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EFFECT OF HIGH PRESSURE PROCESSING ON THE SAFETY, SHELF LIFE AND QUALITY OF RAW MILK

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Keywords

Raw milk, high pressure, safety, shelf life, colour, stability

Abstract

High pressure processing (HPP) was investigated as an alternative to standard raw milk processing. Different pressure levels (400-600 MPa) and exposure times (1-5 min) were tested against artificially inoculated pathogenic *E. coli*, *Salmonella* and *L. monocytogenes*. HPP effectively inactivated bacterial concentration by 5 log CFU/ml. CFU/ml. The most effective HPP conditions in terms of pathogen reduction were subsequently utilised to determine the effect of pressure on microbiological shelf life, particle size and colour of milk during refrigerated storage. Results were compared to pasteurised and raw milk. HPP (600 MPa for 3 min) also significantly reduced the total viable counts, Enterobacteriaceae, lactic acid bacteria and *Pseudomonas* spp. in milk thus prolonging the microbiological shelf life of milk by 1 week compared to pasteurised milk. Particle size distribution curves of raw, pasteurised and HPP milk, showed that raw and HPP milk had more similar casein and fat particle sizes compared to pasteurised milk. The results of this study show the possibility of using HPP to eliminate pathogens present in milk while maintaining key quality characteristics similar to those of raw milk.

1. INTRODUCTION

Recently, a strong preference for food products and ingredients that are natural has emerged amongst consumers (Murphy, Martin, Barbano, & Wiedmann, 2016; Melini, Melini, Luziatelli, & Ruzzi, 2017). Therefore, the demand for fresh-like food, with high nutrient content and high organoleptic quality has steadily increased (Hong & Wang, 2015). In this regard, the consumption of raw milk, and dairy products made from raw milk is increasingly considered desirable by some consumers.

Raw milk has been identified as a cause of foodborne illness outbreaks in many cases (Rodriguez, Arques, Nunez, Gaya, & Medina 2005; Oliver et al. 2005; Tambekar, & Bhutda, 2010). According to the European Food Safety Authority, 27 illness outbreaks took place within the EU between 2007 and 2012 which were linked with the consumption of raw milk (EFSA 2015). The presence and level of pathogens in milk is determined by different factors, such as season, farm size, farm hygiene and management practices and milking (Griffiths, 2010). Transmission to raw milk can take place either from zoonotic pathogens present within animals or from the environment. Specifically, raw milk can become contaminated with pathogenic bacteria by direct passage from the animal's blood into milk and externally via faecal contamination or contamination from humans. Thus, dairy farms are an important reservoir of various foodborne pathogens (Oliver, Jayarao, & Almeida, 2005). Pathogenic *Escherichia coli*, *Salmonella spp.* and *Listeria monocytogenes* are amongst the most common pathogenic bacteria found in milk and some of the most commonly reported gastrointestinal bacterial pathogens in humans in the European Union causing milk-borne infections, intoxications and toxicoinfections (Dhanashekar, Akkinapalli, & Nellutla, 2012; EFSA 2016; Melini et al., 2017). Therefore, pathogens in milk represent a safety risk that needs to be managed.

The majority of the countries require raw milk to undergo some level of thermal processing (e.g. 72 °C for 15 s, 135 - 150 °C for 1-4 s, 105 - 120 °C for 20 - 40 min) in order to be rendered safe for the consumer (Griffiths 2010; Melini et al., 2017). However, conventional thermal treatment can have a detrimental effect on the nutrient content of milk as well as on its organoleptic and physicochemical properties (Buckow, Chandry, Ng, McAuley, & Swanson, 2014). The recent interest in the consumption of raw milk has led to the consideration of alternative processing technologies for production of milk that is safe but also minimally processed in order to be perceived as fresh by the consumer (Román, Sánchez-Siles, & Siegrist, 2017). The utilisation of emerging non-thermal technologies has been explored as means to decrease the negative effects of conventional processing technologies and present promising alternatives for the dairy sector. High-pressure processing (HPP) is a food preservation technology that is a promising alternative to conventional thermal pasteurization as it can inactivate foodborne pathogens while minimising the loss of nutrients, such as vitamins, and maintaining the fresh-like characteristics of food products (Lee & Kaletunç 2010; Yang et al. 2012; Yao et al. 2014; Sheen, Cassidy, Scullen, & Sommers, 2015). HPP, although very efficient in eliminating vegetative microorganisms, has little or no effect on bacterial spores, when applied at ambient temperatures, so it is important to take into consideration that *Bacillus* spp. and other spore-forming microorganisms may not be inactivated in milk or other dairy products and may go on to cause spoilage issues or represent food safety concerns.

HPP can also influence the physicochemical and technological characteristics of milk by modifying the structure of milk components (Patterson, 2005; Cadesky, Walkling-Ribeiro, Kriner, Karwe, & Moraru, 2017). Pressurization can result in conformational changes of milk proteins as it can disrupt milk casein micelles as well as the structure of whey proteins (Chawla, Patil, & Singh, 2011). It does not seem to affect lactose in milk which suggests that

no Maillard or lactose isomerization reaction takes place in milk as a result of pressure treatment (Lopez-Fandino, Carrascosa, & Olano, 1996).

The aim of the present study was to compare the effects of HPP on the microbiological safety, the microbiological shelf life and the quality of raw milk with those of conventional heat pasteurization and an untreated, raw milk control.

2. MATERIALS AND METHODS

2.1. Preparation of *E. coli*, *Salmonella* and *L. monocytogenes* inoculum

5 strain cocktail of the three pathogenic microorganisms was inoculated into raw milk samples separately in three different inoculation studies. The cocktail of *E. coli* consisted of NCTC 11601, NCTC 11602, NCTC 11603, NCTC 9706 and NCTC 9707. The *Salmonella* cocktail consisted of *Salmonella* Senftenberg, *Salmonella* Typhimurium, *Salmonella* Anatum, *Salmonella* Agona and *Salmonella* Saint Paul. The *L. monocytogenes* cocktail consisted of FMT 1750, NCTC 11994, NCTC 5214, NCTC 10888 and NCTC 19118 strains. These cocktails contained some relatively pressure-resistant strains, a *L. monocytogenes* strain associated with an outbreak in soft cheese and a *L. monocytogenes* strain isolated from a dairy processing environment.

