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A microbiological perspective of raw milk preserved at room temperature using hyperbaric storage compared to refrigerated storage

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ABSTRACT

The effects of hyperbaric storage (HS, 50–100 MPa) at room temperature (RT) on endogenous and inoculated pathogenic surrogate vegetative bacteria (*Escherichia coli*, *Listeria innocua*), pathogenic *Salmonella enterica* and bacterial spores (*Bacillus subtilis*) were assessed and compared with conventional refrigeration at atmospheric pressure for 60 days. Milk stored at atmospheric pressure and refrigeration quickly surpassed the acceptable microbiological limit within 7 days of storage, regarding endogenous microbiota, yet 50 MPa/RT slowed down microbial growth, resulting in raw milk spoilage after 28 days, while a significant microbial inactivation occurred under 75–100 MPa (around 4 log units), to counts below 1 log CFU/mL throughout storage, similar to what was observed for *B. subtilis* endospores. While inoculated microorganisms had a gradually counts reduction in all HS conditions. Results indicate that HS can not only result in the extension of milk shelf-life but is also able to enhance its safety and subsequent quality.

Industrial relevance: This new preservation methodology could be implemented in the dairy farm storage tanks, or during milk transportation for further processing, allowing a better microbial control, than refrigeration. This methodology is very promising, and can improve food products shelf-life with a considerable lower carbon footprint than refrigeration.

1. Introduction

Milk is considered one of the most complete foods, rich in essential nutrients including protein, fat, carbohydrates, vitamins, and various mineral needed for a healthy growth and development (Sharabi, Okun, & Shpigelman, 2018), with milk and dairy products widely consumed all over the world, representing an important part of the human diet.

After collection, raw milk temperature is around 38 °C, and thus it needs to be cooled rapidly and kept at refrigeration temperatures, as its rich nutritional profile, near neutral pH and high-water activity makes milk the perfect environment for the proliferation of several microorganisms (Lundén, Tolvanen, & Korkeala, 2004). Microbial composition and diversity in raw milk can be associated to diverse types and origins of contaminations, occurring during pre- or post-harvest. Microorganisms can already be present when milk is excreted (pre-harvest), for example, if the mammary gland is infected (mastitis), which is the most common disease associated with dairy cattle (Angulo, LeJeune, & Rajala-Schultz, 2009). This inflammation cannot always be visible and the source of infection ranges from bacteria, yeasts, mycoplasma, and algae, that can subsequentially be excreted into milk (Bradley, 2002). Also when the milk is being excreted it can come in contact with commensal microbiota that live in the teat skin, or on the epithelial lining of the teat canal or via the lactiferous duct (Isaac et al., 2017). Thereby, by the time the milk leaves the animal, microbial contamination may occur even in a healthy animal.

Post-harvest contamination can derive from the dairy farm environment during production, collection, processing, distribution, and storage of milk. These contaminants may result from faecal, animal feed, mud, water, soil, human handling, farm utensils, distribution pipes, bulk, or transport tanks (Damm, Holm, Blaabjerg, Bro, & Schwarz, 2017). *Staphylococcus, Campylobacter, Listeria*, *Escherichia*, *Salmonella, Micrococcus*, *Clostridium*, *Yersinia enterocolitica* and *Bacillus* (vegetative and spore cells) are commonly associated with milk contamination when improper or poor sanitary conditions are in place (Papademas & Bintsis, 2010). Maintenance of low temperature (4–10 °C) once the milk is collected or transported for further processing remains one of the most

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crucial factors for the overall quality in raw milk, since it slows down microbial growth and chemical deterioration (Koutsoumanis, Pavlis, Nychas, & Xanthiakos, 2010). However, psychrophile microorganisms can proliferate under low temperatures, releasing lipases and proteases responsible for organoleptic changes, like rancidity and bitter off-flavours (McClements, Patterson, & Linton, 2001). Additionally, heat pasteurization is also ineffective in the inactivation of spores commonly found in the farm environment, like *Bacillus cereus* (Heyndrickx, 2011), and thus, spoilage of raw milk can easily occur when proper processing protocols are not followed correctly during pre- or post-harvest of milk (LeJeune & Rajala-Schultz, 2009).

Hyperbaric storage (HS) is a preservation methodology based on high pressure as a hurdle for microbial growth a like refrigeration, that uses moderate pressures ranging from 25 to 100–220 MPa during lengthy periods of time, in fact during the whole storage period (Moreira et al., 2015; Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). This new methodology was accidently discovered when several perishable foods (sandwiches, soups, and apples) were recovered in good consumable conditions from a submersible that was sunken after 10 months at 1540 m depth (~15 MPa) at 3 °C (Jannasch, Eimhjellen, Wirsen, & Farmanfarmalan, 1971). The combination of low temperature and pressure was assumed to be the main cause for the good preservation state observed for those recovered foods, and so, a few studies were subsequently carried out using those combined conditions in different foods (Charm, Longmaid, & Carver, 1977; Mitsuda, 1972). However, the feasibility to use HS at room temperature (RT) re-emerged in the recent decade, as a possibility to substitute refrigeration, since no energy is required to control the temperature throughout the storage (Duarte et al., 2014; Queirós et al., 2014). This novel food preservation methodology is considered environmentally friendlier than conventional refrigeration, as energy is only applied shortly in the compression and decompression phases of the pressure vessel, with considerably lower energy requirements (Bermejo-Prada, Colmant, Otero, & Guignon, 2017; Segovia-Bravo et al., 2012). One of the first studies concerning HS at RT was focused on strawberry juice (low pH) stored under 25, 100 and 220 MPa at 20 °C for 20 days, successfully inhibiting microbial growth even under the lower tested pressure, 25 MPa (Segovia-Bravo et al., 2012).

In the following years, the possibility to store more perishable food products, watermelon, and melon juice (low acidity), was tested under a combination of different pressures (25–150 MPa) at and above RT (25–37 °C), however only during short periods of time, from 8 to 60 h (Fidalgo et al., 2014; Queirós et al., 2014; Santos et al., 2015). All authors were able to consistently observe that for these juices a minimal pressure of 50 MPa was required to inhibit microbial growth, and above 75 MPa microbial inactivation even during short periods of time at and above RT was achieve (Queirós et al., 2014). Later, HS at and above RT was reported to be able to extend the shelf-life of non-liquid highly perishable food products (minced pork meat, whey cheese and fresh salmon) stored for longer periods, 1 to 10 days, under pressures above 75 MPa (Duarte et al., 2017; Fernandes et al., 2018; Fidalgo, Lemos, Delgadillo, & Saraiva, 2018). All these results point to the possible increase of highly perishable foods shelf-life under HS at and above RT, potentially replacing and improving the common refrigeration (RF) preservation effect.

The high investment required for the high pressure equipment acquisition seems to be the main factor limiting HS viability (Bermejo-Prada et al., 2017), however the currently available equipment's for high pressure processing (HPP), are highly more complex and exigent then the ones required of HS. For instance, HPP equipment used in the food industry usually operate at a maximum pressure of 600 MPa, with reduced processing times (3–6 min) that are financially vital for HPP, which results in high performance intensifiers. On the other hand, HS usually has great food preservation results under 75–100 MPa, and the pressurization rate does not represent a critical economic impact in HS as in HPP, pointing to significant costs reduction, which should be properly assessed.

To the best of the authors knowledge, the present study is the first work regarding HS of milk, and so, in this work the effects of HS on endogenous microbiota (total aerobic mesophiles, total aerobic psychrophiles, lactic acid bacteria, Enterobacteriaceae, coliform bacteria, yeasts and moulds), and inoculated surrogate pathogens (*Listeria innocua*, *Escherichia coli*), pathogenic *Salmonella enterica* and bacterial spores (*Bacillus subtilis*) at 50, 62, 75 and 100 MPa in raw milk under naturally variable/uncontrolled RT was evaluated and compared with RF storage under atmospheric pressure (AP). Additionally, in order to evaluate the possible effect of HS on microbial recovery, for the endogenous microbiota, a post-HS study was conducted under AP/RF.

2. Materials and methods

2.1. Raw milk samples preparation and storage

Raw milk was kindly supplied by a local dairy farm association company and milk samples were packed under aseptic conditions, inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) in UV-light sterilized, low permeability polyamide-polyethylene bags (90 μm, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal), and heat-sealed individually, avoiding as much as possible leaving air inside.

