
	0.5	212.6 ± 4.9^{c}	97.2 ± 0.2^{a}	46.0 ± 2.2^{a}	2.8 ± 0.2^{b}	-	-
	1	248.5 ± 6.2^{cd}	92.7 ± 0.5^{ab}	58.4 ± 2.8^{a}	7.3 ± 0.5^{ab}	-	-
	1.5	256.3 ± 11.8^{c}	90.9 ± 2.5^{b}	52.6 ± 4.7^{a}	9.1 ± 2.6^{a}	5344.5 ± 304.8^{a}	1.7 ± 0.8^{a}

- : not detectable

a, b, c, d, e, f, g: Different letters indicate significantly different means (ANOVA; $p<0.05$) in the same column.

Table 2: Total bacteria (TBC), lactic acid bacteria (LAB), coagulase-positive *Staphylococci* (SC+), fecal coliforms (FC) and total coliforms (TC) counts in raw skim milk stored for up to 6 days under refrigerated (0.1 MPa, 4.0 ± 0.5 °C) or hyperbaric conditions (150 MPa, 20 ± 1 °C). Results are expressed as logCFU mL^{-1}.

Storage	Time (days)	TBC	LAB	SC+	FC	TC
Fresh	0	3.89 ± 0.16	3.44 ± 0.42	2.91 ± 0.28	2.38 ± 0.03	2.70 ± 0.22
Refrigerated	1	3.85 ± 0.15	3.82 ± 0.03	2.95 ± 0.12	2.13 ± 0.29	2.66 ± 0.17
	2	3.88 ± 0.07	3.43 ± 0.28	2.57 ± 0.03	2.22 ± 0.01	2.50 ± 0.11
	4	3.80 ± 0.03	3.41 ± 0.52	3.11 ± 0.05	2.72 ± 0.17	2.88 ± 0.08
	6	5.98 ± 0.09	4.12 ± 1.08	2.51 ± 0.22	4.69 ± 0.44	3.56 ± 0.16
Hyperbaric	1	3.41 ± 0.38	3.59 ± 0.05	2.41 ± 0.57	< L.o.D.**	1.70 ± 0.29
	2	3.41 ± 0.30	2.99 ± 0.50	2.10 ± 0.45	< L.o.D.**	< L.o.D.**
	4	2.95 ± 0.31	2.29 ± 0.25	1.95 ± 0.24	< L.o.D.**	< L.o.D.**
	6	< L.o.D.*	< L.o.D.*	< L.o.D.*	< L.o.D.**	< L.o.D.**

*L.o.D.: 1 logCFU mL^{-1}

** L.o.D.: 0 logCFU mL^{-1}

Table 3: Counts of inoculated *E. coli* and *S. aureus* in UHT skim milk stored for up to 6 days under refrigerated (0.1 MPa, 4.0 ± 0.5 °C) or hyperbaric conditions (150 MPa, 20 ± 1 °C). Results are expressed as logCFU mL^{-1}.

Storage	Time (days)	*E. coli*	*S. aureus*
Fresh	0	5.49 ± 0.13	5.33 ± 0.08
Refrigerated	1	5.49 ± 0.16	5.32 ± 0.09
	2	5.56 ± 0.11	5.38 ± 0.00
	4	5.55 ± 0.24	5.29 ± 0.05
	6	5.25 ± 0.09	5.19 ± 0.02
Hyperbaric	1	1.47 ± 0.18	4.94 ± 0.08
	2	< L.o.D.*	4.13 ± 0.18
	4	< L.o.D.*	2.43 ± 0.19
	6	< L.o.D.*	< L.o.D.**

*L.o.D.: 0 logCFU mL^{-1}
** L.o.D.: 1 logCFU mL^{-1}

Table 4: Counts of inoculated *E. coli* and *S. aureus*, and relevant TBC (in brackets) in raw skim milk stored for up to 6 days under refrigerated (0.1 MPa, 4.0 ± 0.5 °C) or hyperbaric conditions (150 MPa, 20 ± 1 °C). Results are expressed as logCFU mL^{-1}.

Storage	Time (days)	*E. coli* (TBC)	*S. aureus* (TBC)
Fresh	0	5.13 ± 0.33 (5.16 ± 0.02)	5.66 ± 0.93 (5.56 ± 0.83)
Refrigerated	1	5.00 ± 0.17 (5.15 ± 0.15)	5.67 ± 1.04 (5.51 ± 0.67)
	2	5.12 ± 0.28 (5.30 ± 0.08)	5.50 ± 0.71 (6.07 ± 1.52)
	4	4.97 ± 0.21 (5.13 ± 0.07)	5.47 ± 0.81 (5.52 ± 0.93)
	6	4.99 ± 0.30 (6.07 ± 0.11)	5.59 ± 0.94 (6.05 ± 0.26)
Hyperbaric	1	2.25 ± 0.25 (3.69 ± 0.04)	5.20 ± 0.92 (5.20 ± 0.85)
	2	< L.o.D.* (3.02 ± 0.17)	3.83 ± 1.86 (4.28 ± 1.27)
	4	< L.o.D.* (2.43 ± 0.19)	2.67 ± 1.02 (2.94 ± 0.08)
	6	< L.o.D.* (<L.o.D.**)	< L.o.D.** (2.10 ± 0.02)

*L.o.D.: 0 logCFU mL^{-1}
** L.o.D.: 1 logCFU mL^{-1}

Table 5: Foaming capacity and foam stability determined by mechanical agitation or steam injection in raw skim milk stored for up to 6 days under refrigerated (0.1 MPa, 4.0 ± 0.5 °C) or hyperbaric conditions (150 MPa, 20 ± 1 °C).

