



2013

Combined Treatment with Mild Heat, Manothermosonication and Pulsed Electric Fields Reduces Microbial Growth in Milk.

Rachel Halpin

Dublin Institute of Technology, rachel.halpin@dit.ie

O. Cregenzán-Alberti

School of Agriculture and Food Science, University College Dublin

P. Whyte

School of Veterinary Medicine, University College Dublin


J.G Lyng

School of Agriculture and Food Science, University College Dublin

F. Noci

School of Agriculture and Food Science, University College Dublin

Follow this and additional works at: <http://arrow.dit.ie/schfsehart>

 Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#), and the [Food Biotechnology Commons](#)

Recommended Citation

Halpin, R. et al. (2013) Combined Treatment with Mild Heat, Manothermosonication and Pulsed Electric Fields Reduces Microbial Growth in Milk. *Food Control*, Volume 34, Issue 2, December 2013, Pages 364-371 DOI :10.1016/j.foodcont.2013.05.008

This Article is brought to you for free and open access by the School of Food Science and Environmental Health at ARROW@DIT. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@DIT. For more information, please contact yvonne.desmond@dit.ie, arrow.admin@dit.ie, brian.widdis@dit.ie.



This work is licensed under a [Creative Commons Attribution-NonCommercial-Share Alike 3.0 License](#)



1 **Combined treatment with mild heat,**
2 **manothermosonication and pulsed electric fields reduces**
3 **microbial growth in milk.**

4 **Halpin, R.M.^{1*}, Cregenzán-Alberti, O.¹, Whyte, P.², Lyng, J.G.¹ and Noci, F.^{1§}**

5 ¹ School of Agriculture and Food Science, University College Dublin, Ireland,

6 ² School of Veterinary Medicine, University College Dublin. Ireland.

7

8 [§] Current address: Hotel School, Galway-Mayo Institute of Technology, Galway Campus,
9 Dublin Road, Galway, Ireland.

10

11 * Corresponding author: Dr. Rachel Halpin.

12 Postal address: School of Agriculture and Food Science, University College Dublin, Belfield,
13 Dublin 4, Ireland.

14 Tel No.: 0035317162851

15 E-mail: rachel.halpin@ucd.ie

16

17

18

19

20

21 **Abstract**

22 In recent years, there has been considerable interest in non-thermal milk processing. The
23 objective of the present study was to assess the efficacy of two non-thermal technologies
24 (manothermosonication; MTS, and pulsed electric fields; PEF) in comparison to thermal
25 pasteurisation, by assessing the microbiological quality of each of these milk samples post-
26 processing. Homogenised milk was subjected to MTS (amplitude; 27.9 μm , pressure; 225
27 kPa) at two temperatures (37°C or 55°C), before being immediately treated with PEF
28 (electric field strength; 32 kV/cm, pulse width; 10 μs , frequency; 320 Hz). Thermal
29 pasteurisation (72°C, 20 s) was included as a control treatment. Microbial content of each
30 milk sample was monitored over a 21-day period. It was determined that milks treated with
31 MTS/PEF at 37°C and 55°C contained lower microbial levels for a certain duration, but after
32 14 days milk which had been pasteurised by conventional methods contained significantly (P
33 <0.05) less microorganisms. However, milks treated with MTS/PEF contained significantly
34 ($P<0.05$) fewer microorganisms than raw milk at each time point. Although not as effective
35 as thermal pasteurisation, the present study demonstrates the ability of MTS/PEF treatment to
36 reduce microbial content of milk, while avoiding prolonged heat exposure to temperatures
37 such as those used during conventional (thermal) pasteurisation.

38

39

40 **Keywords:** non-thermal processing, manothermosonication, pulsed electric fields, milk,
41 microbiological quality.

42

43

44 **1. Introduction**

45 Milk is a nutritious medium which presents an ideal environment for the growth of many
46 pathogenic and spoilage microorganisms (Pereda *et al.*, 2009). Consequently, milk has a
47 relatively short shelf-life. Milk is usually processed by heat treatment in order to prolong its
48 shelf-life, and although these thermal treatments destroy microorganisms, they can also have
49 detrimental effects on the nutritional and organoleptic properties of milk (Odriozola-Serrano,
50 Bendicho-Porta, & Martin-Belloso, 2006). The demand by consumers for fresh, safe foods
51 with favourable sensory properties while also having an adequate shelf-life is a difficult need
52 for the food industry to meet while using thermal treatment (Pereda *et al.*, 2009). Thus, in
53 recent years there has been considerable interest in the non-thermal processing of foods and
54 beverages.

55 Non-thermal processing technologies require significantly less heat than thermal
56 processing, or in some cases no heat at all, and are consequently less energy-intensive and
57 more environmentally friendly than thermal methods (Piyasena, Mohareb, & McKellar,
58 2003). In addition, foods/beverages subjected to non-thermal processing are regarded as
59 being fresh-like, with minimal loss of colour, flavour and nutrients (Bermudez-Aguirre,
60 Mawson, Versteeg, & Barbosa-Canovas, 2009). Examples of such non-thermal processes
61 include microfiltration, pulsed-light inactivation and high hydrostatic pressure. Two non-
62 thermal processing methods of particular relevance to the present study are ultrasonication
63 and pulsed electric field technology.