HS experiments were performed in a high pressure equipment (SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), equipped with a pressure vessel of 30 mm inner diameter and 500 mm height), at variable uncontrolled room temperature (RT). Determination of HS effect on endogenous microbiota was determined in two different sets of experiments. In the first experiment, raw milk samples were stored under 50/62/75 and 100 MPa at variable uncontrolled RT (18–22 °C) during 7, 14, 28, 39 and 60 days, and as a control, at RT and refrigeration both under atmospheric pressure (0.1 MPa, AP). In the second experiment, raw milk samples presented higher initial microbial load than those of the first experiment, and were stored under 0.1, 50, 75 and 100 MPa for 1, 5, 15, 35, 60 and 130 days at RT and, a post-HS experiment under refrigeration was conducted on those samples that had been previously stored for 15, 60 and 130 days under 75 and 100 MPa. For the Post-HS, samples were then stored under RF at AP for 5, 14, 30 and 60 days, and milk samples were subjected to microbiological analysis to evaluate the endogenous microbiota, to assess the possible effect promoted by prolonged high pressure exposure on microbial recovery under AP/RF conditions.

2.2. Inoculated pathogenic surrogate microorganisms

To assess the effect of HS on pathogenic surrogate microorganisms, raw milk was inoculated with two non-pathogenic surrogate strains, *Escherichia coli* ATCC 25922, *Listeria innocua* ATCC 33090, and pathogenic *Salmonella enterica* serovar Senftenberg ATCC 43845. The three microorganisms were previously grown in Tryptic Soy Broth (TSB; Liofilchem, Italy) at 37 °C for 24 h to ensure they reached the stationary phase. Late stationary phase is a well-known higher resistant pressure phase comparatively to the exponential-phase, where cells display a more rigid/thicker membrane and higher nucleoid condensation, which is believed to increase their viability under stress/high pressure (Mañas & Mackey, 2004). The grown microorganisms were inoculated into raw milk from the first experiment to achieve a final concentration around 4–5 log CFU/mL, and placed under different conditions, 50, 75 and 100 MPa at RT and AP/RF for comparison, during 3, 7, 10, 14, 21 and 31 days.

2.3. Bacillus subtilis endospores inoculation

Bacillus subtilis ATCC 6633 endospores preparation was performed as described by Pinto, Santos, Fidalgo, Delgadillo, and Saraiva (2018). Briefly, a liquid culture of *B. subtilis* was grown overnight in brain-heart

infusion (BHI) broth at 30 °C for 24 h, and afterwards spread-plated into BHI-agar plates, which were incubated at 30 °C for 15 days to allow sporulation to occur. Sporulation was confirmed by phase-contrast microscopy, then spores were harvested by flooding the cultures with cold (4 °C) sterile distilled water, and by scratching the agar plates with a bend glass rod, followed by 3-fold centrifugation (10 min at 5000 ×*g* at 4 °C) and stored at 4 °C until use. Endospores were then inoculated into raw milk from the first experiment, in order to reach a final concentration around 5–6 log spores/mL, and described as presumptive *B. subtilis* endospores. Vegetative *Bacillus* spp. and sporeformer bacteria endospores regarding the endogenous loads, as well as the inoculated load were studied throughout the storage, during 1, 4, 7, 21, 31 and 60 days under AP/RF, AP/RT and under 50, 75 and 100 MPa at RT. To clearly distinguish between endospore germination and inactivation, aliquots of milk were heat-treated at 80 °C for 20 min, allowing to inactivate germinated spores and vegetative forms (Black et al., 2005).

2.4. Microbial analyses

After each experiment, samples were serially diluted in Ringer's solution, except for *B. subtilis*, which was serially diluted in physiological solution (0.9% NaCl) and plated on the appropriate media. Total aerobic mesophiles (TAM) and total aerobic psychrophiles (PSY) were enumerated on plate count agar (PCA), incubated at 30 °C and 20 °C for 3 and 5 days, respectively (ISO 4833:2013). Enterobacteriaceae (ENT) counts were determined on violet red bile glucose agar (VRBGA), incubated at 37 °C for 1 day (ISO 21528:2017). Lactic acid bacteria (LAB) counts were determined on de Man Rogosa Sharpe agar (MRS) and incubated at 30 °C for 3 days (ISO 11133:2014). Coliform bacteria (COL) were enumerated on chromocult coliform agar (CCA), incubated at 37 °C for 1 day (ISO 4832: 2007). Yeasts and moulds (YM) were enumerated using rose bengal chloramphenicol agar (RBCA) at 25 °C for 5 days (ISO 21527:2008). *Listeria innocua* ATCC 33090 was determined in PALCAM Listeria agar base with the selective supplement PALCAM (FD061) and incubated at 37 °C for 2 days (ISO 11290-1:2017). *Escherichia coli* ATCC 25922 was enumerated in CCA after incubation at 37 °C for 1 day (ISO 9308-1:2014). *Salmonella enterica* ATCC 43845 was incubated on xylose lysine deoxycholate agar at 37 °C for 1 day (ISO 6579-1:2017). *Bacillus* spp. was enumerated in BHI-agar and incubated at 30 °C for 1 day (ISO 7932:2004). All the results were expressed as decimal logarithm of colony forming units per millilitre of raw milk (log CFU/mL).

2.5. D-value and z_p-value determination

Determination of the D_p- and z_p-values was carried out for the microbial groups analysed in this study for which inactivation was verified and measurable (values below the quantification and detection limits were not considered), and for all cases a first order inactivation kinetics was verified. D_p-value is the time needed at a constant pressure, to reach a decimal reduction in the microbial load (expressed here in days) and was calculated based on the negative inverse of the log linear slope (Eq. 1), while z_p-value, the pressure resistance (here expressed in MPa), was calculated based on the D_p-values of the different HS conditions for a specific microorganism type, determined as the negative reciprocal of the slope as shown Eq. 2:

$$Log\ (N) = Log\ (N_0) - \frac{t}{D_p} \quad (1)$$

N is the microbial load (CFU/ml) under a certain pressure (MPa) for certain time (t) in days, and N_0 is the initial microbial load (CFU/mL). The slop was obtained from the log linear decreased throughout storage, under a certain pressure.

$$\text{Log D} = \text{Log D}_0 - \frac{P - P_0}{z_p} \quad (2)$$

where D and D_0 (in days) are D_p-values at pressures P and P_0 (in MPa), respectively, being P_0 a reference pressure, here considered as zero MPa.

2.6. Statistical analysis

All experiments were carried out in triplicate and all analyses were done in triplicate. The different storage conditions were compared using Analysis of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance.

3. Results/discussion

3.1. Microbial analyses

The acceptable microbial limits for cows raw milk vary between countries legislation (Ledenbach & Marshall, 2009). In the EU, TAM counts below 5 log CFU/mL reflect good milk production hygiene, to be considered for further thermal processing (Nunes, 2009), while raw milk used to produce dairy products, immediately before processing, should also contain TAM counts below 5.5 log CFU/mL (EC Regulation N° 853/2004). This study was divided in two stages, with raw milk samples from the first experiment revealing a microbial load within the limits allowed for raw milk before pasteurization in the EU (TAM counts below 5 log CFU/mL (EC Regulation N° 853/2004)), and those from the second experiment containing a higher microbial load above this limit in order to simulate a worst-case scenario.

Samples from the first study presented initial microbial counts around 4.93 ± 0.05, 3.57 ± 0.02, 2.96 ± 0.06, 2.45 ± 0.11 and 2.40 ± 0.02 log CFU/mL for TAM, LAB, YM, COL and ENT respectively (Fig. 1). It is important to note that in this study, when samples from a storage condition achieved TAM counts above 5.5 log CFU/mL, the acceptable limit considered, the samples were withdrawn from the experiment and no further analyses were performed regarding such storage condition. As expected, samples stored at room temperature and atmospheric pressure (AP/RT) after 7 days of storage presented higher ($p < 0.05$) microbial counts well above the acceptable threshold (≥5.5 log CFU/mL for TAM and LAB), and of >5.00 log CFU/mL for COL, ENT and YM (Fig. 1).