For each *E. coli*, *Salmonella* and *L. monocytogenes* strain used, a loopful of a fresh tryptone soya agar (Oxoid code CM0131) + 0.6% yeast extract (Oxoid code LP0021) (TSAYE) slope culture was inoculated into 10 ml of brain heart infusion broth (BHI) (Oxoid code CM1135) and incubated at 37 °C for 24 h. Subsequently 100 µl of a 10⁻⁴ dilution of this broth was inoculated into another 10 ml BHI broth and incubated at 37 °C for either 24 h or 48 h, until the stationary phase of growth was reached. The final 10 ml cultures were centrifuged at 3600 × g, for 30 min, washed twice in phosphate-buffered saline (PBS) and the pellet re-suspended in a final volume of 1 ml PBS to give approximately 10⁹-10¹⁰ CFU/ml. The suspensions of all 5 strains for each pathogenic microorganism were combined and mixed

well. The combined suspensions were inoculated (100 µl) into different raw milk samples (10 ml), to give a level of approximately 7-8 log CFU/ml. The 10 ml samples were transferred to polyethylene/polyamide pouches (Somerville Packaging, Lisburn, Northern Ireland) and the pouches heat sealed, excluding as much air as possible. For pressure treatment, the pouches were vacuum packed in a larger pouch and the vacuum pouches were packed in an outer bag containing 5% Anistel disinfectant. Inoculated samples were held for 24 h before pressure treatment to allow time for the bacteria to acclimatise to the substrate. 48 h after HPP, three samples in total for each of the 3 different treatments and each pathogenic microorganism were opened aseptically and the contents were aseptically transferred to a sterile plastic test-tube. Required decimal dilutions were prepared in maximum recovery diluent (MRD) (Oxoid code CM733).

2.2. Raw milk sample preparation and processing

Three separate milk batches were supplied by The Village Dairy, Clonmore, Killeslin, Co. Carlow, Ireland. For each batch raw milk samples were placed either in plastic bottles for heat treatment or in polyethylene/polyamide pouches for HPP, then heat sealed, excluding as much air as possible. Inoculated packaged raw milk samples were heat pasteurised (controls) in a water bath at $72\text{ }^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 5 min (with agitation of the bottles). Pressure treatment of inoculated packaged raw milk samples was performed in a commercial-scale high pressure press (Quintus 35L, Avure Technologies, U.S.A.), with a pressure vessel of 35 L volume. The pressure transmission fluid used was potable water. The pressure come-up time was approximately 25 s per 100 MPa and the pressure release time was approximately 10 s. The initial temperature of the water was approximately 18 °C and the temperature increase due to adiabatic heating was approximately 2-3°C per 100 MPa. The samples were pressure treated at 400, 500 and 600 MPa with a hold time at pressure of 1, 3 and 5 min. The heat-treated and HPP milk was stored for 48 h at 4°C before enumeration as this gives a better estimate of

survivors, as injured cells may either recover or die during subsequent cold storage. Unprocessed inoculated samples were enumerated at the time of pressure processing (i.e. 24 h after inoculation).

2.3. Enumeration of *E. coli*, *Salmonella* and *L. monocytogenes*

For enumeration of pathogenic *E. coli* an aliquot of 100 µl of each of the appropriate 10-fold dilutions was spread plated on TBX agar plates (Oxoid, CM0945) and the plates incubated at 37 °C for 24 h. For enumeration of pathogenic *Salmonella* an aliquot of 100 µl of each of the appropriate 10-fold dilutions was spread plated on brilliant green agar plates (Oxoid, CM0329) and incubated at 37 °C for 24 h. For enumeration of *L. monocytogenes* an aliquot of 100 µl of each of the appropriate 10-fold dilutions was spread plated on Palcam agar (Oxoid, code CM0877) supplemented with Palcam selective supplement (Oxoid SR0150) and incubated at 37 °C for 48 h. Each sample was plated in duplicate.

2.4. Microbial shelf-life assessment

After processing, raw, pasteurised and HPP milk was stored in one litre bottles at 4 ± 0.5 °C for the duration of the 28 days shelf life study. Shelf life assessment of samples treated at 600 MPa for 3 min was determined as it was found to be the most promising in terms of pathogen reduction. Ten-fold dilutions of milk samples were prepared in MRD (Oxoid, Basingstoke, Hampshire, U.K.) and serially diluted further. Total mesophilic aerobic bacteria (TVC), were enumerated by spread plating 100 µl from each dilution on standard plate count agar (PCA, Oxoid Ltd., Basingstoke, Hampshire, U.K.). Plates were incubated at 30 °C for 48 ± 2 h. Numbers of *Pseudomonas spp.* were determined by spread plating on Pseudomonas agar base with CFC supplement (Oxoid Ltd., Basingstoke, Hampshire, U.K.) incubated for 72 ± 2 h at 25 °C. *Enterobacteriaceae* were enumerated by pour plating using violet red bile glucose agar (VRBG, Oxoid Ltd., Basingstoke, Hampshire, U.K.) incubated for 24 ± 2 h at 37°C.

Lactic acid bacteria were enumerated on de Man, Rogosa, Sharpe Agar (MRS, Oxoid Ltd., Basingstoke, Hampshire, U.K.), incubated for 48 ± 2 h at 30 °C. Results were reported as Log_{10} CFU ml^{-1} . Samples were taken on days 0, 5, 7, 14, 21 and 28 for microbiological, particle size and color analysis. Day 0 was set as the first day after high pressure treatment.

2.5. Particle size analysis

Particle size analysis was carried out on day 0 and after 7 days of storage for raw, pasteurised and HPP treated milk (600 MPa for 3 min) using a Malvern Mastersizer 3000 laser diffraction particle size analyser (Malvern Instruments, GB). The sample was added in drops (approximately 4-5 drops) into the dispersant (distilled water). Refractive Index (nr) of the sample was 1.33 for the dispersant, 1.38 and 1.45 for casein and fat particle sizes respectively. The particle diameters were expressed as: $D[(3,2)]$, the area mean weighted average surface diameter, which measured spherical particles of the same surface area (Sauter mean diameter, according to eq. 1); $D[(4,3)]$, the volume moment mean weighted average volume diameter, which measure the spherical particles having the same volume (De Brouckere mean diameter, according to eq. 2); $d(0.9)$, indicates that 90 % of the volume distribution is below observed diameter and $d(0,5)$ or median diameter, which indicates that 50 % of the volume distribution is above, and 50 % is below the observed diameter.

$$D(3, 2) = \frac{\sum_i n(i)X d(i)^3}{\sum_i n(i)X d(i)^2} \quad [1]$$

$$D(4, 2) = \frac{\sum_i n(i)X d(i)^4}{\sum_i n(i)X d(i)^3} \quad [2]$$

Where (n) is the number of fat and casein globules having a diameter [m] identical to $d(i)$. Particles size measurements were performed in triplicates at Day 0 and Day 7 for raw, thermally and HPP milk.

