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 the 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, Akkinepalli, & Nellutla, 2012; EFSA 2016; Melini et al., 2017). Therefore, pathogens in milk represent a safety risk that needs to be managed.

58 The majority of the countries require raw milk to undergo some level of thermal processing
59 (e.g. 72 ºC for 15 s, 135 - 150 ºC for 1-4 s, 105 - 120 ºC for 20 - 40 min) in order to be rendered
60 safe for the consumer (Griffiths 2010; Melini et al., 2017). However, conventional thermal
61 treatment can have a detrimental effect on the nutrient content of milk as well as on its
62 organoleptic and physicochemical properties (Buckow, Chandry, Ng, McAuley, & Swanson,
63 2014). The recent interest in the consumption of raw milk has led to the consideration of
64 alternative processing technologies for production of milk that is safe but also minimally
65 processed in order to be perceived as fresh by the consumer (Román, Sánchez-Siles, & Siegrist,
66 2017). The utilisation of emerging non-thermal technologies, has been explored as means to
67 decrease the negative effects of conventional processing technologies and present promising
68 alternatives for the dairy sector. High-pressure processing (HPP) is a food preservation
69 technology that is a promising alternative to conventional thermal pasteurization as it can
70 inactivate foodborne pathogens while minimising the loss of nutrients, such as vitamins, and
71 maintaining the fresh-like characteristics of food products (Lee & Kaletunç 2010; Yang et al.
72 2012; Yao et al. 2014; Sheen, Cassidy, Scullen, & Sommers, 2015). HPP, although very
73 efficient in eliminating vegetative microorganisms,has little or no effect on bacterial spores,
74 when applied at ambient temperatures, so it is important to take into consideration that *Bacillus*
75 spp. and other spore-forming microorganisms may not be inactivated in milk or other dairy
76 products and may go on to cause spoilage issues or represent food safety concerns.

77 HPP can also influence the physicochemical and technological characteristics of milk by
78 modifying the structure of milk components (Patterson, 2005; Cadesky, Walkling-Ribeiro,
79 Kriner, Karwe, & Moraru, 2017). Pressurization can result in conformational changes of milk
80 proteins as it can disrupt milk casein micelles as well as the structure of whey proteins (Chawla,
81 Patil, & Singh, 2011). It does not seem to affect lactose in milk which suggests that no Maillard

82 or lactose isomerization reaction takes place in milk as a result of pressure treatment (Lopez-
83 Fandino, Carrascosa, & Olano, 1996).

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

87 **2. MATERIALS AND METHODS**

88 **2.1. Preparation of *E. coli*, *Salmonella* and *L. monocytogenes* inoculum**

89 5 strain cocktail of the three pathogenic microorganisms was inoculated into raw milk samples
90 separately in three different inoculation studies. The cocktail of *E. coli* consisted of NCTC
91 11601, NCTC 11602, NCTC 11603, NCTC 9706 and NCTC 9707. The *Salmonella* cocktail
92 consisted of *Salmonella* Senftenberg, *Salmonella* Typhimurium, *Salmonella* Anatum,
93 *Salmonella* Agona and *Salmonella* Saint Paul. The *L. monocytogenes* cocktail consisted of
94 FMT 1750, NCTC 11994, NCTC 5214, NCTC 10888 and NCTC 19118 strains. These
95 cocktails contained some relatively pressure-resistant strains, a *L. monocytogenes* strain
96 associated with an outbreak in soft cheese and a *L. monocytogenes* strain isolated from a dairy
97 processing environment.

98 For each *E. coli*, *Salmonella* and *L. monocytogenes* strain used, a loopful of a fresh tryptone
99 soya agar (Oxoid code CM0131) + 0.6% yeast extract (Oxoid code LP0021) (TSAYE) slope
100 culture was inoculated into 10 ml of brain heart infusion broth (BHI) (Oxoid code CM1135)
101 and incubated at 37 °C for 24 h. Subsequently 100 µl of a 10^{-4} dilution of this broth was
102 inoculated into another 10 ml BHI broth and incubated at 37 °C for either 24 h or 48 h, until
103 the stationary phase of growth was reached. The final 10 ml cultures were centrifuged at 3600 ×
104 *g*, for 30 min, washed twice in phosphate-buffered saline (PBS) and the pellet re-suspended in
105 a final volume of 1 ml PBS to give approximately 10^9-10^{10} CFU/ml. The suspensions of all 5
106 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. If 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, Killeshin, 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 ºC ± 0.5ºC 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 ºC 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 4ºC 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