Refrigerated storage (AP/RF) was able to slow down ($p > 0.05$) the microbial growth of TAM, LAB and COL bacteria up to the 7th day of storage, while ENT and YM presented higher counts ($p < 0.05$), comparatively to the initial ones, 3.46 ± 0.10 and 5.48 ± 0.06 log CFU/mL, respectively. After 14 days at AP/RF storage, raw milk was microbiologically unacceptable, with TAM, ENT, YM and COL counts reaching values above 5.5 log CFU/mL, while LAB counts presented an overall increase ($p < 0.05$) of approximately 1 log unit. Previous works reported that even under AP/RF, TAM and psychotropic bacteria (PSY) are capable of proliferate in milk and release extracellular hydrolytic enzymes (some thermoresistant) resulting in overall nutritional quality losses (Pinto, Martins, & Vanetti, 2006); YM metabolism may produce by-products that cause off-odours and unpleasant flavours later on when transformed into dairy foods (Giudici, Masini, & Caggia, 1996); and LAB, COL and ENT may produce gas, and several metabolites that favour the development of off-flavours (Bintsis, 2018; Frank, 2007).

Regarding HS, at the 7th day of storage at the lower pressure (50 MPa), all studied microorganisms were affected, being reduced either to counts below the quantification limit of 2 log CFU/mL, in the case of COL, ENT and YM, or as observed for TAM and LAB counts, undergoing a decrease ($p < 0.05$) of approximately 1 log unit. At the 14th day of storage TAM, LAB and COL were able to grow to values similar to the initial ones (still within the acceptable limit), extending the microbial shelf-life of raw milk under 50/RT comparatively to AP/RF, and it was only at the 28th day of storage that microbial counts reached values above the acceptable limit. This behaviour of TAM and LAB was also observed when watermelon juice and fresh salmon were stored under 50 MPa at 15 °C, after 3 and 6 days of storage, respectively (Fidalgo

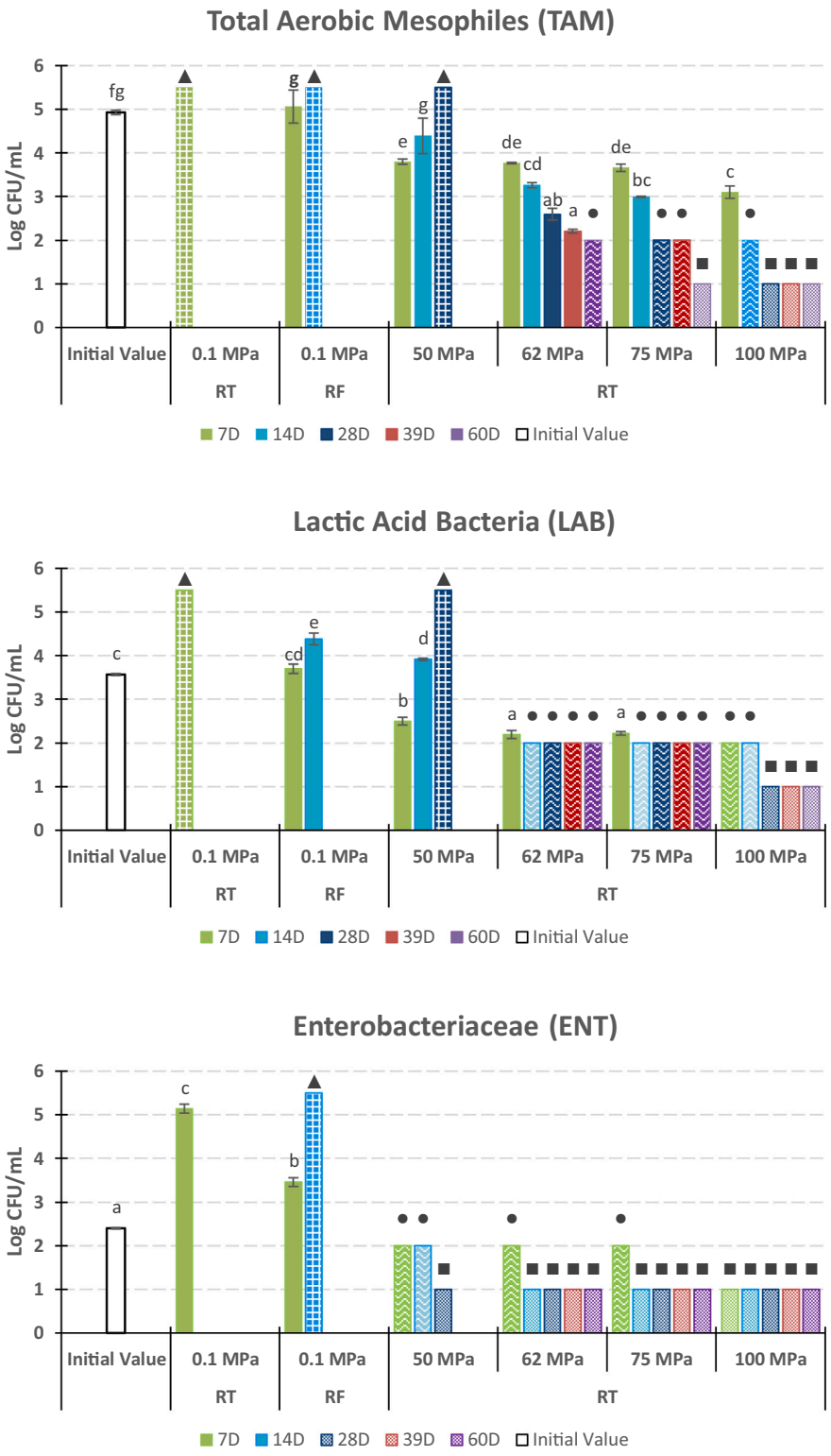

Fig. 1. TAM, LAB, ENT, COL and YM microbial evolution during HS at uncontrolled room temperature (RT) of raw milk used in the first set of experiments, and comparison with storage under refrigeration (RF) and RT at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where ▲, • and ■ represent counts above the acceptable (5.5 log CFU/mL), and below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively.

et al., 2019; Lemos, Ribeiro, Fidalgo, Delgadillo, & Saraiva, 2017). TAM microbial group is very heterogeneous, since for example, several microorganisms in milk can grow on PCA medium, from gram-positive, like *Bacillus* spp., to gram-negative bacteria like *E. coli* and other coliforms, and so, the reduction in gram-negative bacteria groups such ENT and COL could be responsible for the initial decrease in TAM microbial counts, since gram-negative bacteria tend to be more sensitive to high pressure (Tomasula et al., 2014).

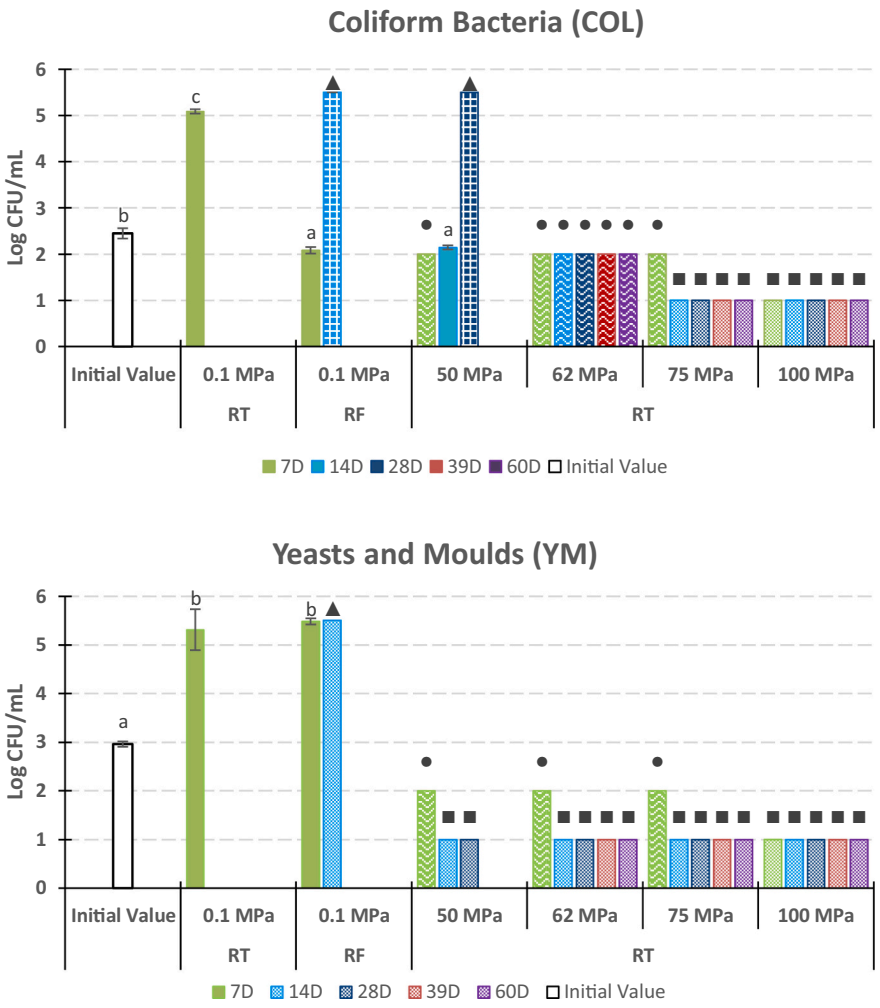

Fig. 1. (*continued*).