64 Ultrasound (US) is defined as sound waves with frequencies above the threshold of
65 human hearing (>16 kHz) (Manas & Pagan, 2005), and this technology can be used to
66 damage or disrupt microbial cells (Dolatowski, Stadnik, & Stasiak, 2007). During sonication,
67 a sonic wave meets a liquid medium and regions of alternating compression and expansion

68 are created. These regions of pressure change cause cavitation, and bubbles are formed in the
69 medium. During the expansion cycle, the bubbles expand, and a point is reached where the
70 bubble implodes and rapid condensation occurs. The condensed molecules collide violently
71 and shock waves are created, which are of very high pressures and temperatures of up to
72 50MPa and 5500°C, respectively (Dolatowski, Stadnik, & Stasiak, 2007). These resulting
73 changes in pressure and ‘hot zones’ can kill bacteria, with the former being the main
74 contributor to microbial inactivation (Piyasena, Mohareb, & McKellar, 2003). In order to
75 increase antimicrobial efficacy, sonication can be combined with heat (thermosonication;
76 TS), pressure (manosonication; MS), or heat and pressure (manothermosonication; MTS).
77 However, the effectiveness of ultrasound is dependent on the type of microorganism targeted,
78 the amplitude of the ultrasonic waves, treatment time and the volume and composition of the
79 food being treated (Dolatowski, Stadnik, & Stasiak, 2007). With regard to microbial
80 susceptibility, Gram positive bacteria are generally more sensitive than Gram negative
81 microorganisms, and spores are considered to be more resistant to treatment with ultrasound
82 than vegetative cells (Burgos, 1998).

83 Another non-thermal inactivation method which is gaining popularity in terms of
84 commercial interest is pulsed electric field (PEF) technology (Devlieghere, Vermeiren, &
85 Debevere, 2004). This method is based on a pulsing power delivered to a food/beverage
86 placed between a set of electrodes within a chamber. Essentially, a series of high voltage
87 pulses are applied between the set of electrodes, which causes disruption to and subsequent
88 permeabilisation of microbial cell membranes; a process known as ‘electroporation’
89 (Hamilton, & Sale, 1967, Devlieghere, Vermeiren, & Debevere, 2004; Walkling-Ribeiro *et*
90 *al.*, 2009a). The electrical fields can be applied for durations varying from nanoseconds to
91 microseconds, and depending on the electrical field strength, pulse duration and pulse
92 number, permeabilisation can be either reversible or irreversible (Aronsson, Borch, Stenlof,

93 & Ronner, 2004). The effectiveness of PEF treatment for the purpose of microbial
94 inactivation is a reflection of these parameters (i.e. EFS and overall treatment time). In
95 general, Gram-positive bacteria are more resistant to treatment with PEF than Gram negative
96 bacteria, while yeasts are more sensitive than bacteria (Devlieghere, Vermeiren, & Debevere,
97 2004).

98 The objective of the present study was to compare the efficacy of MTS/PEF when
99 applied at two temperatures (37°C and 55°C) to thermal pasteurisation. After milk had been
100 subjected to processing with MTS/PEF or high temperature short time (HTST) pasteurisation,
101 specific microorganisms were monitored over a 21-day period, and comparisons made
102 between the microbiological qualities of each milk sample after processing.

103 **2. Materials and Methods**

104 **2.1 Milk Sourcing and Preparation**

105 Fresh raw bovine milk from a bulk tank was obtained from Lyons Research Farm
106 (Newcastle, Co. Dublin) and transported to the laboratories at UCD Dublin within 40 min.
107 Three batches of raw whole milk were obtained on three separate occasions. Milk was stored
108 at 4°C until required. On the same day as collection from the farm, milk was preheated to
109 55°C and homogenized at a total pressure of 21 MPa. A two-stage high-pressure
110 homogenizer (Model No. Panda 2682, Niro Soavi, Parma, Italy) was used for this purpose,
111 and pressures of 18 MPa and 3MPa were applied in the primary and secondary stages,
112 respectively. Milk was subsequently processed using non-thermal methods or conventional
113 pasteurisation.

114 **2.2 Treatment of Milk with MTS/PEF**

115 Previous experiments in this laboratory examining the inhibitory effect of (i) MTS and (ii)
116 PEF as individual treatments showed that optimal microbial inactivation levels were achieved

117 using power levels of 27.9 μm (225 kPa) and 32 kV/cm for MTS and PEF, respectively (data
118 not shown). Thus, these were the power settings used in the present study. Other parameters
119 applied were as follows: for MTS treatment, the frequency was set to 20kHz, and the total
120 pressure was 225kPa, while for PEF the frequency was set at 320kHz, and the pulse width
121 was 10 μs .