156 °C. *Enterobacteriaceae* were enumerated by pour plating using violet red bile glucose agar
157 (VRBG, Oxoid Ltd., Basingstoke, Hampshire, U.K.) incubated for 24±2 h at 37°C. Lactic acid
158 bacteria were enumerated on de Man, Rogosa, Sharpe Agar (MRS, Oxoid Ltd., Basingstoke,
159 Hampshire, U.K.), incubated for 48±2 h at 30 °C. Results were reported as Log_{10} CFU ml^{-1}.
160 Samples were taken on days 0, 5, 7, 14, 21 and 28 for microbiological, particle size and color
161 analysis. Day 0 was set as the first day after high pressure treatment.

162
163 **2.5. Particle size analysis**

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

176 $$D\,(3,2) = \frac{\sum_i n(i) X\, d(i)^3}{\sum_i n(i) X\, d(i)^2} \quad [1]$$

177 $$D\,(4,2) = \frac{\sum_i n(i) X\, d(i)^4}{\sum_i n(i) X\, d(i)^3} \quad [2]$$

178 where (*n*) is the number of fat and casein globules having a diameter [m] identical to *d(i)*.
179 Particles size measurements were performed in triplicates at Day 0 and Day 7 for raw, thermally
180 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 ($\Delta E = \text{sqrt}\,(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2$). 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

206 phase to simulate the worst case scenario. In some cases, HPP can result in sub-lethally injured
207 cells which cannot be detected on selective media. These cells can potentially repair themselves
208 and cause disease. Repair of foodborne pathogens during storage is important for HPP low-
209 acid foods such as milk because it can cause overestimation of safety (Jordan, Pascual, Bracey,
210 & Mackey, 2001; Russell, 2002). It has also been shown that in some cases sub-lethally injured
211 pathogens such as *E. coli* can recover even in a nutrient-free environment (Koseki &
212 Yamamoto, 2006). To tackle that in the present study the pressure-treated milk was held for 48
213 h at 4^{o}C to allow time for sub-lethally injured cells to either recover or die off. These samples
214 were then enumerated. Here, raw milk was inoculated with individual cocktails of the three
215 pathogenic *bacteria* at a high level in order to determine which pressure conditions are able to
216 give a 5-log reduction in CFU. Specifically, *E. coli*, *Salmonella* and *L. monocytogenes* were
217 inoculated at 8.11, 8.33 and 7.19 log CFU/ml of milk, respectively. Pasteurisation resulted in
218 a reduction of *E. coli*, *Salmonella* and *L. monocytogenes* below the detection limit, which
219 corresponds to a >7.11, >7.33 and >6.19 log CFU/ml reduction, respectively.

220 **3.2. Influence of HPP on the inactivation of *E. coli*, *Salmonella* and *L. monocytogenes*.**

221 The effect of increasing pressure (400-600 MPa) and exposure time (1-3 min) from 400 to
222 600 MPa on the survival of the three artificially inoculated pathogens in raw milk is presented
223 in Fig. 1. In general, for all three microorganisms a more pronounced inactivation was obtained
224 with increasing pressure levels and increasing exposure time ($P < 0.05$). In all cases, HPP
225 application even at the lower pressure level (400 MPa) and exposure time (1 min) resulted in a
226 significant reduction ($P < 0.05$) in the levels of *E. coli*, *Salmonella spp*. and *L. monocytogenes*
227 (0.85, 1.09 and 1.42 log reduction, respectively) compared to the control (raw milk). With
228 regards to pathogenic *E. coli*, although HPP at 400 MPa and 500 MPa for 1 min did not result
229 in statistically significant differences in reduction levels, at longer exposure times (3 and 5 min)
230 there was a significantly higher reduction between the 400 and 500 MPa treatments.