For HS at 62 and 75 MPa, a similar effect on the microbial load was observed for both storage conditions and after 7 days of storage, LAB, YM, COL and ENT counts were reduced microbial counts below 2 log CFU/mL, with a significant ($p < 0.05$) reduction for TAM, which presented similar values between these two storage conditions (3.77 ± 0.02 and 3.66 ± 0.08 log CFU/mL at 62 and 75 MPa, respectively). TAM counts were gradually reduced ($p < 0.05$) throughout the storage period, reaching the quantification limit at the 60th day of storage at 62/RT. This inactivation effect was faster for samples stored at 75/RT, which reached counts lower than 2 log CFU/mL right after 28 days of storage, and below 1 log CFU/mL (detection limit) at the 60th day of storage, which is in agreement with previous observations reported by Santos, Castro, Delgadillo, and Saraiva (2020), who observed a greater inactivation effect at 75 MPa over 60 MPa, for TAM and LAB counts in raw bovine minced meat throughout storage. When raw milk was stored at 100 MPa just after 7 days, ENT, YM and COL bacteria were all inactivated below the detection limit, LAB were inactivated below 2 log CFU/mL, and TAM were significantly ($p < 0.05$) reduced to 3.10 ± 0.14 log CFU/mL (2 log units reduction). Overall, the microbial load of those samples remained low with LAB and TAM achieving counts below 1 log CFU/mL at the 28th day of storage, with no further changes until the end of the study.

As observed in Fig. 1, for 50 MPa the results were comparable to RF (microbial growth slowdown) but to a greater extent, thus pointing to a possible longer microbial shelf-life extension. Additionally, higher pressures (62–100 MPa) resulted in progressively higher microbial inactivation and so better microbial proliferation control throughout storage, pointing to a minimal pressure of 62–75 MPa to maintain raw milk microbiologically stable, for at least 60 days of storage without temperature control. Noteworthy, at 100 MPa all studied microbiological groups were at least below the quantification limit after 14 days and below the detection limit onwards. Thus, all HS conditions resulted in better microbial preservation than AP/RF and it is important to highlight, HS yielded these results at RT with no energetic costs throughout storage with considerable microbial inactivation.

In the second part of the experiment, raw milk with a higher microbial load was used to simulate a worst-case scenario in order to study the effect of HS on samples with higher microbial spoilage levels and for longer storage time (130 days) at 50, 75 and 100 MPa (since in the previous study, 62 and 75 MPa storage achieved comparable results, only HS at 75 MPa was further selected) at RT and compared to storage under AP at 4 °C. The initial microbial load was 6.73 ± 0.16, 6.49 ± 0.17, 4.90 ± 0.12, 3.26 ± 0.05 and 2.79 ± 0.03 log CFU/mL for TAM, PSY, COL, ENT and YM respectively (Fig. 2). As mentioned previously, TAM counts in raw bovine milk above 5.5 log CFU/mL are beyond the acceptable limit, so in this part of the study a higher microbial limit was considered (6.5 log CFU/mL) for experiment interruption. Due to the higher spoilage levels of the milk used in the second set of experiments, initially shorter sampling periods were selected, 1 and 5 days, comparatively to 7 days studied in the first set. Just after 1 day, even at lower temperatures (AP/RF) a significant increase in COL, ENT and YM counts were observed ($p < 0.05$), which was significantly more pronounced

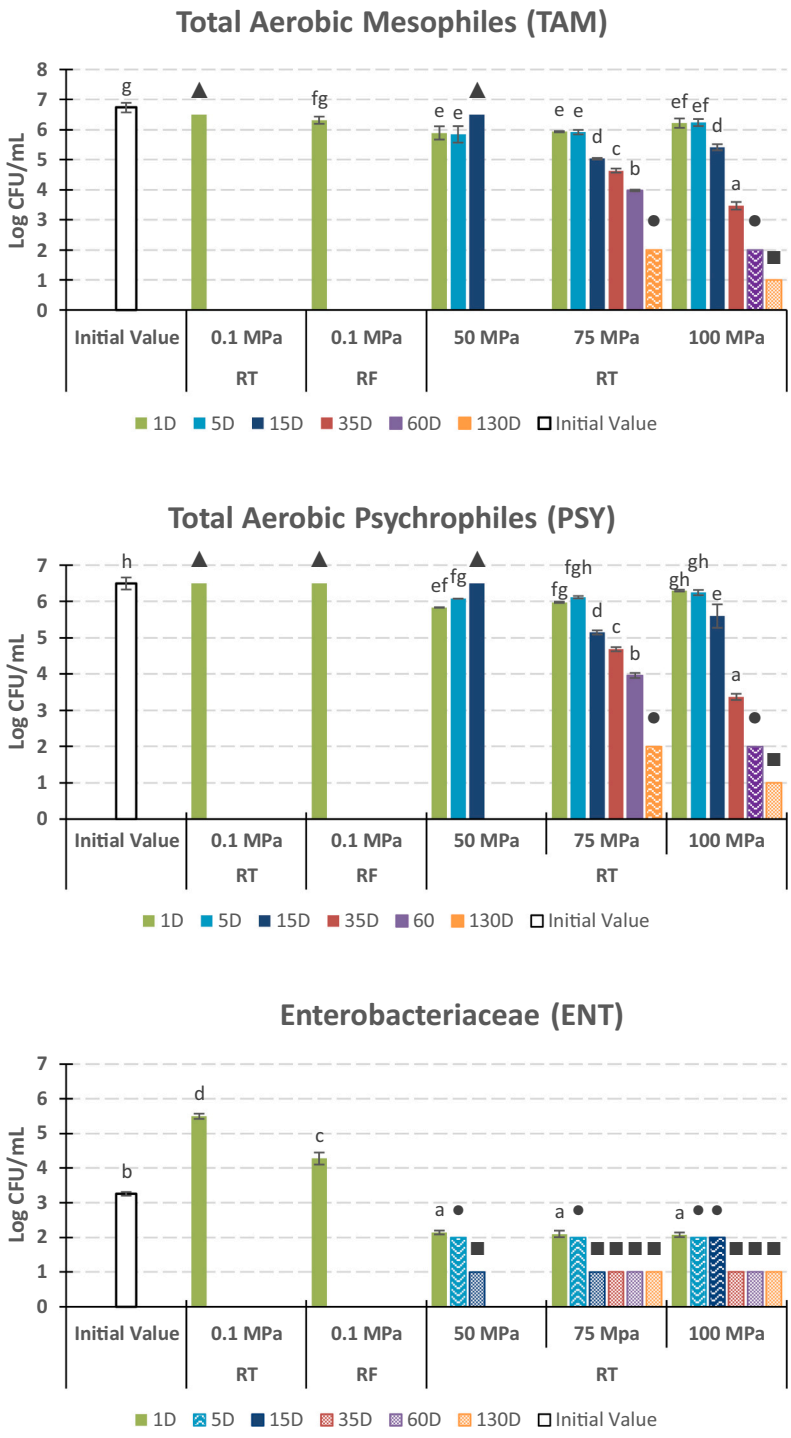

Fig. 2. TAM, PSY, ENT, COL and YM microbial evolution during HS at uncontrolled room temperature (RT) of raw milk used in the second set of experiments, and comparison with storage under refrigeration (RF) and RT at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively. While ▲, represent counts above the limit defined for this storage experiment interruption (6.5 log CFU/mL), due to considerable initial spoilage.

under storage at AP/RT, above 1 log unit (Fig. 2).

Differently and interestingly, even at the lowest pressure, 50 MPa, COL and ENT were significantly affected ($p < 0.05$) just after 1 day of storage, with a reduction of 2.70 and 1.12 log units, respectively and with YM counts being reduced to below the quantification level. As observed in the first set of experiments, for this pressure level, the more baro-resistant microbial groups (TAM and PSY) also underwent significant reductions ($p < 0.05$) in the first days, with a reduction of approximately 1 and 0.7 log units in the first day of storage, respectively. TAM and PSY growth was slowed down up to the 5th day of storage ($p < 0.05$), presenting counts around 5.84 ± 0.27 and 6.08 ± 0.01 log CFU/mL respectively, however, at the 15th day both reached counts above 6.5 log CFU/mL, while ENT, COL and YM reached counts below the detection limit.