2.6. Color Measurement

Instrumental colour analysis was performed at day 0, 5, 7, 14, 21 and 28 of storage at 4°C for all the samples. Before each measurement samples were mixed by shaking and 200 ml of milk poured into a 50 mm glass bottle so that it was filled to the top. Colour readings were taken in triplicate by emptying and refilling the bottle at each measurement. Measurements were performed using a dual beam spectrometer Hunter Lab system (UltraScan XE, Hunter Lab., VA, USA). Measurements were reported as distribution of CIE L* (lightness), a* (redness) and b* (yellowness) and the value used to calculate the total color difference between the samples ($\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$). Depending on the value of ΔE the color difference between treated and untreated samples could be estimated such as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great (6.0–12.0) according to Cserhalmi, Sass-Kiss, Tóth-Markus, and Lechner (2006).

2.7. Statistical analysis

The entire experiment was randomised and replicated on three different occasions. Data were subjected to a analysis of variance (ANOVA) with treatment and storage time as the main effects and their interaction. Differences between groups were assessed by the Tukey's test. A significance level of 0.05 was used.

3. RESULTS AND DISCUSSION

3.1. Initial considerations on experimental design

Literature has shown that bacterial cells in the stationary phase exhibit greater pressure tolerance than exponentially-growing cells (Hayman, Anantheswaran, & Knabel, 2007; McClements, Patterson, & Linton, 2001). Therefore, bacteria were inoculated at the stationary phase to simulate the worst case scenario. In some cases, HPP can result in sub-lethally injured cells which cannot be detected on selective media. These cells can potentially repair themselves and cause disease. Repair of foodborne pathogens during storage is

important for HPP low-acid foods such as milk because it can cause overestimation of safety (Jordan, Pascual, Bracey, & Mackey, 2001; Russell, 2002). It has also been shown that in some cases sub-lethally injured pathogens such as *E. coli* can recover even in a nutrient-free environment (Koseki & Yamamoto, 2006). To tackle that in the present study the pressure-treated milk was held for 48 h at 4°C to allow time for sub-lethally injured cells to either recover or die off. These samples were then enumerated. Here, raw milk was inoculated with individual cocktails of the three pathogenic *bacteria* at a high level in order to determine which pressure conditions are able to give a 5-log reduction in CFU. Specifically, *E. coli*, *Salmonella* and *L. monocytogenes* were inoculated at 8.11, 8.33 and 7.19 log CFU/ml of milk, respectively. Pasteurisation resulted in a reduction of *E. coli*, *Salmonella* and *L. monocytogenes* below the detection limit, which corresponds to a >7.11, >7.33 and >6.19 log CFU/ml reduction, respectively.

3.2. Influence of HPP on the inactivation of *E. coli*, *Salmonella* and *L. monocytogenes*.

The effect of increasing pressure (400-600 MPa) and exposure time (1-3 min) from 400 to 600 MPa on the survival of the three artificially inoculated pathogens in raw milk is presented in Fig. 1. In general, for all three microorganisms a more pronounced inactivation was obtained with increasing pressure levels and increasing exposure time ($P < 0.05$). In all cases, HPP application even at the lower pressure level (400 MPa) and exposure time (1 min) resulted in a significant reduction ($P < 0.05$) in the levels of *E. coli*, *Salmonella spp.* and *L. monocytogenes* (0.85, 1.09 and 1.42 log reduction, respectively) compared to the control (raw milk). With regards to pathogenic *E. coli*, although HPP at 400 MPa and 500 MPa for 1 min did not result in statistically significant differences in reduction levels, at longer exposure times (3 and 5 min) there was a significantly higher reduction between the 400 and 500 MPa treatments. Application of pressure at 600 MPa for 3 and 5 min resulted in a reduction of 5.6 and 6.8 log CFU/ml, respectively. Linton, McClements and Patterson (2001) observed that

pressure inactivation of pathogenic *E. coli* in skimmed milk varied between 3.4 and 6.7 log using a pressure treatment of 600 MPa for 15 min. Ramaswamy, Jin, & Zhu, (2009) demonstrated that HPP at 200 MPa for 15 min or 300 MPa for 5 min resulted in similar reduction of *E. coli* K12 counts (approx. 1.2 logs) in milk. In general, *Salmonella* exhibited the same trend as pathogenic *E. coli* (Fig. 1B). Reduction for 400 MPa for 1-5 min ranged from 1.09 to 2.36 log CFU/ml and for 500 MPa for 1-5 min ranged from 1.17 to 3.28 log CFU/ml. Significantly higher reductions were achieved at 600 MPa compared to the lower pressure levels ($P < 0.05$). Specifically, HPP at 600 MPa for 1, 3 and 5 min resulted in 2.48, 5.06 and 6.27 log CFU reduction in *Salmonella* counts, respectively. Similar results were obtained by Guan, Chen, & Hoover (2005) when pressure treated UHT whole milk. They found that *S. typhimurium* was reduced by 0.6, 1.8, and 5.0 log₁₀ CFU/ml, at pressures of 350, 400, and 450 MPa for 30 min, respectively. Whereas pressures of 500, 550, and 600 MPa for 10 min reduced counts of *S. typhimurium* by approx. 4.5 - 5.1 logs.