122 A peristaltic pump (Masterflex [®] L/S [®], Model No. 77250-62, Cole-Parmer
123 Instrumental Company, IL, USA) was used to pass milk through a submerged coil in a heated
124 water bath so that the milk reached a temperature of 37°C or 55°C prior to entering the
125 sonicators. This heating process was quite rapid, taking less than one or two minutes to reach
126 37°C or 55°C, respectively. A flow rate of 160 ml/min was used. For MTS processing, two
127 ultrasonic processors were employed (Model No. UIP 1000hd, Hielscher, Germany). These
128 sonicators were connected in a row, and had an operational frequency of 20 kHz. Two
129 sonotrodes (Model No. BS2d40, Hielscher), each of which had a 40 mm frontal face diameter
130 were used. Also, boosters were used to strengthen the amplitude (Model No. B2-1.8,
131 Hielscher). The volume of each sonication chamber was 168ml, and the total residence time
132 of milk within the chamber was determined to be on average 2.1 min; this duration was found
133 to be adequate for microbial inactivation in preliminary studies carried out to optimise
134 processing conditions (data not shown). In addition, the chambers of each sonicator were
135 surrounded by jackets which contained flowing water so that the temperature within the
136 chamber could be maintained at 37°C or 55°C. The temperatures of the milk entering and
137 leaving the sonicators were monitored using T-type thermocouples (Model No. SQ2020,
138 Grant Instruments, Cambridge, UK).

139 Immediately following treatment with MTS, milk was subjected to PEF treatment. A
140 continuous laboratory scale PEF unit (ELCRACK HVP 5, DIL, German Institute of Food
141 Technologies, Quackenbruck, Germany) was used. The treatment module consisted of three

142 co-linear treatment chambers with a refrigerated cooling module integrated; these cooling
143 sections were present following the first and third chambers. Each chamber held two co-
144 linear stainless steel electrodes separated by a 5.0 mm gap, with the electrode diameter being
145 3.0 mm, which resulted in a total treatment volume of 0.106 cm³. PEF treatment was applied
146 using monopolar square-wave pulses (10 µs pulse width, 320 Hz frequency). The EFS was
147 32kV/cm and the total treatment time was determined to be 127 µs. The product temperature
148 was recorded using thermocouples (Testo 925, type-K probe, Testo AG, Lenzkirch,
149 Germany) both before and after the treatment module, and was never allowed to exceed 37°C
150 or 55°C depending on the treatment being applied. Following treatment with MTS/PEF, milk
151 was collected in a sterile glass bottle and stored at 4°C until required.

152 **2.3 Thermal Pasteurisation of Milk**

153 Raw, homogenised milk was thermally pasteurised using a tubular heat exchanger (Model
154 No. FT74 UHT/HTST Processing System, Armfield Technical Education Co. Ltd,
155 Ringwood, U.K.). Milk was heated at 72°C for 20 s. An attached cooling system ensured the
156 temperature of the milk was below 10°C after treatment. Milk was collected in a sterile glass
157 bottle, and stored at 4°C for microbial analysis for the duration of the microbiological
158 assessment.

159 **2.4 Microbiological Analysis**

160 The microbial content of all milk samples (i.e. raw and post-processing with MTS/PEF or
161 thermal pasteurisation) was monitored at defined time points (days 0, 2, 5, 7, 9, 14, 21). The
162 total viable count (TVC) was quantified by preparing decimal dilutions of each milk sample
163 in sterile quarter-strength Ringer's solution (Oxoid, Basingstoke, U.K.). An aliquot (100 µl)
164 of appropriate dilutions was then spread-plated onto plate count agar (PCA; Scharlau,
165 Barcelona, Spain) in duplicate, and plates were incubated at 37°C for 48 hours. The following
166 microorganisms were also quantified:

- 167 (a) Yeasts and moulds: on potato dextrose agar (PDA; Oxoid; Basingstoke, U.K.),
168 incubated at 30°C for 48 h.
- 169 (b) *Staphylococcus aureus*: on Baird Parker agar (BPA; Merck, Darmstadt, Germany),
170 incubated at 37°C for 48 h.
- 171 (c) *Enterobacteriaceae*: serial decimal dilutions (1 ml) of milk samples were pour-plated
172 in violet red bile lactose dextrose agar (VRBLD; Oxoid, Basingstoke, U.K.) with an
173 over-layer, and incubated at 37°C for 24 h.
- 174 (d) Lactic acid bacteria: on DeMan, Rogosa and Sharpe agar (MRS; Oxoid, Basingstoke,
175 U.K.), incubated at 30°C for 48 h.
- 176 (e) Pseudomonads: on *Pseudomonas* specific agar (Oxoid, Basingstoke, U.K.)
177 supplemented with cetrimide, fucidin and cephaloridine (CFC supplement, Oxoid,
178 Basingstoke, U.K.), incubated at 30°C for 48 h.
- 179 (f) Total psychrotrophic count: serially-diluted milk samples in sterile quarter-strength
180 Ringer's solution (1 ml) were pour-plated in PCA with an over-layer, and incubated at
181 4°C for a period of 10 days. The quantity of psychrotrophic bacteria was monitored
182 on days 0, 7, 14 and 21 only.