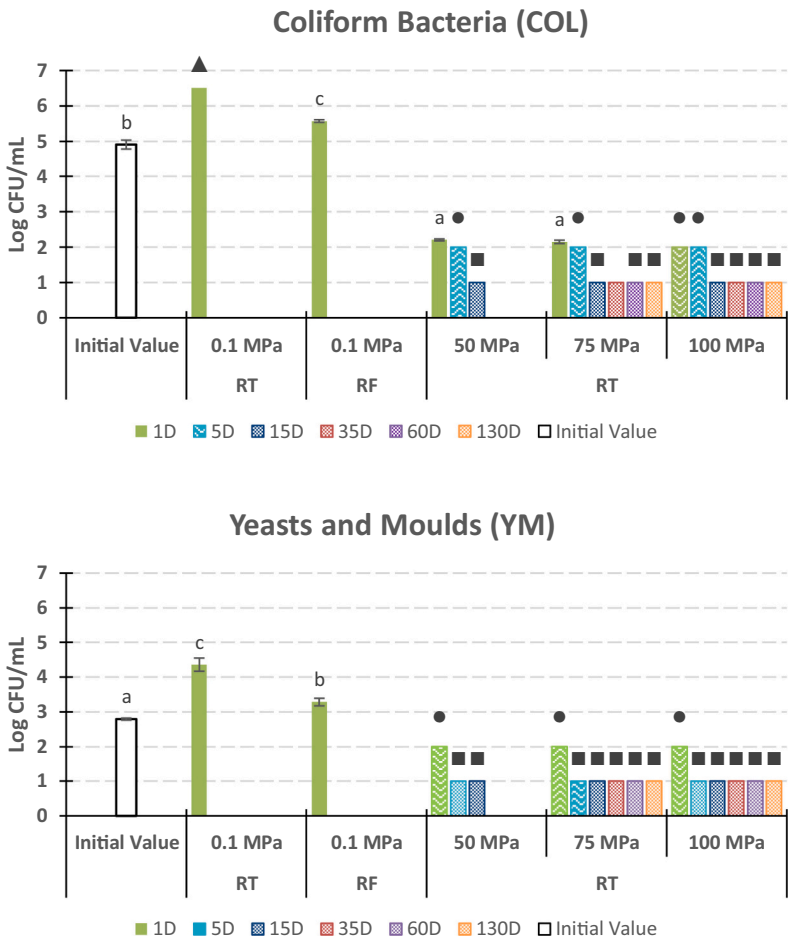

Fig. 2. (*continued*).

As for storage at 75 MPa, again ENT, COL and YM were highly susceptible to pressure, presenting counts below the detection limit by the 15th day of storage. Concurrently, storage at 75/RT in the first day reduced TAM counts around 0.80 log units ($p < 0.05$), staying stable until the 5th day, followed by a gradual reduction ($p < 0.05$) throughout storage, reaching the quantification level at the 130th day. A similar inactivation effect on PSY counts was observed, which were gradually reduced over time ($p < 0.05$), noteworthy the remarkable reduction of $\geq$4.5 log units at the 130th day of storage, when compared to the initial load. It is relevant to note the importance in quality and proper management of raw milk, and its impact in the initial microbial load, as it took more than 4 times longer for samples used in the second experiment to reach the quantification limit, when compared to samples used in the first experiment (initial load of 4.93 $\pm$ 0.05 and 6.73 $\pm$ 0.16 log CFU/mL, regarding TAM counts, respectively). As for 100/RT the inactivation effect was more pronounced when compared to 75/RT (Fig. 2), with TAM and PSY counts inactivated faster throughout the storage, reaching values below the detection limit after 130 days of storage.

Differences in the inactivation rates between the different HS conditions can also be assessed by the calculated D_p-values (*supplementary material, Fig. S1 and Table S1*). Storage under 75 and 100 MPa resulted in D_p-values of 25.6 and 12.8 days for TAM, respectively, similarly to PSY, D_p-values of 25.6 and 13.0 days at 75 and 100 MPa, respectively. When comparing the D_p-values of TAM and PSY under 100/RT over 75/RT, the inactivation was 2 times faster under 100/RT, with both microbial groups reaching counts below the quantification level at day 60 and 130 of storage under 100 and 75 MPa, respectively.

3.1.1. Post-hyperbaric storage

A post hyperbaric storage (PHS) was carried out after samples (used in the second set of experiments) had been under HS and consisted in storing them at AP/RF, to evaluate possible impairment in microbial recovery. The samples selected for PHS were the ones stored first under 75 and 100 MPa for 15, 60 and 130 days at RT, presenting distinct levels of exposure and intensity to pressure. Samples stored under HS for 15 days at both 75 and 100 MPa (75 MPa/15d and 100 MPa/15d), showed reduced PSY microbial growth over time when stored at AP/RF (Fig. 3), increasing around 1 and 0.6 log units after 15 days ($p < 0.05$), respectively, which is still lower than the acceptable limit selected in the PHS study (6.5 log CFU/mL).

Samples stored under 75 MPa/60d presented no signs of microbial proliferation in the first 5 days under AP/RF ($p > 0.05$), however at the 30th day of storage, PSY and TAM reached counts of 6.58 $\pm$ 0.20 and 6.65 $\pm$ 0.17 log CFU/mL, respectively ($p < 0.05$) (Fig. 3 and *supplementary material, Fig. S2*, respectively). On the other hand, for the higher pressure, samples that initially presented counts below the quantification limit (100 MPa/60d), remained low even after 60 days under AP/RF, regarding both TAM and PSY counts. The same behaviour was observed for samples stored at 75 MPa/130d and 100 MPa/130d, to which TAM and PSY counts remained below the quantification and detection limit, respectively, after 28 days under AP/RF. Other microbial groups (ENT, COL and YM) that were already below the detection limit for samples initially stored under 75 and 100 MPa for 15, 60 and 130 days at RT, remained undetectable ($\leq$1 log CFU/mL) during the PHS period (*data not shown*).

Low HP (20–200 MPa) has proven to interfere with several mechanisms associated to cellular viability, affecting, for instance, membrane

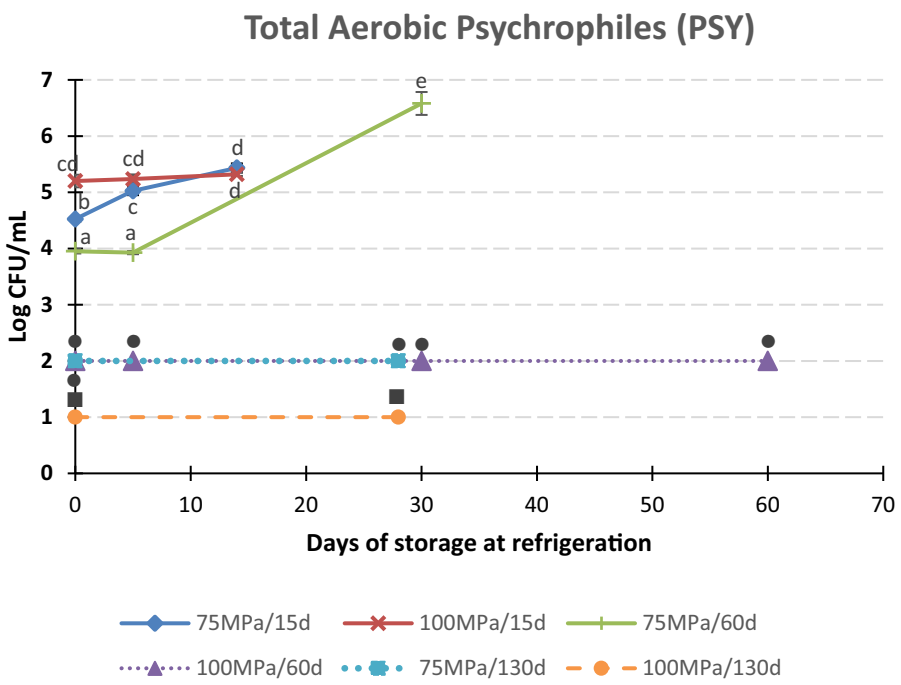

Fig. 3. PSY microbial evolution during PHS under refrigeration (4 °C) of raw milk used in the second set of experiments, stored under HS of 75 and 100 MPa for 15, 60 and 130 days at room temperature (RT). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively.

stability, ribosomes association, nutrient uptake, gene expression such as replication and transcription (Abe, 2007). The magnitude to which these effects may result in cellular death, are not only related to the intensity of HP, but also on other factors such as HP duration, pH, and medium composition (Bull, Hayman, Stewart, Szabo, & Knabel, 2005). After prolonged exposure to pressure during HS, the remaining viable microbial cells would supposedly require more time and resources for full cellular recovery, which would allow microbial growth after the imposed sub-lethal damage. As reported before, temperature plays a crucial role in cell recovery after HPP, in a study conducted with *E. coli* (Koseki & Yamamoto, 2006) and another with *Listeria monocytogenes* (Bull et al., 2005), both microorganisms presented a better recovery rate when incubated at 25 and 15 °C, respectively, when compared to post incubation under AP/RF.