L. monocytogenes survival after HPP is presented in Fig. 1C. In this case as well, increasing pressure and exposure time resulted in more pronounced pathogen reduction. The milder conditions that could achieve a higher than 5 log reduction in the pathogen levels were 500 MPa for 5 min (5.48 logs) and 600 MPa for 3 min (5.65 logs). Pressure applied at 600 MPa for 5 min resulted in 5.91 log CFU/ml which did not differ significantly to the 600 MPa for 3 min treatment ($P > 0.05$). The most pronounced reduction was observed when 600 MPa was applied to the raw milk. However, there were no statistically significant differences between the *L. monocytogenes* counts at 600 MPa for 3 min and 600 MPa for 5 min ($P > 0.05$). This suggests that *L. monocytogenes* was more sensitive to increasing pressure than increasing exposure time (Erkmen & Dogan 2004), at least in the higher pressure levels. Possibly this is because *L. monocytogenes* is Gram-positive, so may behave differently in response to higher pressures compared to the other two Gram-negative species tested. Koseki, Mizuno, &

Yamamoto, (2008) found that *L. monocytogenes* cells artificially inoculated in milk ($7 \log_{10}$ CFU/ml) can be reduced after HPP at 500 MPa for 5 min by 5 log CFU/ml. Whereas, HPP above 550 and 600 MPa reduced the number of *L. monocytogenes* cells to below the limit of detection (<1 CFU/ml) immediately after treatment. According to Erkmen & Dogan, (2004), HPP at 400 and 600 MPa for 10 min resulted in 2.76 and 6.47 log CFU/ml reduction in *L. monocytogenes* counts in raw milk. Misiou, van Nassau, Lenz, & Vogel (2017) inoculated *L. monocytogenes* in milk at similar inoculum level (7.4 log CFU/ml) as in the present study and found that 300 MPa for 10 min did not have any effect on the pathogen counts. When pressures of 400 and 500 MPa were applied reductions of approx. 4.7 and 6.2 logs were observed, respectively. Based on these results, the lowest HPP condition set that were capable of reducing the levels of all three pathogenic bacteria by >5 log was the 600 MPa for 3 min set. These conditions were therefore assessed in subsequent experiments.

3.3. Effect of HPP on microbiological shelf life

Spoilage of raw milk occurs as a result of both the endogenous spoilage microbiota present in the milk and by spoilage microorganisms introduced from the environment. These microorganisms can affect the nutritional and organoleptic characteristics of milk (Melini et al. 2017). The TVC, Enterobacteriaceae, lactic acid bacteria (LAB) and *Pseudomonas* spp. counts of raw milk were determined immediately after treatment and during refrigerated storage (Fig. 2). The TVC counts for the raw milk were approx. 6 log CFU/ml at the beginning of storage. Pasteurisation led to a significant reduction of 1.19 log CFU/ml whereas HPP (600 MPa at 3 min) led to a more pronounced decrease of 3.95 log CFU/ml, immediately after treatment. After 5 days storage, the TVC of the pasteurised milk, did not differ significantly compared to the raw milk ($P > 0.05$) for the remaining storage period. The TVC for HPP milk was always lower compared to the other two treatments with the TVC in HPP milk reaching 7.05 log CFU/ml after 28 days compared to raw and pasteurised milk

which took 14 days to reach >7 log. Pasteurisation also resulted in a significant reduction in *Enterobacteriaceae* counts by approx. 1.7 log CFU/ml compared to the raw milk and reached 7.87 log CFU/ml after 21 days. On the other side, HPP was able to reduce the levels to below the detection limit, and the counts remained at this level throughout storage. LAB levels in raw milk were 4.26 log CFU/ml at the beginning of storage and reached 7.93 log CFU/ml after 14 days. Pasteurisation reduced the LAB counts by 2.2 log CFU/ml and increased during storage reaching 7.92 log CFU/ml after 21 days. On the other hand, HPP reduced the LAB levels below the detection limit and were detected again at 14 days storage, reaching 7.17 log CFU/ml after 28 days, which was significantly lower ($P < 0.05$) compared to LAB levels of the pasteurised milk at day 21. *Pseudomonas spp.* in the untreated raw milk increased during storage and reached 8.16 log CFU/ml after 14 days. Pasteurisation reduced *Pseudomonas spp.* by 1.28 log CFU/ml immediately after treatment. Its levels increased during storage and after 21 days it reached 7.45 log CFU/ml. On the other hand, HPP reduced the *Pseudomonas spp.* to below the detection limit, where it remained for at least 7 days. After 21 days, *Pseudomonas spp.* levels were 5.63 log CFU/ml, which was significantly lower compared to the pasteurised milk. At 28 days, *Pseudomonas spp.* counts reached 6.91 log CFU/ml for the HPP treatment. Results clearly showed that HPP (600 MPa for 3 min) was able to significantly reduce TVC, *Enterobacteriaceae*, LAB, and *Pseudomonas spp.* and prolong the microbiological shelf life of milk by 7 days compared to pasteurised milk. Erkmen & Dogan (2004) found that HPP at 400 and 600 MPa for 10 min could reduce the aerobic bacteria counts in raw milk by 2.09 and 5.09 log CFU/ml, respectively. High pressure homogenisation has also been applied to raw milk to increase its shelf life and has been found to reduce psychrotrophs, lactococci, and total bacteria count by approx. 4 log CFU/ml in raw milk. When the high pressure homogenised milk was stored at 4°C, the microbiological shelf

life was 14-18 days, similar to that of pasteurised milk (90°C for 15 s) (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007).

3.4. Effect of HPP on casein particles

It is well known that HPP can affect milk constituents such as proteins and fat whereas compounds such as vitamins, amino acids, simple sugars and flavour compounds tend to remain unaffected (Chawla et al., 2011). The effects of HPP on the particle sizes of milk are particularly important since they influence its microstructure and define many properties such as colloidal stability, texture, colour etc. Differences in milk particle size can significantly affect milk quality and its further processing.

Average volume diameter $D[(4,3)]$ and average surface diameter $D[(3,2)]$ for all the three treatments tested, along with the percentile values of distribution $d(0.5)$ and $d(0.9)$ are presented in Table 1. For casein particle sizes, HPP treatment significantly ($P < 0.05$) increased all size parameters at day 0 and day 7, compared to thermally treated milk, showing similarities in $D[(4,3)]$ and $D[(3,2)]$ to those observed for raw milk. From the particle size distribution curve of raw, thermal and HPP treated milk, it can be seen that raw and HPP milk had similar peaks at 2.2 μm and $\sim 2 \mu\text{m}$, while pasteurised milk has a major peak at $\sim 0.5 \mu\text{m}$ corresponding to the smaller casein micelles (Fig. 3). A similar pattern was observed after 7 days of storage for raw and HPP milk showing the same peaks at 1.88 μm , while the peak for pasteurised milk appeared was at 0.46 μm , suggesting that the effect of HPP on casein sizes are irreversible during storage time. It has been previously reported that when HPP is applied the size and number of casein micelles tend to increase due to the dissociation of casein micelle into sub-micelles (Huppertz, Fox, de Kruif, & Kelly, 2006). However, diverse effects on milk proteins have been reported based on different pressures and holding times; for example, the average size of casein micelles of milk treated at 100–200 MPa at ambient