231 Application of pressure at 600 MPa for 3 and 5 min resulted in a reduction of 5.6 and 6.8 log
232 CFU/ml, respectively. Linton, McClements and Patterson (2001) observed that pressure
233 inactivation of pathogenic *E. coli* in skimmed milk varied between 3.4 and 6.7 log using a
234 pressure treatment of 600 MPa for 15 min. Ramaswamy, Jin, & Zhu, (2009) demonstrated that
235 HPP at 200 MPa for 15 min or 300 MPa for 5 min resulted in similar reduction of *E. coli* K12
236 counts (approx. 1.2 logs) in milk. In general, *Salmonella* exhibited the same trend as pathogenic
237 *E. coli* (Fig. 1B). Reduction for 400 MPa for 1-5 min ranged from 1.09 to 2.36 log CFU/ml and
238 for 500 MPa for 1-5 min ranged from 1.17 to 3.28 log CFU/ml. Significantly higher reductions
239 were achieved at 600 MPa compared to the lower pressure levels ($P < 0.05$). Specifically, HPP
240 at 600 MPa for 1, 3 and 5 min resulted in 2.48, 5.06 and 6.27 log CFU reduction in *Salmonella*
241 counts, respectively. Similar results were obtained by Guan, Chen, & Hoover (2005) when
242 pressure treated UHT whole milk. They found that *S. typhimurium* was reduced by 0.6, 1.8,
243 and 5.0 $\log_{10}$ CFU/ml, at pressures of 350, 400, and 450 MPa for 30 min, respectively. Whereas
244 pressures of 500, 550, and 600 MPa for 10 min reduced counts of *S. typhimurium* by
245 approx. 4.5 - 5.1 logs.

246 *L. monocytogenes* survival after HPP is presented in Fig. 1C. In this case as well, increasing
247 pressure and exposure time resulted in more pronounced pathogen reduction. The milder
248 conditions that could achieve a higher than 5 log reduction in the pathogen levels were 500
249 MPa for 5 min (5.48 logs) and 600 MPa for 3 min (5.65 logs). Pressure applied at 600 MPa for
250 5 min resulted in 5.91 log CFU/ml which did not differ significantly to the 600 MPa for 3 min
251 treatment ($P>0.05$). The most pronounced reduction was observed when 600 MPa was applied
252 to the raw milk. However, there were no statistically significant differences between the *L.*
253 *monocytogenes* counts at 600 MPa for 3 min and 600 MPa for 5 min ($P> 0.05$). This suggests
254 that *L. monocytogenes* was more sensitive to increasing pressure than increasing exposure time
255 (Erkmen & Dogan 2004), at least in the higher pressure levels. Possibly this is because *L.*

256 *monocytogenes* is Gram-positive, so may behave differently in response to higher pressures
257 compared to the other two Gram-negative species tested. Koseki, Mizuno, & Yamamoto,
258 (2008) found that *L. monocytogenes* cells artificially inoculated in milk (7 $\log_{10}$ CFU/ml) can
259 be reduced after HPP at 500 MPa for 5 min by 5 log CFU/ml. Whereas, HPP above 550 and
260 600 MPa reduced the number of *L. monocytogenes* cells to below the limit of detection
261 (<1 CFU/ml) immediately after treatment. According to Erkmen & Dogan, (2004), HPP at 400
262 and 600 MPa for 10 min resulted in 2.76 and 6.47 log CFU/ml reduction in *L. monocytogenes*
263 counts in raw milk. Misiou, van Nassau, Lenz, & Vogel (2017) inoculated *L. monocytogenes*
264 in milk at similar inoculum level (7.4 log CFU/ml) as in the present study and found that 300
265 MPa for 10 min did not have any effect on the pathogen counts. When pressures of 400 and
266 500 MPa were applied reductions of approx. 4.7 and 6.2 logs were observed, respectively.
267 Based on these results, the lowest HPP condition set that were capable of reducing the levels
268 of all three pathogenic bacteria by >5 log was the 600 MPa for 3 min set. These conditions
269 were therefore assessed in subsequent experiments.

270

271 **3.3. Effect of HPP on microbiological shelf life**

272 Spoilage of raw milk occurs as a result of both the endogenous spoilage microbiota present in
273 the milkand by spoilage microorganisms introduced from the environment.

274 . These microorganisms can affect the nutritional and organoleptic characteristics of milk
275 (Melini et al. 2017). The TVC, Enterobacteriaceae, lactic acid bacteria (LAB) and
276 *Pseudomonas* spp. counts of raw milk were determined immediately after treatment and during
277 refrigerated storage (Fig. 2). The TVC counts for the raw milk were approx. 6 log CFU/ml at
278 the beginning of storage. Pasteurisation led to a significant reduction of 1.19 log CFU/ml
279 whereas HPP (600 MPa at 3 min) led to a more pronounced decrease of 3.95 log CFU/ml,
280 immediately after treatment. After 5 days storage, the TVC of the pasteurised milk, did not