Storage	Time (days)	Mechanical agitation		Steam injection	
		Foaming capacity (%)	Foam stability (%)	Foaming capacity (%)	Foam stability (%)
Fresh	0	72.5 ± 4.4^{d}	72.6 ± 4.1^{a}	112.7 ± 6.0^{b}	50.2 ± 6.6^{ab}
Refrigeration	4	83.4 ± 9.5^{d}	62.3 ± 15.5^{a}	-	-
	6	92.8 ± 5.1^{d}	71.4 ± 6.8^{a}	106.6 ± 6.9^{b}	60.2 ± 0.8^{a}
Hyperbaric	1	119.5 ± 7.9^{c}	75.0 ± 5.6^{a}	N.D.	N.D.
	2	123.4 ± 8.5^{c}	79.6 ± 6.6^{a}	122.4 ± 0.7^{ab}	51.6 ± 0.7^{ab}
	4	197.2 ± 6.5^{b}	71.2 ± 0.8^{a}	127.4 ± 2.9^{ab}	54.9 ± 3.5^{ab}
	6	267.3 ± 15.7^{a}	71.7 ± 1.5^{a}	147.5 ± 15.3^{a}	49.3 ± 2.2^{b}

a, b, c, d: Different letters indicate significantly different means (ANOVA; $p<0.05$) in the same column.

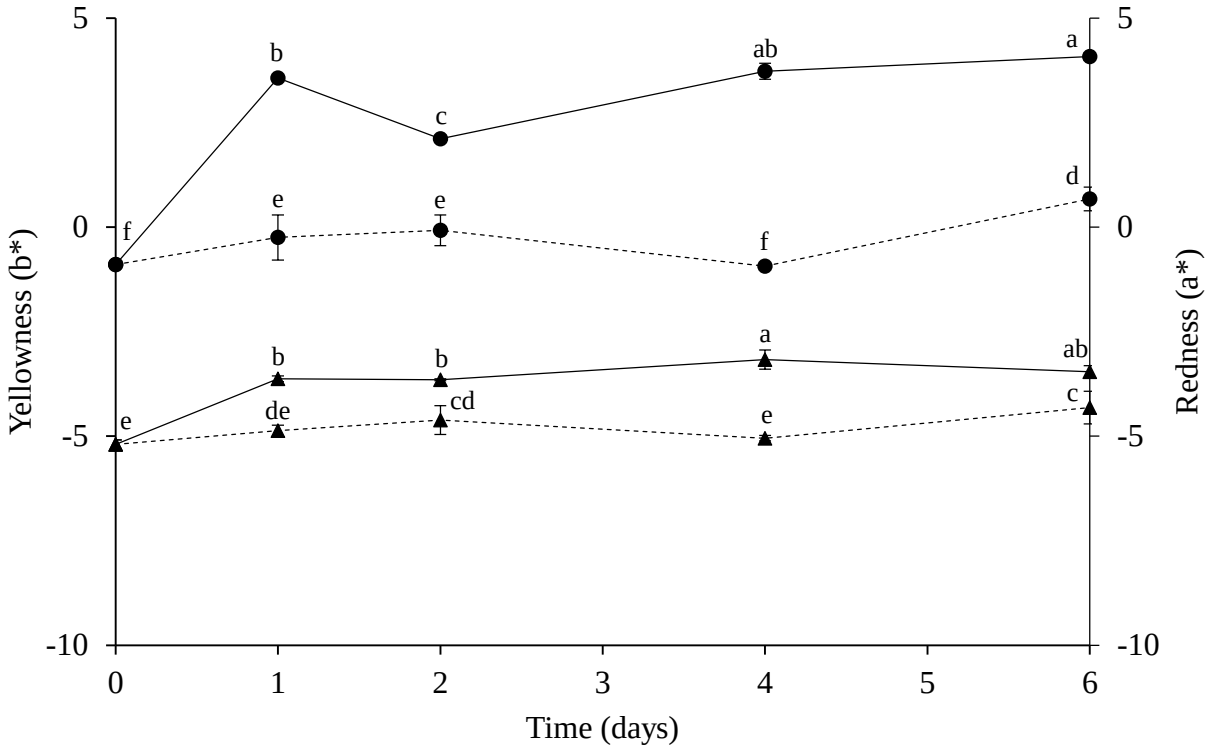

Figure 1: Redness (a*) and yellowness (b*) of raw skim milk stored for up to 6 days under refrigerated (0.1 MPa, 4.0 ± 0.5 °C) or hyperbaric conditions (150 MPa, 20 ± 1 °C).
a, b, c, d, e, f: Different letters for the same colour parameter indicate significantly different means (ANOVA; $p < 0.05$).