temperature was comparable to untreated milk, while a pressure of 250 MPa, yielded considerably larger casein micelles than untreated milk (Huppertz, Fox, & Kelly, 2004; Regnault, Thiebaud, Dumay, & Cheftel, 2004). Decreases in micelle diameter were observed after treatment of raw or pasteurized skim milk at 400 and 600 MPa, with treated samples having ~50% smaller casein micelles than those in untreated milk (Needs, et al., 2000; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Regnault et al., 2004). However, increases in average casein micelle size were observed after treatment at 200 MPa for 60 min at 30 or 40 °C or after treatment at 300 MPa for 5 min at 40 °C (Anema, Lowe, & Stockmann, 2005). Cadesky et al. (2017) reported similar changes in particle sizes as a result of pressure treatment at pressures greater than 250 MPa; increasing the pressure in low milk proteins concentration (2.5%) resulted in progressively smaller particle sizes, while for higher protein concentration (10%) a significant increase in particle size was observed. Increase in the average micelle size induced by HPP is most likely due to the presence of large casein aggregates in the milk; the results of the present study seem to support this view and are consistent with other studies where the presence of large casein aggregates in HPP treated milk was determined by electron microscopy (Considine, Patel, Anema, Singh, & Creamer, 2007; Garcia-Risco, Olano, Ramos, & Lopez-Fandino, 2000; Gaucheron et al., 1997; Needs et al. 2000).

3.5. Effect of HPP on fat particles

The particle size of the fat droplets present in dairy products is important in defining properties such as flavor release, mouth feel and the emulsion stability. Along with changes in milk proteins, HPP has been also linked with modifications of fat globules. In particular, the use of HPP has been observed to contribute to homogenization of dairy products due to a reduction of fat globule size; smaller globules cannot form large enough clusters for creaming to occur, resulting in an increased shelf-life for the milk. According to the literature, typical

parameters for the size distributions of particles for homogenized milk at pressure of 100 MPa for D [(4, 3)] and a D [(3, 2)] are of about 0.5 μm and 0.2 μm . For non-homogenized milk, respective values of 4.5 μm and 1 μm are usually observed (Tobin, Heffernan, Mulvihill, Huppertz, & Kelly, 2015). Table 2 shows the fat particle size distribution of raw, pasteurised and HPP milk samples after 0 and 7 days of storage at 4°C. In the present study, HPP of milk at 600 MPa for 3 min did not result in a significant reduction of the fat particle size. Pasteurised milk displayed significant smaller ($P < 0.05$) average size distribution for fat globules compared to raw and HPP milk, (Fig. 3). Studies have shown that minimum fat particle sizes are observed after pressure application at 200-250 MPa (Picart et al., 2006; Serra, Trujillo, Quevedo, Guamis, & Ferragut, 2007), while above 250 MPa the size of the fat globules may actually increase. This has been attributed to the formation of a too large surface area which would cause the formation of cluster between the fat globules (Pereda et al., 2007; Serra et al., 2007).

3.6. Colour evaluation

The white colour of milk is due to scattering of light particles by fat globules and casein micelles and generally, the Hunter Luminance value (L^* value) is used as a measure of the whiteness of a liquid (Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003). As discussed previously, different treatments can cause changes in the size of fat particles and micelle disintegration, resulting in different light scatter and therefore differences in colour. Results of the colour parameters distribution during the storage time of milk samples are shown in Table 3. Pasteurised milk presented the highest L^* values; significant changes ($P < 0.05$) could be detected after HPP with L^* value closer to raw milk L^* values. This is in agreement with Chawla et al. (2011) and Tao, Sun, Hogan, and Kelly (2014). A similar trend was found by Naik, Sharma, & G. (2013) in skimmed milk after treatment at 250–450 MPa, where a significant decrease in the L^* values was observed, and in ewe's milk, by Gervilla, Ferragut,

& Guamis (2001). Also, Harte et al. (2003) reported that milk subjected to HPP or thermal treatment followed by high pressure, loses its white colour and turns yellowish. Significant differences ($P < 0.05$) were observed in the colour parameter $-a^*$ (greenness) of raw milk (-0.34 ± 0.05) compared to HPP (-0.61 ± 0.08) and thermal treated (-0.72 ± 0.06) milk. For the $+b^*$ value (yellowness), HPP caused a significant (14.03 ± 0.30) increase ($P < 0.05$) compared to raw milk (12.49 ± 0.26) and to pasteurised milk samples (9.79 ± 0.19). The total colour difference (ΔE) parameter is used to indicate the degree of colour difference between treated/untreated samples or before/after storage (Barba, Esteve, & Frígola, 2012) and values can be classified as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great (6.0–12.0) (Cserhalmi et al., 2006).. According to this, noticeable colour differences could be observed at the beginning of the shelf life between HPP and raw milk (ΔE 2.82) and between raw and thermally-treated milk (ΔE 2.95), while well visible differences could be seen between HPP and thermally-treated milk (ΔE 5.69). Moving towards the end of shelf life (based on LAB bacterial count), the perceived colour difference between HPP and raw milk decreased to slightly noticeable (ΔE 1.41) while remained in the range of well visible for HPP compared to thermally treated milk (ΔE 4.98) and raw to thermal milk samples (ΔE 3.65). These observations are in line with previous studies where optical parameters were reported not to be affected after treatment of milk at 100-200 MPa, but were reduced progressively with treatment pressures of 200–400 MPa, with further reduction when pressures > 400 MPa was applied. Moreover, changes in optical parameters became irreversible during subsequent storage at 5 °C (Huppertz et al. 2004; Huppertz et al., 2006). Further studies on the sensory profile and consumer acceptance of the HPP milk should be conducted to confirm the quality results found in this study and investigate in more depth the effect on the sensory attributes (Schiano et al. 2017).