281 differ significantly compared to the raw milk ($P > 0.05$) for the remaining storage period. The
282 TVC for HPP milk was always lower compared to the other two treatments with the TVC in
283 HPP milk reaching 7.05 log CFU/ml after 28 days compared to raw and pasteurised milk which
284 took 14 days to reach >7 log. Pasteurisation also resulted in a significant reduction in
285 *Enterobacteriaceae* counts by approx. 1.7 log CFU/ml compared to the raw milk and reached
286 7.87 log CFU/ml after 21 days. Whereas HPP was able to reduce the levels to below the
287 detection limit, and the counts remained at this level throughout storage. LAB levels in raw
288 milk were 4.26 log CFU/ml at the beginning of storage and reached 7.93 log CFU/ml after 14
289 days. Pasteurisation reduced the LAB counts by 2.2 log CFU/ml and increased during storage
290 reaching 7.92 log CFU/ml after 21 days. On the other hand, HPP reduced the LAB levels below
291 the detection limit and were detected again at 14 days storage, reaching 7.17 log CFU/ml after
292 28 days, which was significantly lower ($P <0.05$) compared to LAB levels of the pasteurised
293 milk at day 21. *Pseudomonas spp*. in the untreated raw milk increased during storage and
294 reached 8.16 log CFU/ml after 14 days. Pasteurisation reduced Pseudomonas spp. by 1.28 log
295 CFU/ml immediately after treatment. Its levels increased during storage and after 21 days it
296 reached 7.45 log CFU/ml. On the other hand, HPP reduced the *Pseudomonas spp*. to below the
297 detection limit, where it remained for at least 7 days. After 21 days, *Pseudomonas spp*. levels
298 were 5.63 log CFU/ml, which was significantly lower compared to the pasteurised milk. At 28
299 days, *Pseudomonas spp.* counts reached 6.91 log CFU/ml for the HPP treatment. Results
300 clearly showed that HPP (600 MPa for 3 min) was able to significantly reduce TVC,
301 Enterobacteriaceae, LAB, and *Pseudomonas spp*. and prolong the microbiological shelf life of
302 milk by 7 days compared to pasteurised milk. Erkmen & Dogan (2004) found that HPP at 400
303 and 600 MPa for 10 min could reduce the aerobic bacteria counts in raw milk by 2.09 and 5.09
304 log CFU/ml, respectively. High pressure homogenisation has also been applied to raw milk to
305 increase its shelf life and has been found to reduce psychrotrophs, lactococci, and total bacteria

306 count by approx. 4 log CFU/ml in raw milk. When the high pressure homogenised milk was
307 stored at 4°C, the microbiological shelf life was 14-18 days, similar to that of pasteurised milk
308 (90°C for 15 s) (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007).

309
310 **3.3. Effect of HPP on casein particles**

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

317 Average volume diameter D[(4,3)] and average surface diameter D[(3,2)] for all the three
318 treatments tested, along with the percentile values of distribution d (0.5) and d (0.9) are
319 presented in Table 1. For casein particle sizes, HPP treatment significantly ($P<0.05$) increased
320 all size parameters at day 0 and day 7, compared to thermally treated milk, showing similarities
321 in D[(4,3)] and D[(3,2)] to those observed for raw milk. From the particle size distribution
322 curve of raw, thermal and HPP treated milk, it can be seen that raw and HPP milk had similar
323 peaks at 2.2 μm and ~ 2 μm, while pasteurised milk has a major peak at ~0.5 μm corresponding
324 to the smaller casein micelles (Fig. 3). A similar pattern was observed after 7 days of storage
325 for raw and HPP milk showing the same peaks at 1.88 μm, while the peak for pasteurised milk
326 appeared was at 0.46 μm, suggesting that the effect of HPP on casein sizes are irreversible
327 during storage time. It has been previously reported that when HPP is applied the size and
328 number of casein micelles tend to increase due to the dissociation of casein micelle into sub-
329 micelles (Huppertz, Fox, de Kruif, & Kelly, 2006). However, diverse effects on milk proteins
330 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).