In the present work, the results obtained during the PHS period may indicate that the degree of intracellular injury could be related to the duration and intensity of HS. For instance, samples kept at 75 MPa/15d and 100 MPa/15d, when stored under RF, presented different growth rates. Regarding PSY counts, 75 MPa/15d showed significant growth ($p < 0.05$) both at day 5 and 15, while on the other hand, PSY counts of 100 MPa/15d condition, remained stable during the 15 days ($p > 0.05$) at AP/RF. This may indicate, since no information in available regarding the effect on microbial recovery after such longer exposure times to HP, that longer periods under HS increase the intensity of sub-lethal damages, which will decrease the ability to recover afterwards. Samples kept at 100 MPa/60d, when placed at AP/RF, presented a stable microbial load evolution, even after 60 days at AP/RF. This may indicate a greater microbial stability of raw milk when stored at AP/RF after HS, which can also contribute to an extended shelf-life under PHS.

3.2. Inoculated microorganisms

Campylobacter, *L. monocytogenes*, pathogenic *E. coli* and *Salmonella* are among the most commonly and important epidemiological pathogens found in milk, which contamination may derive mainly from improper raw milk handling or processing (LeJeune & Rajala-Schultz, 2009; Quigley et al., 2013). Raw milk was inoculated with two pathogenic-surrogate microorganisms, *E. coli* ATCC 25922 and *L. innocua* ATCC 33090, with pathogenic *S. enterica* ATCC 43845 (to a final concentration around 5 log CFU/mL) and then stored under AP/RF, and also under 50, 75 and 100 MPa at RT. Prior to raw milk inoculation, evaluation of endogenous *E. coli, Salmonella* and *Listeria* was conducted, with the last ones being undetected (below the detection limit), while *E. coli* was below the quantification level (2 log CFU/mL).

After inoculation, the initial load for *E. coli*, *S. enterica* and *L.innocua* was 5.07 ± 0.04, 4.85 ± 0.04 and 5.02 ± 0.13 log CFU/mL, respectively (Fig. 4). When stored under refrigeration, all microorganisms were initially affected ($p < 0.05$), with a reduction around 0.4, 1.0 and 0.4 log units on day 3, for *E. coli*, *S. enterica* and *L. innocua*, respectively. This could result from difficulties in adaptation for the inoculated microorganisms to the new environment (raw milk), and since raw milk was not heat treated, this initial decrease could be related to the competition between the endogenous microbiota, like lactic acid bacteria with the inoculated microorganisms (Arias, Monge-Rojas, Chaves, & Antillón, 2001). *Escherichia coli* was able to retain its counts at constant levels during refrigerated storage without significant growth after the 7th day ($p > 0.05$), similarly to that observed by Zapico, Gaya, Nuñez, and Medina (1995) and Guraya, Frank, and Hassan (1998), wherein *E. coli* stored at AP/RF maintained similar counts from the beginning until the last days of storage, 7th and 35th days, respectively. Indeed, this microorganism is able to survive and maintain high viable cell numbers, even after longer storage periods at refrigerated temperatures (Guraya et al., 1998).

When placed under HS, at the 3rd day, *E. coli* counts were gradually reduced ($p < 0.05$) at the lowest pressure (50 MPa), reaching counts bellow the quantification and detection limits at day 10 and 21 of storage, respectively, corresponding to a D_p-value of 3.7 days (*supplementary material, Fig. S3 and Table S1*). The inactivation effect was stronger for 75 MPa, reaching values below the detection limit at the 10th day, remaining constant throughout the storage. Under 100 MPa, *E. coli* counts were already absent at day 3 (and even after 31 days of storage), highlighting the fast inactivation effect of this storage condition, when compared to the lowest one studied. *Escherichia coli* O157:H7 is the most prominent pathogenic strain of *E. coli*, which can cause food

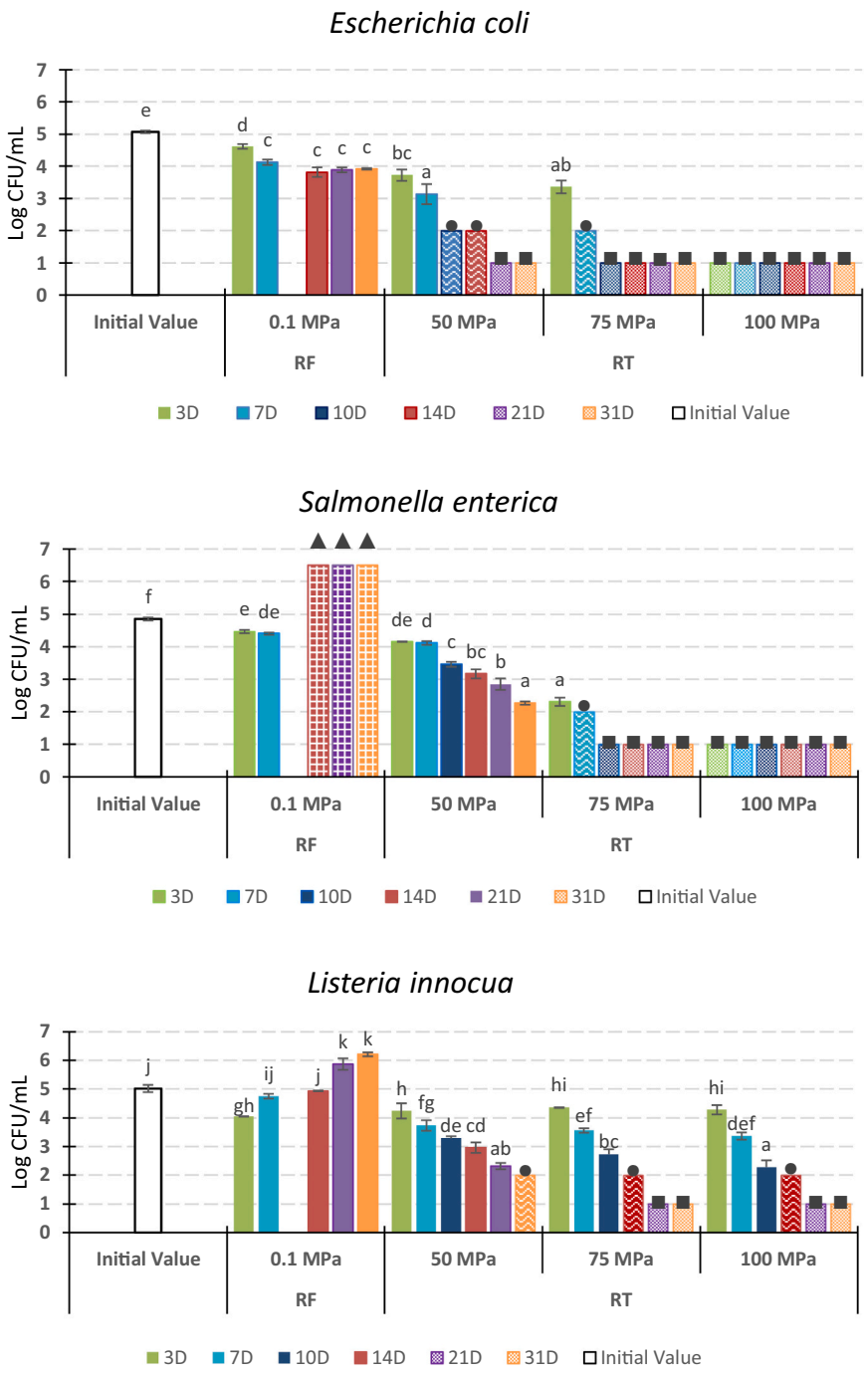

Fig. 4. Evolution of viable cell numbers of inoculated *Escherichia coli*, *Salmonella enterica* and *Listeria innocua* during HS at room temperature (RT) of raw milk, and comparison with storage under refrigeration (RF) at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively. While ▲ represent counts above the limit defined for this storage experiment interruption (6.5 log CFU/mL).

poisoning illness even in low numbers (that could be as low as 10 cells), and thus, if *E. coli* O157:H7 survives pasteurization, it is important to keep this microorganism absent (Bolton, Crozier, & Williamson, 1996; Phillips, 1999).