183 After the appropriate incubation period, colonies were counted and the number of colony
184 forming units per ml (CFU/ml) was determined for each specific microorganism in all of the
185 milk samples.

186 **2.5 Statistical analysis**

187 Statistical analysis was performed using the PROC GLM function of SAS version 9 (SAS
188 Institute, Cary, NC). For all treatments, data from three different batches of milk (i.e. n=3)
189 were analysed. Results were considered significantly different if $P < 0.05$.

190 **3. Results**

191 Firstly, temperatures of milk before and after MTS and PEF treatments were noted
192 during each experiment and the average calculated (n=3). For milk treated at 37°C, milk
193 entered the MTS chamber at 37°C ($\pm 0^\circ\text{C}$) and left the MTS system at 36.5°C ($\pm 0.5^\circ\text{C}$). The
194 milk then cooled to 25.7° C ($\pm 0.5^\circ\text{C}$) before entering the PEF system, and the average
195 temperature post-PEF processing was 32.1°C ($\pm 3^\circ\text{C}$). In the case of milk treated at 55°C,
196 milk entered the MTS system at this temperature ($\pm 0^\circ\text{C}$), and left the MTS chamber at 57°C
197 ($\pm 0.4^\circ\text{C}$). The milk subsequently cooled, before entering the PEF system at a temperature of
198 40.5°C ($\pm 0.4^\circ\text{C}$). Temperatures post-PEF treatments were found to be on average 32.1°C (\pm
199 5.1°C).

200 All processed milks (i.e. 37°C or 55°C MTS/PEF-treated and thermally pasteurised)
201 significantly reduced the TVC of milk when compared to raw milk ($P<0.05$) over the 21-day
202 period (Figure 1). On days 5, 7 and 9 the TVC of milk treated at 37°C was not found to be
203 significantly different from the TVC of milk that had undergone conventional pasteurisation
204 ($P>0.05$). However, after 21 days, the 37°C and 55°C MTS/PEF treatments were found to
205 have a significantly higher TVC than milk that had been pasteurised using the HTST method
206 ($P<0.05$). The TVC of raw milk was found to be *c.* 2×10^8 CFU/ml after 21 days. The average
207 TVC of milks treated at 37°C and 55°C after 21 days were found to be 9.34×10^6 and
208 6.58×10^6 , respectively. These values were significantly ($P<0.05$) higher than that of
209 pasteurised milk (1.26×10^2 CFU/ml). Nonetheless, both the 37°C and 55°C MTS/PEF
210 treatments resulted in a *c.* 4 log reduction of TVC when compared to raw milk after 21 days.
211 However, thermal pasteurisation resulted in a log reduction of 6.8.

212 Similarly, all treated milks contained a significantly ($P<0.05$) lower number of yeasts
213 and moulds than raw milk over the entire 21-day period (Figure 2). On average, raw milk
214 contained *c.* 7.4×10^5 CFU/ml on day 0, rising to *c.* 1.93×10^8 CFU/ml on day 21. All
215 treatments reduced the level of yeasts and moulds in the milk over the 21-day period. On

216 days 2 and 5, no significant difference was found between any of the treatments at the 5%
217 significance level. On days 7 and 9, no significant difference was found between the 37°C
218 MTS/PEF treatment and conventional pasteurisation ($P>0.05$). By day 21, thermally
219 pasteurised milk was found to contain the least number of yeasts and moulds ($P<0.05$).

220 With regard to *S. aureus*, raw milk generally contained a significantly higher level of
221 this microorganism over the 21-day period than all processed milks (Figure 3). The average
222 initial level of *S. aureus* in raw milk was determined to be 4.13×10^4 CFU/ml, and this level
223 reached 2.09×10^6 CFU/ml by day 21, with all treatments significantly reducing the level of *S.*
224 *aureus* in milk ($P<0.05$). However, on day 21 the 37°C MTS/PEF treatment was found to
225 have a similar level of *S. aureus* as raw milk. On day 0, pasteurisation reduced the amount of
226 *S. aureus* by 3 log cycles when compared to raw milk, while treatment with MTS/PEF at
227 37°C and 55°C resulted in log reductions of 1.4 and 2.7, respectively. On days 2, 5, 7 and 9,
228 no significant differences were observed between the *S. aureus* content of all treated milk
229 samples (i.e. MTS/PEF or pasteurised) at the 5% significance level. On days 14 and 21,
230 pasteurised milk was found to contain significantly lower levels of *S. aureus* than milks
231 treated with MTS/PEF at 37°C and 55°C.

232 In the case of *Enterobacteriaceae* (Figure 4), raw milk contained greater levels of
233 *Enterobacteriaceae* than all other milks ($P<0.05$) at each of the time points examined. On
234 days 0, 2 and 9, no significant difference was observed between the milks treated with
235 MTS/PEF at both temperatures (i.e. at 37°C and 55°C). On days 5, 7, 14 and 21, no
236 significant difference was observed between the *Enterobacteriaceae* content of milks treated
237 with MTS/PEF at 37°C and conventional pasteurisation ($P<0.05$). On day 21, the level of
238 *Enterobacteriaceae* in raw milk had reached 2.4×10^7 CFU/ml, whereas the levels present in
239 milks treated at 37°C and 55°C with MTS/PEF were reduced by 4.4 and 4.1 log cycles,

240 respectively. Thermal pasteurisation appeared to almost completely prevent growth of
241 *Enterobacteriaceae* for the duration of the study.