3.4. 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,

356 resulting in an increased shelf-life for the milk. According to the literature, typical parameters
357 for the size distributions of particles for homogenized milk at pressure of 100 MPa for D [(4,
358 3)] and a D [(3, 2)] are of about 0.5 μm and 0.2 μm. For non-homogenized milk, respective
359 values of 4.5 μm and 1 μm are usually observed (Tobin, Heffernan, Mulvihill, Huppertz, &
360 Kelly, 2015). Table 2 shows the fat particle size distribution of raw, pasteurised and HPP milk
361 samples after 0 and 7 days of storage at 4ºC. In the present study, HPP of milk at 600 MPa for
362 3 min did not result in a significant reduction of the fat particle size. Pasteurised milk displayed
363 significant smaller ($P < 0.05$) average size distribution for fat globules compared to raw and
364 HPP milk, (Fig. 3). Studies have shown that minimum fat particle sizes are observed after
365 pressure application at 200-250 MPa (Picart et al., 2006; Serra, Trujillo, Quevedo, Guamis, &
366 Ferragut, 2007), while above 250 MPa the size of the fat globules may actually increase. This
367 has been attributed to the formation of a too large surface area which would cause the formation
368 of cluster between the fat globules (Pereda et al., 2007; Serra et al., 2007).

369
370 **3.5. Colour evaluation**

371 The white colour of milk is due to scattering of light particles by fat globules and casein
372 micelles and generally, the Hunter Luminance value (L* value) is used as a measure of the
373 whiteness of a liquid (Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003). As discussed
374 previously, different treatments can cause changes in the size of fat particles and micelle
375 disintegration, resulting in different light scatter and therefore differences in colour. Results of
376 the colour parameters distribution during the storage time of milk samples are shown in Table
377 3. Pasteurised milk presented the highest L^* values; significant changes ($P<0.05$) could be
378 detected after HPP with L^* value closer to raw milk L^* values. This is in agreement with
379 Chawla et al. (2011) and Tao, Sun, Hogan, and Kelly (2014). A similar trend was found by
380 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).

406

407 3. CONCLUSION

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

417 **Acknowledgements:** The authors would like to thank Joan Tollerton (AFBI) for facilitating
418 the research conducted with the High Pressure Processing equipment.

419

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584

585 # Tables

586

587 **Table 1.** Casein particle size (μm) of raw, thermally treated and HPP milk samples after 0 and

588 7 days of storage at 4ºC.

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]

589 [a-c] Mean value ± standard deviation; values without common superscripts were significantly

590 different ($P < 0.05$).

591 * D (0.5): diameter below which 50% of the volume of particles are found, D (0.9): diameter

592 below which 90% of the volume of particles are found, D[(4,3)]: volume-weighted mean

593 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 4ºC.

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

602 **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]

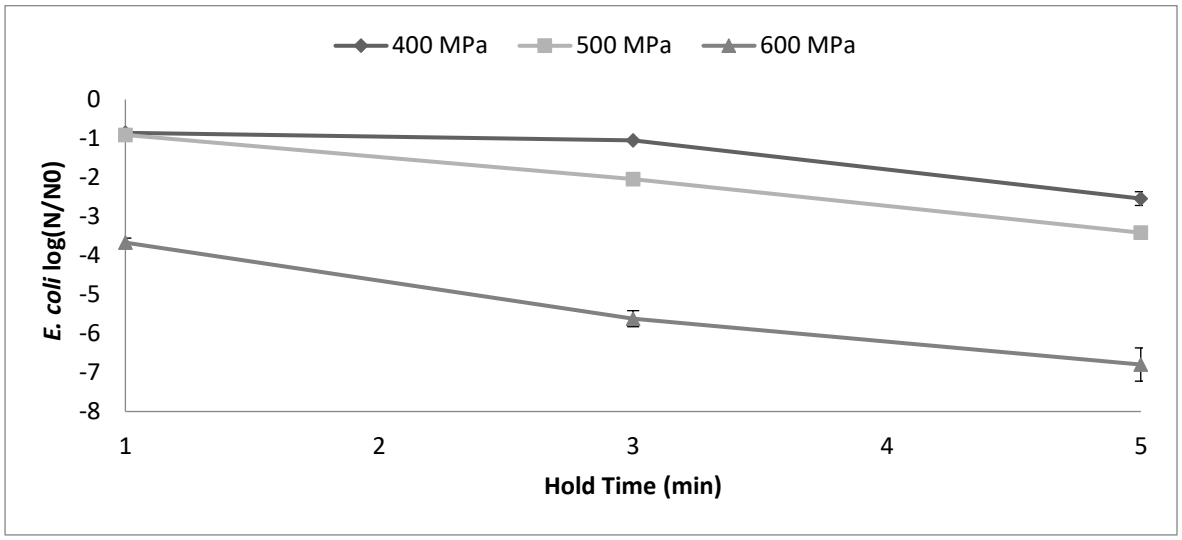
603 [a-c] Mean value ± standard deviation; values without common superscripts were significantly

604 different ($P < 0.05$).