Salmonella enterica, the other gram-negative microorganism studied, presented a similar behaviour under HS, being inactivated below the detection limit after 10 and 3 days when stored under 75 and 100 MPa, respectively. At 50/RT, *S. enterica* counts were gradually reduced ($p < 0.05$) along storage, reaching a minimum of 2.27 ± 0.05 log CFU/mL at the end of storage experiments (Fig. 4), resulting in a calculated D_p-value of 12.7 days (*supplementary material in Fig. S3 and Table S1*). While some species of *Salmonella* do not grow at temperatures below 6 °C, others are able to grow although at a slower rate (Muir, 1996) and, despite the initial decrease in *S. enterica* counts when placed under refrigeration, it ended up reaching higher counts (≥6.5 log CFU/mL) at the 14th day, outlining the need to implement suitable preservation methods capable to inhibit the growth or even inactivate several critical pathogenic microorganisms that can grow under AP/RF before/after pasteurization, and thus preventing food safety issues.

Listeria monocytogenes is a well-known gram-positive psychrophilic microorganism, capable to grow under refrigerated temperatures as low as 0.4 °C and up to 42 °C (Muir, 1996; Sergelidis et al., 1997). In the present study, after the 3rd day of storage under AP/RF, *L. innocua* was able to increase its counts slowly, surpassing the initial load on day 21, reaching around 6.22 ± 0.07 log CFU/mL at the 31 day of storage ($p < 0.05$), presenting in this case a rate increase of 0.076 log CFU/mL per day (*supplementary material, Fig. S3 and Table S1*).

Initially, inoculated samples stored under HS presented similar

values at day 3 when compared to AP/RT, however, *L. innocua* counts decreased continuously in the following days under all three HS conditions ($p < 0.05$). *Listeria innocua* counts were gradually reduced under 50/RT throughout the storage ($p < 0.05$), reaching values below 2 log CFU/mL at day 31. This trend is quite interesting considering that, in previous studies performed by Pinto et al. (2017) it was demonstrated that *L.innocua* was able to proliferate in watermelon juice stored under HS at 50 MPa (for 10 days), reaching values above 6 log CFU/mL. The difference between this study and the aforementioned one, may be due to the common presence of lactic acid bacteria in milk, which has shown to contribute to the inhibition of spoilage and pathogenic microorganisms, present in the composition of dairy products (Grattepanche, Miescher-Schwenninger, Meile, & Lacroix, 2008).

Storage under 75 and 100/RT caused similar reductions between these two storage conditions on *L.innocua* counts ($p > 0.05$) relatively to similar storage periods, with the exception being on day 10, where 100/RT samples presented a significant reduction around 0.5 log units ($p < 0.05$), comparatively to samples stored under 75/RT. As mentioned, all HS conditions were able to inactivate *L.innocua*, however at different rates, with D_p-values of 8.6, 4.5 and 3.74 days for 50/RT, 75/RT, and 100/RT respectively (*supplementary material, Fig. S3 and Table S1*), with a Z_p of 138.9 MPa. Listeria monocytogenes is stated in the literature to have a minimal dose that may cause food poisoning of around 10 to 100 cells (Golnazarian, Donnelly, Pintauro, & Howard, 1989; Schlech, 1988) with milk and other dairy products considered one of the main vehicles types for human infection, with a lethality around 30% caused from listeriosis (Barancelli, Silva-Cruz, Porto, & Oliveira, 2011; Rocourt, BenEmbarek, Toyofuku, & Schlundt, 2003). Even after processing, *L. monocytogenes* can recover at lower temperatures during storage, as described by Ritz, Pilet, Jugiau, Rama, and Federighi (2006), where *L. monocytogenes* was able to recover and grow when placed under AP/RF after HPP of 400 MPa/10 min.

Listeria is a persistent problem in the food industry, mainly due to its ability to produce biofilms, a three-dimensional matrix of extracellular polymeric substances, that acts as a reservoir for *Listeria* colonies, offering protection against antimicrobial agents (Djordjevic, Wiedmann, & McLandsborough, 2002). These protected reservoirs can also allow the growth of spoilage bacteria, being located in places where water is abundant and where cleaning in not performed adequately (Borucki, Peppin, White, Loge, & Call, 2003). Considering the aforementioned and the results obtained regarding *L. innocua*, this may be a good indication for the implementation of HS in the future.

Under HS (75 and 100 MPa), *L. innocua* appears to be more pressure resistant than the other ones studied, with *L.innocua* counts reaching values below the detection limit under 75/RT and 100/RT at day 21 for both conditions, comparatively to raw milk inoculated with *E. coli* and *S. enterica* that reached the same level of inactivation on day 3 and 7 for 100/RT and 75/RT, respectively. As mentioned before, one of the main targets of HP for pasteurization is the microbial membrane (Georget et al., 2015; Morimatsu, Inaoka, Nakaura, & Yamamoto, 2019), affecting its fluidity, stability, and integrity of membrane-bound protein, compromising the normal membrane functions that can result in no osmotic response and in intercellular material leakage (Abe, 2007; Huang, Lung, Yang, & Wang, 2014). Gram-positive microorganisms are characterized by a thicker peptidoglycan layer when compared to gram-negative microorganisms, which reflects a greater pressure resistance (Alpas et al., 1999; Patterson, Quinn, Simpson, & Gilmour, 1995).

3.3. *B. subtilis* vegetative and endospores load

Bacillus spp. are widely present in the natural microbiota of raw milk and can be introduced from soil, bedding materials, silage, faeces, water, and feed (Magnusson, Christiansson, & Svensson, 2007; Slaghuis, Te Giffel, Beumer, & André, 1997). *Bacillus cereus* is of high interest in the dairy industry since this pathogen can form heat-resistant endospores and produce toxins (Gopal et al., 2015). *Bacillus subtilis* is also commonly found in dairy environments and has been used as surrogate endospores form of *B. cereus* in several food inactivation models (Jagannath & Tsuchido, 2003). Non inoculated raw milk was microbiologically evaluated, as a control, for *Bacillus* spp. total endogenous vegetative and total endogenous sporeformer bacteria endospores loads, the ones that survived the heat treatment (80 °C for 20 min), in all tested storage conditions. After inoculation, raw milk contained both endogenous and inoculated endospores.

Initially it was observed that the endogenous vegetative load of *Bacillus* spp. was naturally high in raw milk samples, ranging from 6.06 ± 0.04 to 6.22 ± 0.02 log CFU/mL, with also an endogenous sporeformer bacteria endospores load of 3.26 ± 0.07 log spores/mL, which is within the values reported in the literature (Magnusson et al., 2007), increasing after inoculation to counts of 5.59 ± 0.10 log spores/mL regarding endospores (Fig. 5). Endogenous sporeformer bacteria endospores load in control samples, presented a similar behaviour in all storage conditions, comparatively to the inoculated endospores ones, and thus are not represented in Fig. 5.

At AP/RT storage, regarding inoculated samples, both vegetative and endospores load increased significantly after 1 day ($p < 0.05$), around 2 and 0.2 log units respectively, reaching a total of 8.54 ± 0.02 log CFU/mL and 6.40 ± 0.36 log spores/mL, respectively, on the 4th day of storage ($p < 0.05$). This increase in overall *Bacillus* spp. load was also observed in non-inoculated samples, possibly due to the increase of microbial population in raw milk samples leading to nutrient depletion and pH decrease which often initiate endospores complex development (Coorevits et al., 2011).

Under AP/RF, inoculated samples of *Bacillus* spp. vegetative load increased around 1.6 log units after 7 days ($p < 0.05$), remaining constant ($p > 0.05$) until the end of the storage period (8.07 ± 0.09 log spores/mL), with the endospores load remaining relatively constant throughout storage ($p > 0.05$).