4. CONCLUSION

This study demonstrated that HPP was effective in achieving 5 log reductions for pathogenic *E. coli*, *Salmonella* and *L. monocytogenes* respectively. It is evident that HPP prolonged the shelf life of raw milk by reducing TVC, Enterobacteriaceae, LAB and *Pseudomonas* spp. levels compared to those in pasteurized milk and raw milk. The particle size and color analysis of HPP milk compared to raw and pasteurized milk, revealed that HPP milk seem to preserve the quality attributes which characterize raw unprocessed milk, such as color and mouth feel sensation due to particle size. Since the demand for unpasteurized raw milk appears to be growing, HPP could be a viable alternative for the dairy industry in order to produce microbiologically safe milk with fresh-like characteristics.

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Tables

Table 1. Casein particle size (μm) of raw, thermally treated and HPP milk samples after 0 and 7 days of storage at 4°C.

Day 0	d(0.5)	d(0.9)	D[(4,3)]	D[(3,2)]
Raw	0.96±0.01 ^b	3.44±0.02 ^b	1.49±0.01 ^b	0.53±0.01 ^a
Thermal	0.39±0.00 ^c	0.99±0.00 ^c	0.49±0.00 ^c	0.27±0.00 ^b
HPP	1.21±0.19 ^a	4.05±0.21 ^a	2.15±0.15 ^a	0.54±0.14 ^a
Day 7	d(0.5)	d(0.9)	D[(4,3)]	D[(3,2)]
Raw	1.01±0.01 ^b	4.12±0.09 ^a	2.19±0.13 ^a	0.54±0.01 ^b
Thermal	0.40±0.00 ^c	1.01±0.01 ^c	0.61±0.07 ^c	0.28±0.00 ^c
HPP	1.17±0.01 ^a	3.72±0.04 ^b	1.67±0.01 ^b	0.71±0.00 ^a

^{a-c} Mean value ± standard deviation; values without common superscripts were significantly different ($P < 0.05$). * d (0.5): diameter below which 50% of the volume of particles are found, d (0.9): diameter below which 90% of the volume of particles are found, D [(4,3)]: volume-weighted mean diameter, D[(3,2)]: surface-weighted mean diameter.

Table 2. Fat particle size (μm) of raw, thermally treated and HPP milk samples after 0 and 7 days of storage at 4°C .

Day 0	d(0.5)	d(0.9)	D[(4,3)]	D[(3,2)]
Raw	$1.60 \pm 0.11^{\text{b}}$	$6.07 \pm 0.09^{\text{b}}$	$2.88 \pm 0.27^{\text{b}}$	$0.12 \pm 0.00^{\text{a}}$
Thermal	$0.32 \pm 0.01^{\text{a}}$	$0.96 \pm 0.00^{\text{a}}$	$0.43 \pm 0.00^{\text{a}}$	$0.13 \pm 0.00^{\text{a}}$
HPP	$3.26 \pm 0.42^{\text{c}}$	$7.50 \pm 0.36^{\text{c}}$	$4.79 \pm 0.91^{\text{c}}$	$0.27 \pm 0.14^{\text{a}}$
Day 7	d(0.5)	d(0.9)	D[(4,3)]	D[(3,2)]
Raw	$2.38 \pm 0.06^{\text{b}}$	$8.78 \pm 0.76^{\text{a}}$	$4.24 \pm 0.47^{\text{a}}$	$0.14 \pm 0.00^{\text{a}}$
Thermal	$0.42 \pm 0.03^{\text{c}}$	$1.42 \pm 0.20^{\text{b}}$	$3.03 \pm 1.31^{\text{a}}$	$0.22 \pm 0.04^{\text{a}}$
HPP	$3.19 \pm 0.29^{\text{a}}$	$8.57 \pm 2.19^{\text{a}}$	$5.62 \pm 1.51^{\text{a}}$	$0.23 \pm 0.06^{\text{a}}$

^{a-c} Mean value \pm standard deviation; values without common superscripts were significantly different ($P < 0.05$). *d(0.5): diameter below which 50% of the volume of particles are found, d(0.9): diameter below which 90% of the volume of particles are found, D[(4,3)]: volume-weighted mean diameter, D[(3,2)]: surface-weighted mean diameter.

Table 3. Distribution of the colour values of milk samples in CIE *Lab* system

	L*	a*	b*
HPP	77.29±0.35 ^c	-0.61±0.08 ^a	14.03±0.30 ^c
Raw	78.94±0.31 ^b	-0.34±0.05 ^b	12.49±0.26 ^b
Thermal	80.80±0.32 ^a	-0.72±0.06 ^a	9.79±0.19 ^a

^{a-c} Mean value ± standard deviation; values without common superscripts were significantly different ($P < 0.05$).