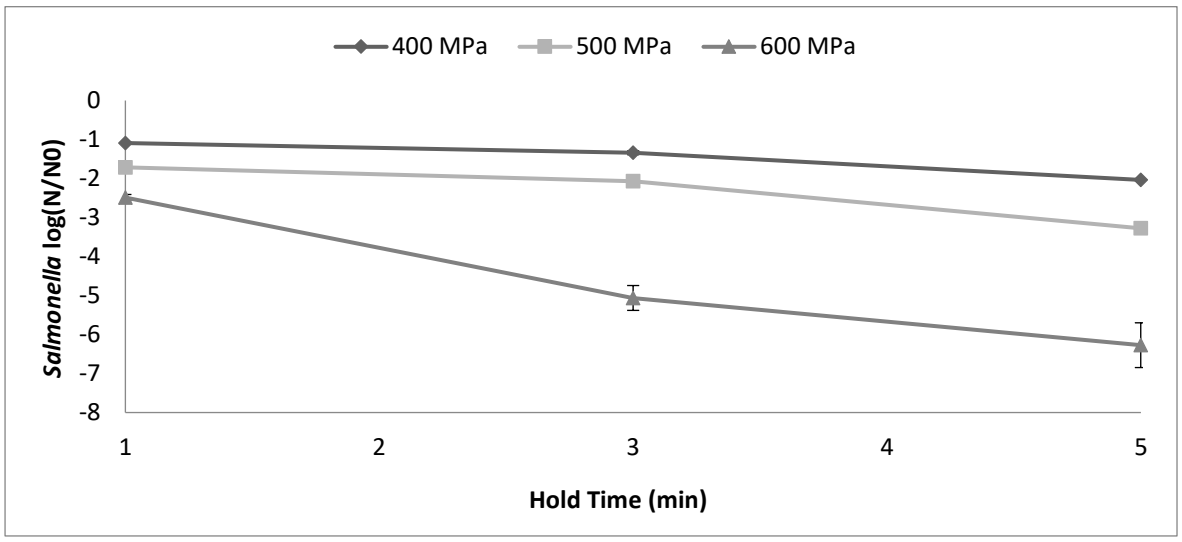
Figures

Figure 1

A



B



C

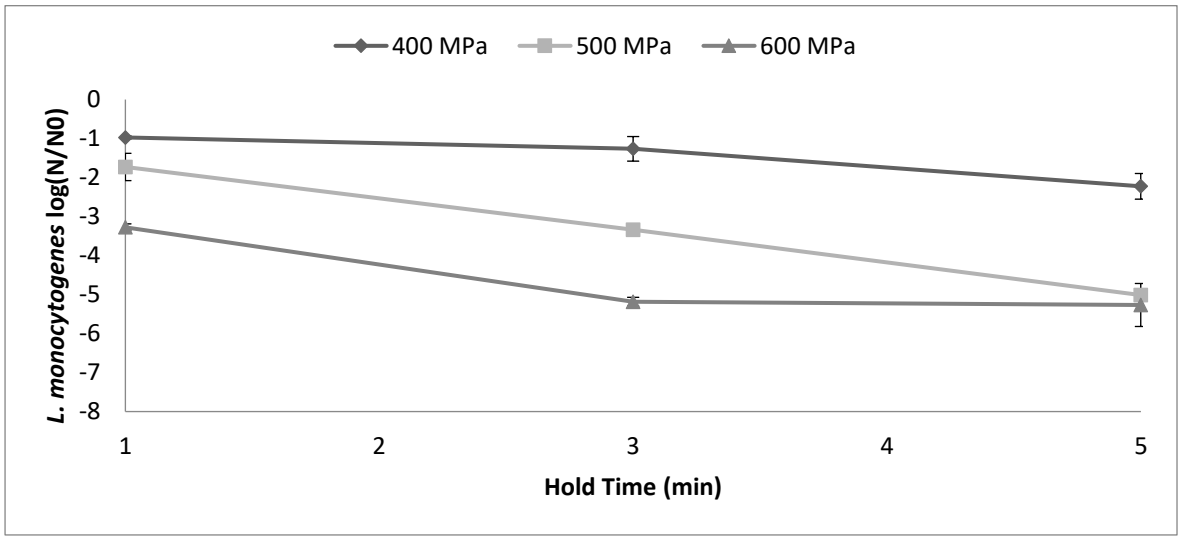