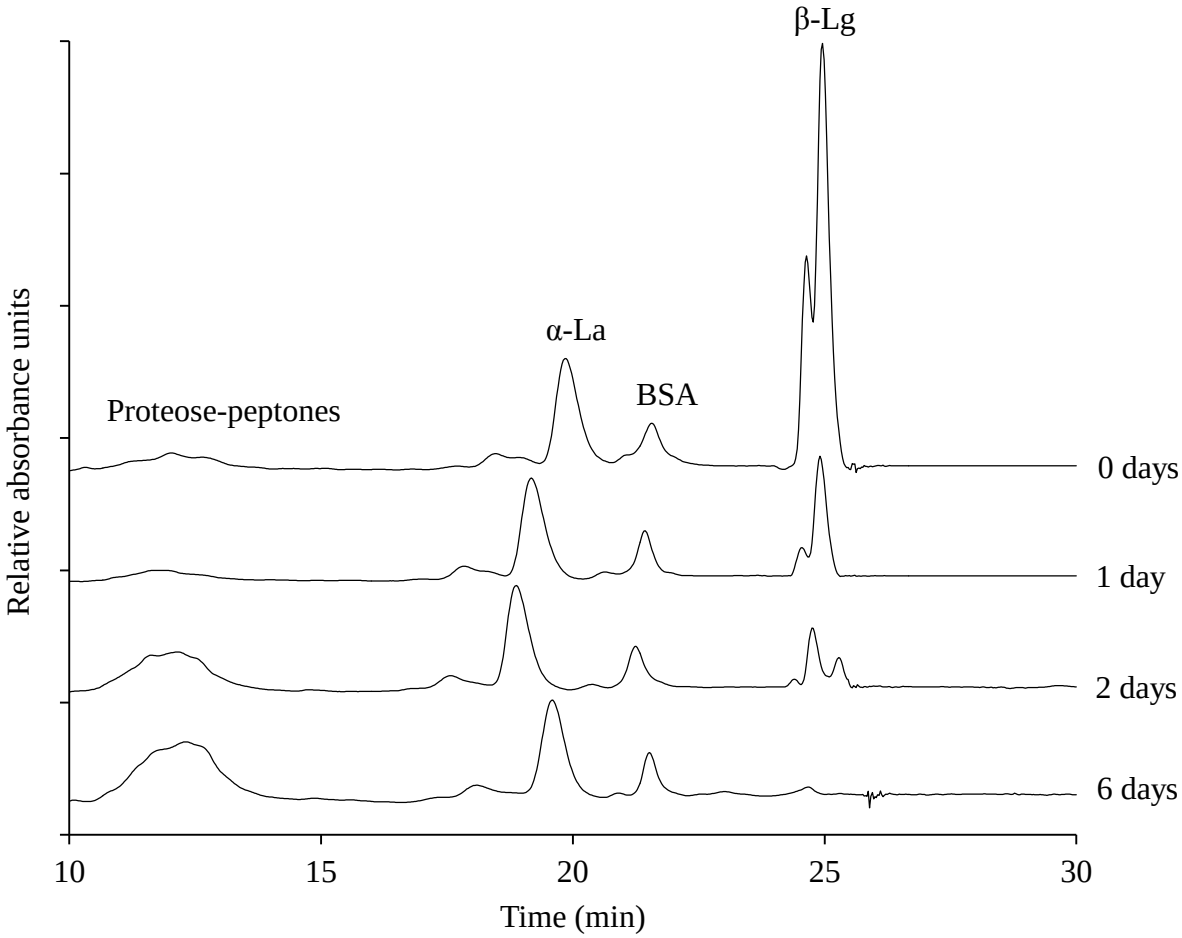

Figure 2: RP-HPLC chromatogram of raw skim milk samples during HS at 150 MPa (20 ± 1 °C °C) for up to 6 days. Peak assignment of proteose-peptones, α-La, BSA and β-Lg is also displayed.

Credit Author Statement

Federico Basso: Investigation, Formal analysis, Data curation, Writing - Original Draft, Visualization; **Michela Maifreni**: Investigation, Formal analysis, Data curation, Writing - Original Draft; **Nadia Innocente**: Conceptualization, Data curation, Writing - Review & Editing; **Lara Manzocco**: Conceptualization, Data curation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision; **Maria Cristina Nicoli**: Conceptualization, Resources, Writing - Review & Editing, Supervision.

Conflict of Interest and Authorship Confirmation Form

The Authors declare that:

- ✓ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

- ✓ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

- ✓ The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.