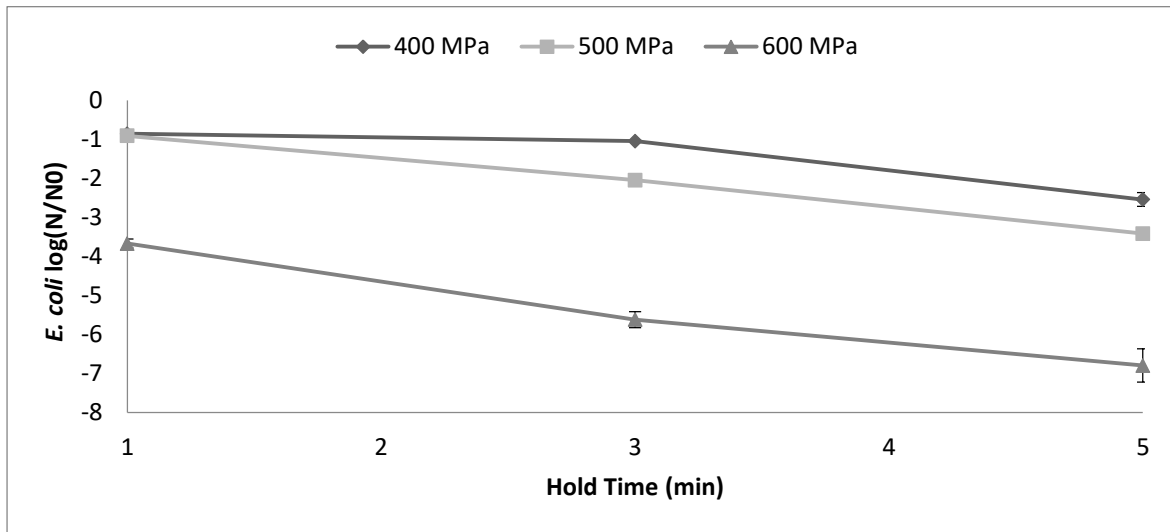
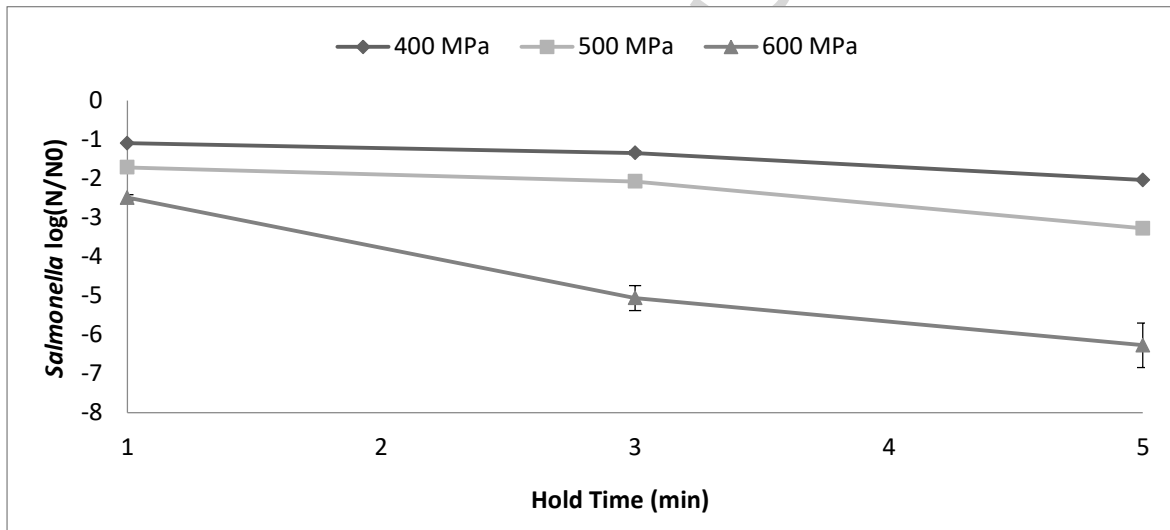
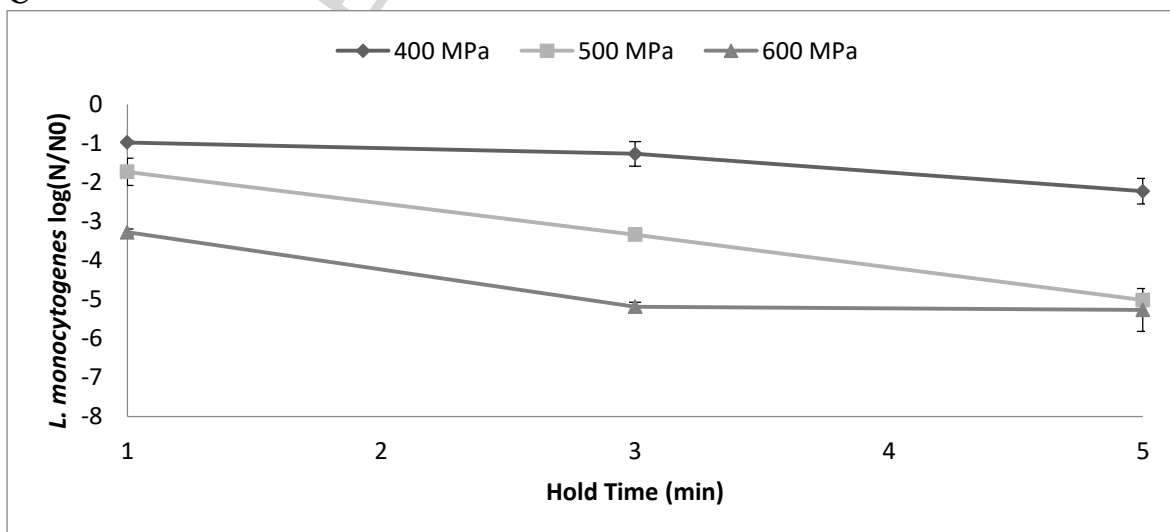
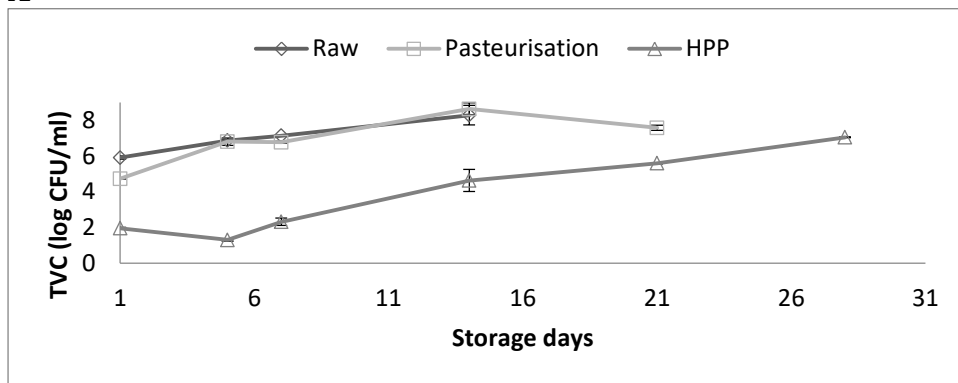
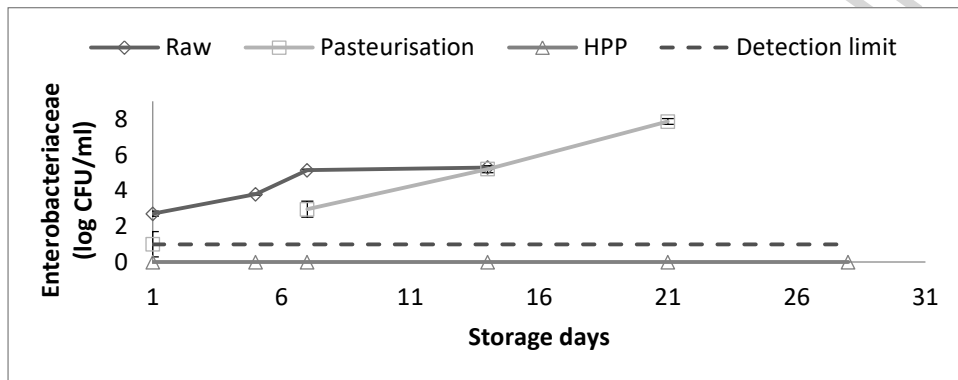
Figures
Figure 1**A****B****C**