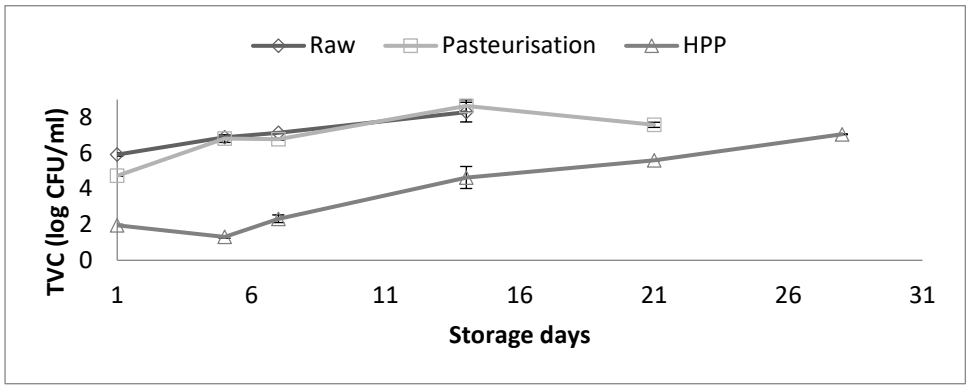
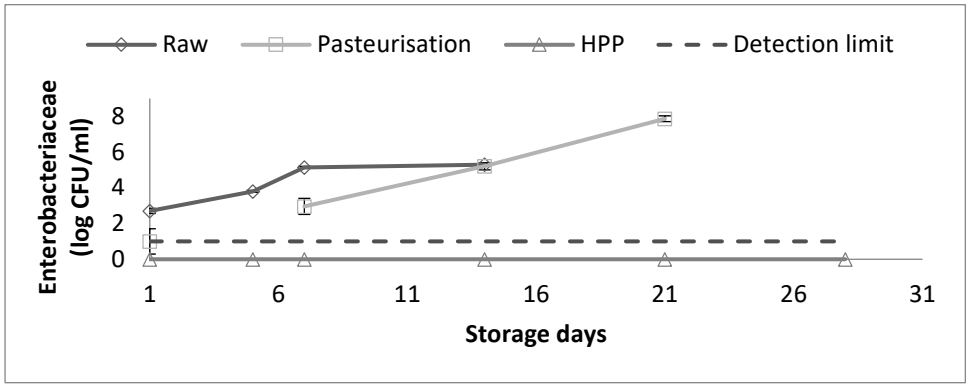