242 Raw milk was also found to contain significantly more lactic acid bacteria (LAB) at
243 each time point than any of the other processed milk samples ($P<0.05$) (Figure 5). On days 2,
244 5 and 7, no significant difference ($P>0.05$) was observed between any of the treatments, but
245 on days 14 and 21, conventionally pasteurised milk contained less lactic acid bacteria than
246 milk heated at 37°C and 55°C prior to MTS/PEF treatment. By day 21, raw milk was found
247 to contain *c.* 5.4×10^7 CFU/ml LAB, while treatments where milk was heated to 37°C and
248 55°C before being subjected to MTS/PEF caused log reductions of 2.9 and 2, respectively,
249 with conventional pasteurisation resulting in a log reduction of 5.4.

250 With the exception of days 9 and 14, raw milk was found to contain significantly
251 more pseudomonads than all of the processed milk samples ($P<0.05$) (Figure 6). Also, on
252 days 0, 2, 5, 7 and 9, milk which had been treated at 55°C followed by MTS/PEF contained a
253 similar level of pseudomonads to thermally pasteurised milk ($P>0.05$). No significant
254 difference was found between milks treated at 37°C and 55°C followed by MTS/PEF at any
255 of the time points over the 21 days ($P<0.05$). By day 21, the level of pseudomonads had
256 reached 1×10^9 CFU/ml, but treating milk by heating at 37°C and 55°C prior to MTS/PEF
257 resulted in log reductions of 1.9 and 1.8, respectively. However, thermal pasteurisation was
258 found to show a substantially higher log reduction (6.6) in the level of pseudomonads in
259 comparison to raw milk than all other treatments by day 21 ($P<0.05$).

260 Finally, the levels of psychrotrophic bacteria in raw milk, MTS/PEF treated milk
261 samples and pasteurised milk were measured at four time intervals over the 21-day period
262 (Figure 7). As had been determined for all other microbial species examined throughout this
263 study, raw milk was found to contain higher levels of psychrotrophs than milks which had
264 been subjected to MTS/PEF or conventional pasteurisation. The average initial level of

265 psychrotrophic bacteria from the three batches of raw milk was estimated to be 1.6×10^5
266 CFU/ml, and this level reached 7×10^7 CFU/ml by day 21. Plate count results from day 0
267 showed there was a minimum of a 3.5 log reduction in the level of psychrotrophic bacteria in
268 milk after treatment with MTS/PEF. On days 14 and 21, thermally pasteurised milk contained
269 significantly lower levels of psychrotrophs than milks treated with MTS/PEF at either 37°C
270 or 55°C ($P < 0.05$).

271 **4. Discussion**

272 Raw milk contains a wide range of microorganisms including yeasts and moulds, both Gram
273 positive and Gram negative bacteria, and a range of bacterial spores (Shamsi, Versteeg,
274 Sherkat, & Wan, 2008). The present study has shown that combining two non-thermal
275 technologies (i.e. MTS and PEF) can decrease the microbial populations of raw milk, and
276 consequently improve the microbiological quality. A study by Noci *et al.* (2009) reported
277 inactivation of *Listeria innocua* by up to *c.* 7 log cycles following treatment with
278 thermosoniaction immediately followed by pulsed electric fields. No other studies have been
279 undertaken to assess the microbiological quality of milk following treatment with a
280 combination of manothermosonication and pulsed electric fields. Thus, no direct comparisons
281 can be made between the present study and other published studies. However, details of some
282 studies which applied ultrasound and pulsed electric fields as separate entities for the purpose
283 of microbial inactivation have been reported.

284 Milk processing by high intensity pulsed electric fields has been reviewed previously
285 (Bendicho, Barbosa-Canovas, & Martin, 2002), and many researchers have reported how
286 PEF can be used specifically for microbial inactivation in milk (Odriozola-Serrano,
287 Bendicho-Porta, & Martin-Belloso, 2006; Cserhalmi, Sass-Kiss, Toth-Markus, & Lechner,
288 2006; Craven *et al.*, 2008; Shamsi, Versteeg, Sherkat, & Wan, 2008; Sepulveda, Gongora-
289 Nieto, Guerrero, & Barbosa-Canovas, 2009; Walkling-Ribeiro, Rodriguez-Gonzalez,

290 Jayaram, & Griffiths, 2011). In addition, PEF is a popular method for extending the shelf-life
291 of fruit juices (Walkling-Ribeiro *et al.*, 2009a, 2009b, 2010; Yeom, Streaker, Zhang, & Min,
292 2000) and fruit juice-milk based beverages (Walkling-Ribeiro *et al.*, 2008; Sampedro *et al.*,
293 2009; Salvia-Trujillo, Morales-de la Pena, Rojas-Grau, & Martin-Belloso, 2011). Therefore,
294 combining PEF with other non-thermal technologies could represent an alternative approach
295 to improving microbiological quality and consequently prolonging the shelf-life of many
296 beverages, both dairy and non-dairy based.