Interestingly, storage under HS/RT presented different results, regarding the lower (50 MPa) and the higher pressures (75 and 100 MPa). As for the vegetative load of inoculated samples, 50/RT was insufficient to inhibit the growth of *Bacillus* spp., allowing a significant growth ($p < 0.05$) throughout the studied period, reaching counts of 7.14 ± 0.02 log CFU/mL on the 31st day. Storage at 75/RT and 100/RT, were able to successfully inactivate *Bacillus* spp. vegetative load along the storage time ($p < 0.05$), being the inactivation superior for 100/RT, with both storage conditions allowing a gradual decrease in microbial counts to 3.08 ± 0.05 and 2.40 ± 0.17 log CFU/mL after two months of storage, under 75/RT and 100/RT, respectively. These two storage conditions presented a D_p-value of 21.7 and 16.0 days, regarding *Bacillus* spp. vegetative load inactivation, under 75 and 100 MPa, respectively (*supplementary material, Fig. S4 and Table S1*).

Regarding the presumptive *B. subtilis* endospores load, storage at 50/RT slightly reduced their counts until the 21st day ($p < 0.05$), to 4.73 ± 0.10 log spores/mL, which increased at the 31st day, to 5.10 ± 0.08 log spores/mL ($p > 0.05$), while a significant reduction in the endospores load ($p < 0.05$) was observed at 75/RT, about 1 log unit just after one day and inactivation to counts below the quantification level (2.30 log spores/mL) on the 21st day, reaching counts below the detection level (1.30 log spores/mL) on the 60th day. A faster inactivation effect was observed for 100/RT ($p < 0.05$), reducing the presumptive *B. subtilis* endospores counts below the quantification level on the 7th day, and reaching undetectable counts on the 31st day, remaining thereafter constantly low, until the end of the storage period. In fact, 100/RT was more than two-fold faster at inactivating the development of the endospores, with a D_p-value of 2.4 days, comparatively to 75/RT, D_p-value of 6.7 days, while a higher D_p-value was achieved under 50/RT of 27.0 days, resulting in a z_p of 47.6 MPa (*supplementary material, Fig. S4 and Table S1*).

Endospores are highly resistant to extreme conditions such as pressure, extreme heat or cold, drought, biocides, and UV irradiation (Gopal et al., 2015), although low pressure (40–100 MPa) has been proved to

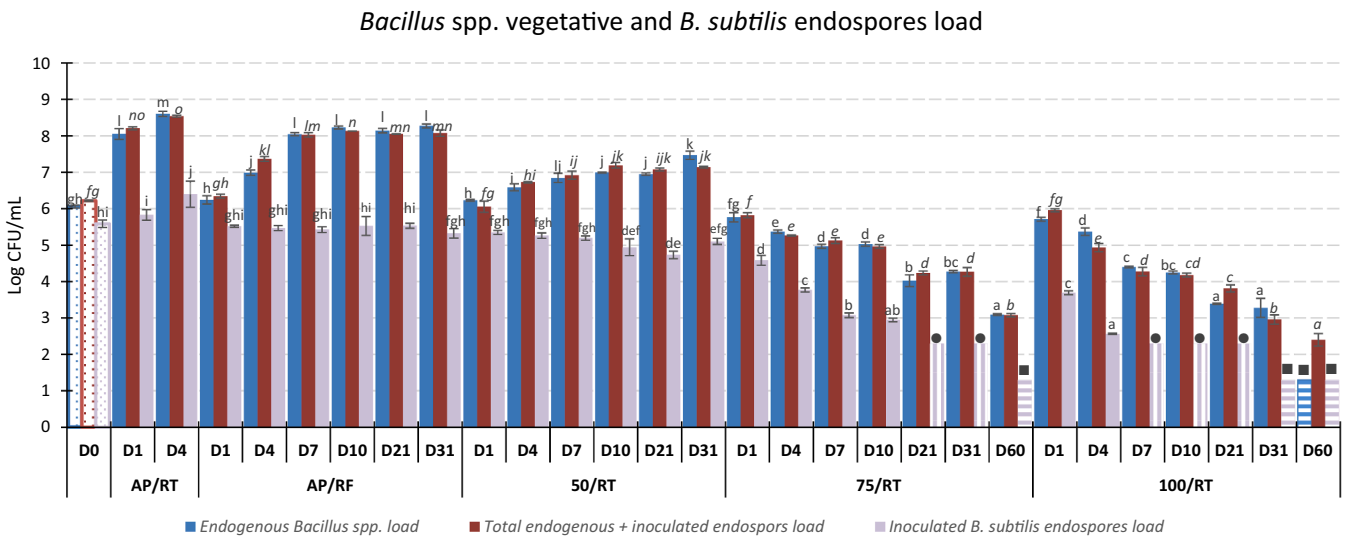

Fig. 5. Total *Bacillus* spp. vegetative endogenous load, total vegetative endogenous plus inoculated endospores load and inoculated *B. subtilis* endospores load (non-germinated) evolution during HS (50, 75 and 100 MPa) at room temperature (RT) in raw milk, and comparison with storage under refrigeration (RF) and RT under atmospheric pressure (AP). Vegetative population of *Bacillus* spp. is expressed in log CFU/mL, while *B. subtilis* endospores load is expressed in log spores/mL. Different letters denote statistically significant differences ($p < 0.05$) between storage conditions at the respective storage period, where • and ■ represent counts below 2.30 and 1.30 log units, respectively.

induce germination in combination with the available nutrients, through activation of the nutrient-like receptors gerA gerB and gerD, by inducing conformational changes in their active sites (Wuytack, Soons, Poschet, & Michiels, 2000). Therefore, HS may trigger endospores germination, followed by outgrowth inhibition due to pressure, and thus resulting in endospore inactivation under pressures equal to above of 75 MPa, as observed in the present work for raw milk.

The level of pressure required to promote endospores inactivation seems to be related to the products pH value and overall nutritional composition, since as Pinto et al. (2019) observed for *Alicyclobacillus acidoterrestris* spores in apple juice (pH 3.50), a minimum of 25 MPa at RT was sufficient for both endospores and vegetative load inactivation, while on a more optimal growth matrix (like BHI-broth, pH 6), higher pressures (≥50 MPa) were required in order to achieve the same microbiological effect (Pinto et al., 2018). Noteworthy, storage under 100 MPa successfully reduced the high levels of presumptive *B. subtilis* endospores, at a rate of 1 log unit per 2.4 days, to constant undetectable levels from the 31st day, until the end of the storage period. Interestingly, D_p-values for presumptive *B. subtilis* endospores were found to be lower than for its vegetative form, which might be hypothesised above, HS may trigger endospores germination, thus stimulating them to germinate, followed by outgrowth inhibition due to pressure. As far as the authors are aware, this was the first study that allowed the determination of D_p and Z_p-values in some of the endogenous microbiota, inoculated pathogenic surrogate vegetative bacteria and in *B. subtilis* endospores, studied under HS conditions.

4. Conclusions

In this study, despite the raw milk level of spoilage, HS at uncontrolled RT performed much better than RF, requiring pressures between 62 and 75 MPa, to not only inhibit the microbial growth of TAM, PSY, LAB, ENT, COL and YM, but also to promote microbial inactivation to undetectable levels at least for two months. Post-hyperbaric storage of samples under 75 and 100 MPa, points to HS capacity to slow down microbial recovery from sub-lethal damage, when stored further under AP/RF, leading to a more microbial stable product after HS. Also, HS was able to restrain the growth of the surrogate pathogenic microorganisms studied, contributing to a microbiological safer product, even under 50 MPa. Furthermore, it is noteworthy the capacity of HS (≥75 MPa) to inactivate *B. subtilis* endospores, a highly resistant bacterial to thermal treatment and very relevant endospore in the food industry. The good microbial preservation of raw milk under HS allowed additionally to maintain the majority of the physicochemical, rheological, nutritional and enzymatic profiles of raw milk (*data not shown – under publication)*, even after 60 days at variable room temperature.

Despite the need for further scientific and technological research, HS could have a significant impact when applied to raw milk contributing significantly to its increased microbial safety and considerable enhanced shelf-life, compared to refrigeration (up to at least two months, the longest storage period studied in this work). In addition, being *quasi*-energetically costless, comparatively to refrigeration and so it deserves further studies, namely in what concerns the nutritional and sensorial quality.

CRediT authorship contribution statement

Ricardo V. Duarte: Conceptualization, Investigation, Writing – original draft. **Carlos A. Pinto:** Investigation, Writing – review & editing. **Ana M. Gomes:** Supervision, Resources, Writing – review & editing. **Ivonne Delgadillo:** Supervision, Resources, Writing – review & editing. **Jorge A. Saraiva:** Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2022.103019.

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