Figure 2

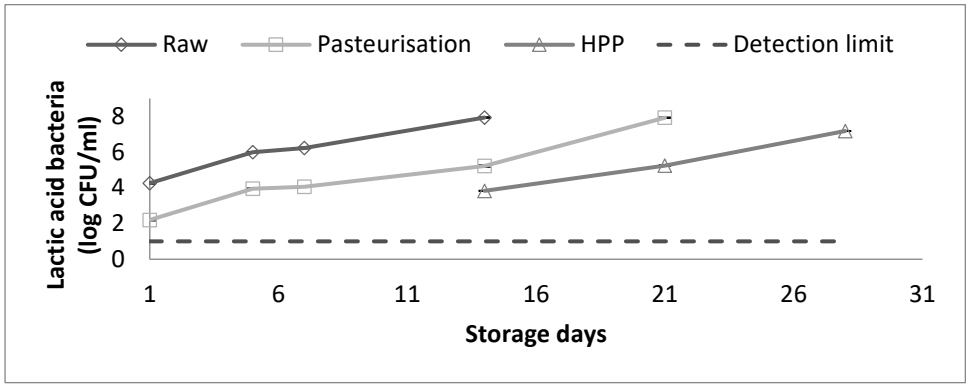
A



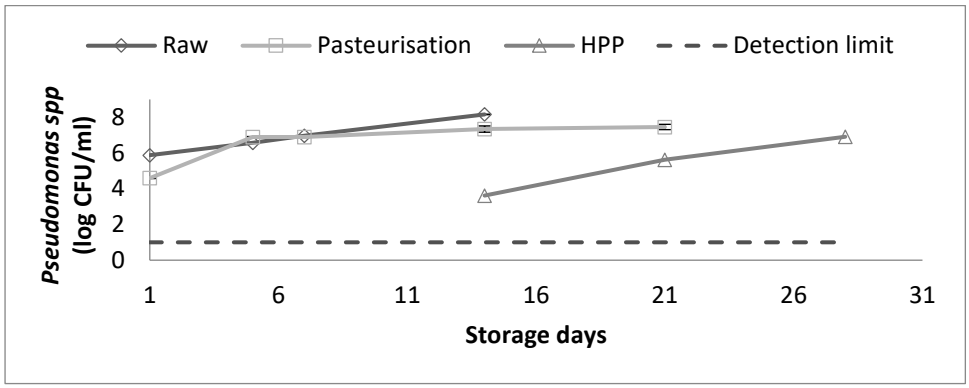
B



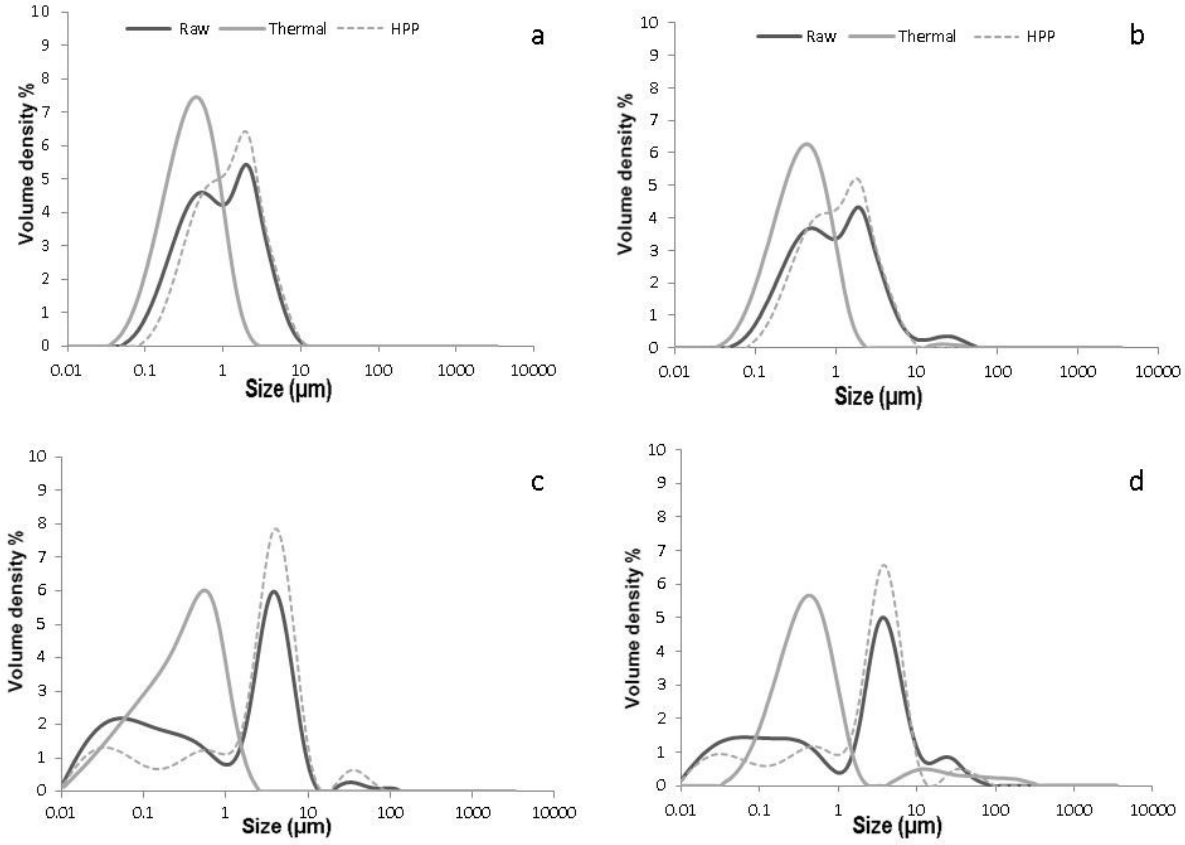
C



D



623 **Figure 3**


Raw
Thermal
HPP
a
b
c
d
Volume density %
Size (µm)
0.01
0.1
1
10
100
1000
10000