297 Under the experimental conditions used in the present study for MTS and PEF, the
298 TVC values were reduced considerably following processing at both temperatures (37°C and
299 55°C), producing milk with a similar TVC to that of conventionally pasteurised milk. A study
300 by Villamiel and de Jong (2000) which assessed the total bacterial count of milk following
301 treatment with ultrasound (20 kHz) in a continuous-flow system reported reductions of 0.2,
302 0.6 and 2.9 log cycles following treatment with US at residence times of 34, 56 and 102
303 seconds, respectively, inside the treatment chamber. In addition, the temperatures reached
304 during these treatments were 48.6°C, 62°C and 76°C, respectively. These inactivation values
305 are much lower than those of the present study, where only mild heat was applied during
306 MTS/PEF treatment (i.e. 37°C and 55°C). In the same study by Villamiel and de Jong (2000),
307 milks which had been processed at 62°C in a continuous flow ultrasound system or in a
308 conventional heating system were compared over a five day period. It was determined that
309 the number of bacteria remained constant at *c.* 4 log CFU/ml until the third day of storage,
310 and then went on to reach *c.* 5 log CFU/ml after five days, with the increase in total bacteria
311 counts being similar for both US-treated milk and thermally treated milk. These values for
312 inactivation are in contrast to those observed in the present study, where MTS/PEF processed
313 milk treated at 37°C and 55°C had TVC levels of *c.* 1.8 and 2.7 log CFU/ml, respectively,

314 seven days after treatment. This demonstrates the benefits of combining MTS with PEF so
315 that higher levels of microbial inactivation can be achieved.

316 Another study by Chouliara, Georgogianni, Kanellopoulou, & Kontominas (2010)
317 examined TVC levels in full fat milk following treatment with US over an eight day period.
318 Ultrasound was applied at 24 kHz (amplitude was not specified) and after eight days, it was
319 determined that raw milk contained 4.3 log CFU/ml, but values were reduced to 3.8, 3.1, 2.9
320 and 2.9 log CFU/ml following 2, 4, 8 and 16 mins of sonication, respectively. Similar studies
321 have also been conducted by Bermudez-Aguirre and co-workers where microbial levels of
322 milk following treatment with US (Bermudez-Aguirre, Mawson, Versteeg, & Barbosa-
323 Canovas, 2009) and PEF (Bermudez-Aguirre *et al.*, 2011) were assessed. In one study, these
324 authors examined the total mesophilic count of raw whole milk over a 16-day period
325 (Bermudez-Aguirre, Mawson, Versteeg, & Barbosa-Canovas, 2009). The mesophilic count
326 was determined to be 4.7 log CFU/ml on day 0, and after treatment with US (24 kHz, 108
327 μm , 63°C, 30 min) this was reduced to *c.* 2 log CFU/ml and <1 log CFU/ml on days 0 and 16,
328 respectively. Thermally treated milk contained *c.* 5.8 log CFU/ml after 16 days under
329 refrigeration at 4°C. In addition, these researchers applied ultrasound to milk at 36 μm , 72
330 μm , 108 μm and 120 μm , and no more than 2 log cycles of mesophiles were detected over the
331 16-day period. However, it should be taken into consideration that milk was heated at 63°C
332 for 30 min while being treated with ultrasound during these experiments. In the present study,
333 ultrasound was applied for *c.* 2.1 mins, and milk was heated at 37°C or 55°C. Immediately
334 after processing (i.e. on day 0), milks heated at these temperatures (37°C, 55°C) during MTS
335 treatment still yielded reductions of 2.6 and 3.6 log cycles, respectively, in spite of relatively
336 mild and moderate heat treatments applied.

337 With regard to PEF, a shelf-life study by Odriozola-Serrano, Bendicho-Porta, &
338 Martin-Belloso (2006) reported a reduction of mesophilic bacteria of between 1 and 2 log