Figure 2

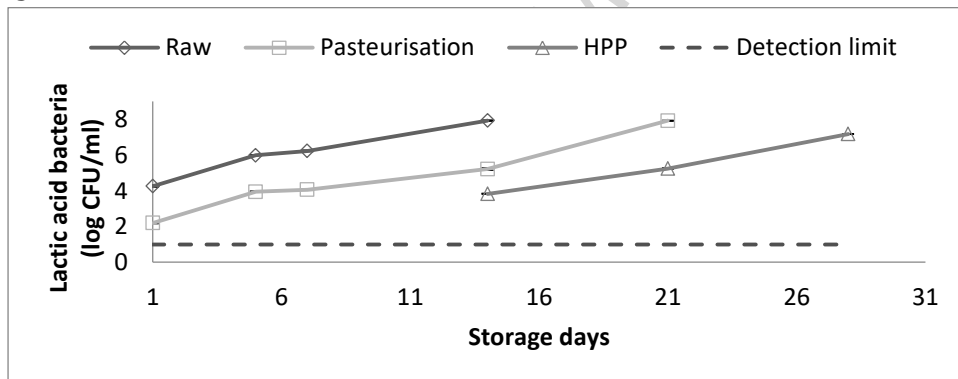
A



B



C



D

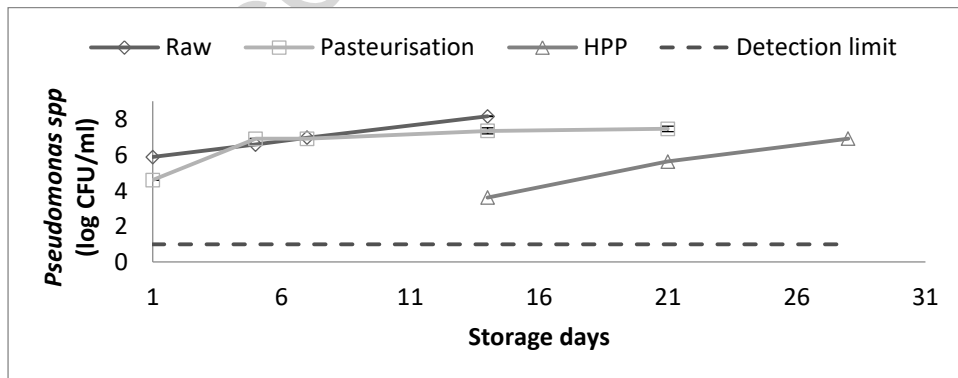
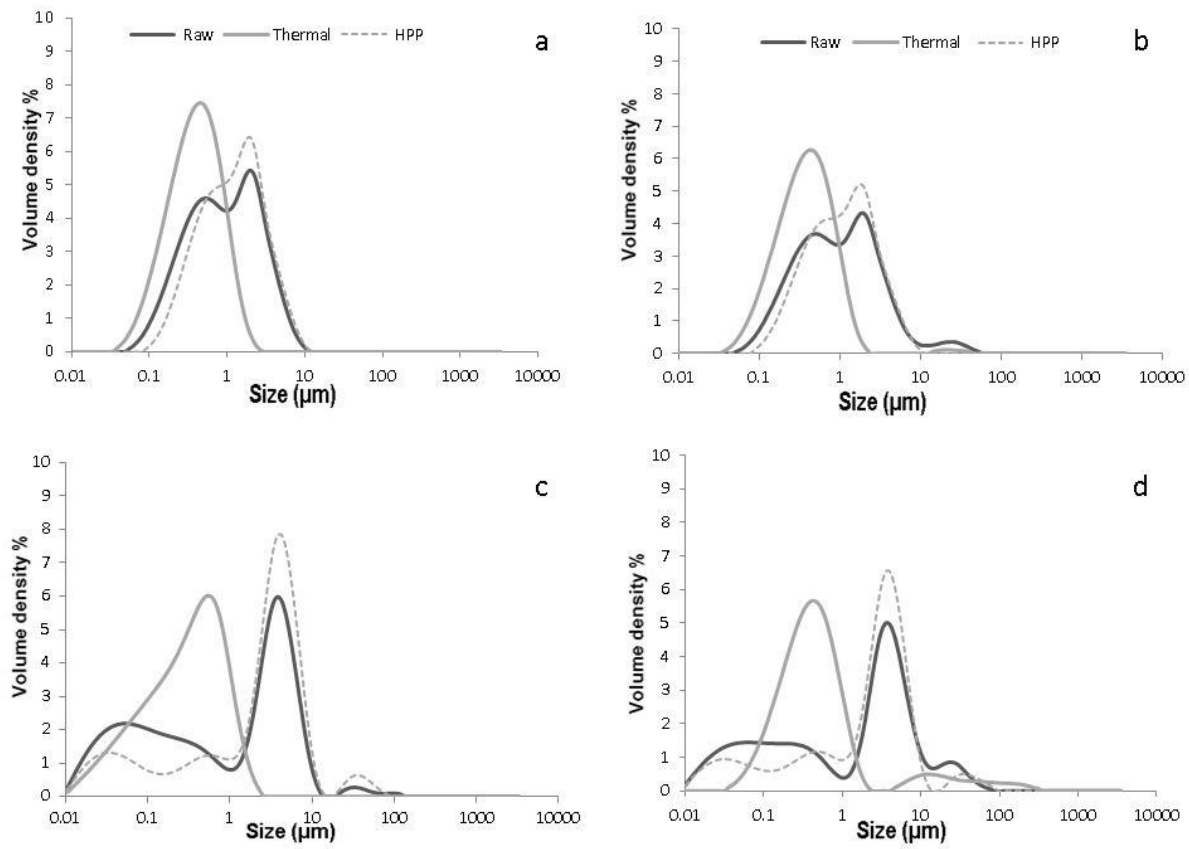


Figure 3



ACCEPTED

Highlights

- HPP effectively achieved 5 log reductions for pathogenic *E. coli*, *Salmonella* and *L. monocytogenes*.
- HPP prolonged the shelf life of raw milk by reducing TVC, Enterobacteriaceae, LAB and *Pseudomonas* spp.
- HPP preserved the quality attributes typical of raw unprocessed milk.
- HPP could be an alternative to produce microbiologically safe milk with fresh-like characteristics.