339 cycles in raw whole milk treated at 35.5 kV/cm, temperature of <40°C and pulse width of 7
340 µs. A separate study by Bermudez-Aguirre *et al.* (2011) examined the effect of PEF (46.15
341 kV/cm, 200 kHz, 30 pulses × 2 µs, flow rate 1 L/min) on microbial inactivation in whole fat
342 milk. It was determined that mesophilic and psychrotrophic bacteria were reduced by 0.4 and
343 0.8 log cycles, respectively, after treatment with PEF at 40°C. It was observed during this
344 study that in general, mesophilic bacteria were more resistant than psychrotrophic bacteria to
345 PEF thermal treatment, although inactivation of both of these types of microorganisms was
346 not considered to be high. A shelf-life study carried out by Bermudez-Aguirre *et al.* (2011)
347 showed that mesophiles grew rapidly during storage at 4°C in milk after treatment with PEF
348 at 20°C, 30°C and 40°C, with the level of mesophilic bacteria exceeding 6 log cycles after the
349 first week of storage. A similar growth pattern was observed for psychrophilic bacteria,
350 regardless of the PEF process temperature (Bermudez-Aguirre *et al.*, 2011). In addition, a
351 shelf-life study carried out by Odriozola-Serrano, Bendicho-Porta, & Martin-Belloso (2006)
352 where whole raw milk was subjected to PEF (35.5 kV/cm, 1000 µs, 111 Hz) in a continuous
353 flow system (60 ml/min) showed that after eight days of storage, PEF-treated milk was found
354 to contain similar levels of mesophilic aerobic microorganisms as milk which had been
355 treated with thermal pasteurisation ($P>0.05$).

356 Other researchers have reported US to have no effect on the levels of yeasts and
357 moulds in milk. In an investigation carried out by Engin and Karagul-Yuceer (2012), no
358 significant difference was found between the levels of yeasts and moulds in US treated (20
359 kHz, 5°C, 75 W, 15 min) milk and raw milk ($P>0.05$). In this study the effect of US on
360 *Staphylococcus* sp. and *E. coli* were also assessed, and it was determined that under the
361 specified conditions these microorganisms were reduced by 0.8 and 1.15 log cycles,
362 respectively. A separate study by Herceg, Jambrak, Lelas, & Thagard (2012) examined the
363 inactivation of *S. aureus* and *E. coli* in raw whole fat (4%) cow's milk immediately following

364 treatment with US (20 kHz, 120 μm). It was determined that after treatment with US for 12
365 mins at 60°C, *S. aureus* and *E. coli* were reduced by 1.5 and 3.1 log cycles, respectively.
366 However, in the present study, where sonication was only applied for approximately 2 mins,
367 *S. aureus* was found to be reduced by 1.4 and 2.7 log cycles following treatment with
368 MTS/PEF at respective temperatures of 37°C and 55°C, immediately following processing.
369 The reduction of *E. coli* as a single microorganism was not quantified in the present study,
370 but the levels of *Enterobacteriaceae* were reduced by 3.9 and 3.63 log cycles following
371 treatment with MTS/PEF at 37°C and 55°C, respectively. The levels of inactivation observed
372 in the current study were achieved using a lower amplitude of US (27.9 μm compared to 120
373 μm), and a shorter treatment time (*c.* 2.1 mins). Taking into consideration the inactivation of
374 *S. aureus*, it appears that although ultrasound alone caused up to 1.49 log cycles of
375 inactivation (Herceg, Jambrak, Lelas, & Thagard, 2012) under sonication conditions different
376 (i.e. higher amplitude, higher treatment temperature) to those applied in the present study,
377 greater inactivation of *S. aureus* was achieved (*c.* 2.7 log cycles at 55°C) in the current study
378 when MTS/PEF was applied in combination. This suggests that either the addition of (i)
379 pressure or (ii) PEF resulted in an increase in the antimicrobial efficacy of ultrasound.

380 A shelf-life study by Juraga, Salamon, Herceg, & Jambrak, (2011) using whole fat
381 (4%) cow's milk showed that after five days of refrigeration, the levels of *Enterobacteriae*
382 were reduced by 0.2, 2.7 and 3.1 log cycles when compared to raw milk following treatment
383 with US (20 kHz, 120 μm) for 6, 9 and 12 mins, respectively. These reductions were
384 increased to 2.12, 4.63 and 3.05 log cycles when US was combined with heat (60°C). The
385 inactivation levels of *Enterobacteriaceae* observed in the present study were found to be far
386 greater (a minimum of 3.2 log cycles) than those reported by Juraga, Salamon, Herceg, &
387 Jambrak, (2011), even though a lower amplitude level was used in our study (27.9 μm), and
388 lower temperatures also (37°C and 55°C). This shows the benefits of combining ultrasound

389 with pressure and mild heat (i.e. MTS), and also the increased levels of inactivation which
390 can be achieved when MTS and PEF technologies are combined.

391 A publication by Villamiel and de Jong (2000) discussed the effect of US (20 kHz) on
392 the viability of *Pseudomonas fluorescens* in raw cow's milk following sonication in a
393 continuous flow system. Reductions of 0.6, 0.8 and 3.1 log cycles were observed, but
394 temperatures of 43.1°C, 49°C and 61.6°C were applied, respectively, in order to achieve
395 these levels of inactivation. Experiments previously carried out in our laboratory using *P.*
396 *fluorescens* showed after subjecting this microorganism to treatment with either PEF or a
397 combination of US/PEF, some *P. fluorescens* cells were not killed but merely sub-lethally
398 injured (unpublished data). This may explain why, in the present study, the levels of
399 pseudomonads reached such high levels (c. 7 logs) after 21 days following treatment with
400 MTS/PEF at 37°C and 55°C. Milk which had undergone thermal pasteurisation contained
401 only c. 1 log of pseudomonads after 21 days, showing that thermal pasteurisation kills this
402 microorganism without sub-lethally injuring these bacterial cells. In addition, a study by
403 Craven *et al.* (2008) showed that pseudomonads could be reduced by >5 log cycles following
404 treatment with PEF (31 kV/cm) and mild heat of up to 55°C. In the present study, lower
405 inactivation values of pseudomonads were recorded for MTS/PEF under our experimental
406 conditions, but it should be noted that the temperature did not exceed 40°C during PEF
407 treatment.

408 In the study by Chouliara, Georgogianni, Kanellopoulou, & Kontominas (2010),
409 psychrotrophic bacteria were also quantified. On day 0, raw milk which had not been
410 subjected to sonication contained 3.3 log CFU/ml psychrotrophic bacteria, but these levels
411 were reduced to 3.2, 2, 2.2 and 1.9 following 2, 4, 8 and 16 mins of sonication, respectively
412 (24 kHz, amplitude not specified). After eight days, milk which had been sonicated for 16
413 mins contained significantly fewer psychrotrophic bacteria than all other milk samples

414 ($P < 0.05$). However, it should be noted that these milk samples were not subjected to
415 sonication in a continuous-flow system. In the current study, levels of psychrotrophic bacteria
416 reached *c.* 4 log CFU/ml in milk treated with MTS/PEF at 37°C and 55°C after 21 days of
417 storage at 4°C. This increase in microbial levels may have been due to the presence of some
418 Gram-positive thermotolerant bacteria and spores, which are known to have the potential to
419 survive thermal pasteurisation and/or PEF and ultrasound processing (Shamsi, Versteeg,
420 Sherkat, & Wan, 2008). It may be the case that a greater number of thermotolerant bacteria are
421 killed by thermal pasteurisation than by processing with manothermosonication and PEF, but
422 further investigation would be required in order to determine the exact nature of the
423 inactivation of such microorganisms. Some preliminary studies carried out in this laboratory
424 have shown that treatment with MTS/PEF caused sub-lethal injury of *E. coli* K12 and *Ps.*
425 *fluorescens* (data not shown), and it may be the case that other microorganisms are merely
426 injured following treatment with these non-thermal technologies, and are not actually 'killed'.
427 Nonetheless, the present study shows that inactivation of psychrotrophic bacteria is
428 achievable by MTS/PEF.

429 **5. Conclusion**

430 The present study has shown that treating milk with MTS/PEF can inhibit microbial growth
431 without the use of excessive heat, such as the temperatures employed during thermal
432 pasteurisation. Although conventional pasteurisation appears to be the most effective method
433 of prolonging the shelf-life of milk, the results for microbial inactivation following treatment
434 with MTS/PEF are promising. However, further optimisation of processing conditions would
435 be required in order to improve the microbiological quality of milk treated with MTS/PEF so
436 that microbial content is on a par with those typical of thermally pasteurised milk. Also, it
437 would be worthwhile to investigate the efficacy of these technologies for control of other

438 pathogens associated with raw milk, such as *Listeria monocytogenes*, *Yersinia enterocolitica*
439 and *Campylobacter jejuni*.

440 **Acknowledgements**

441 This work was supported by SMARTMILK, a 2 year R&D project funded by the Seventh
442 Framework Programme of the EC under the “Research for SMEs” sub-programme. Grant
443 agreement No. 261591. The authors would also like to thank Lyons Research Farm
444 (Newcastle, Co. Dublin) for supplying the milk used throughout this study.

445

446 **Literature cited**

- 447 Aronsson, K., Borch, E., Stenlof, B., & Ronner, U. (2004) Growth of pulsed electric field
448 exposed *Escherichia coli* in relation to inactivation and environmental factors.
449 *International Journal of Food Microbiology*, 93 (1), 1-10.
- 450 Bendicho, S., Barbosa-Canovas, G.V., & Martin, O. (2002) Milk processing by high intensity
451 pulsed electric fields. *Trends in Food Science and Technology*, 13, 195-204.
- 452 Bermudez-Aguirre, D., Mawson, R., Versteeg, K., & Barbosa-Canovas, G.V. (2009)
453 Composition properties, physicochemical characteristics and shelf-life of whole milk
454 after thermal and thermo-sonication treatments. *Journal of Food Quality*, 32 (3), 283-
455 302.
- 456 Bermudez-Aguirre, D., Fernandez, S., Esquivel, H., Dunne, P.C., & Barbosa-Canovas, G. V.
457 (2011) Milk processed by pulsed electric fields: Evaluation of microbial quality,
458 physicochemical characteristics, and selected nutrients at different storage conditions.
459 *Journal of Food Science*, 76 (5), S289-S299.
- 460 Chouliara, E., Georgogianni, K.G., Kanellopoulou, N., & Kontominas, M.G. (2010) Effect of
461 ultrasonication on microbiological, chemical and sensory properties of raw, thermized
462 and pasteurized milk. *International Dairy Journal*, 20 (5), 307-313.
- 463 Craven, H.M., Swiergon, P., Ng, S., Midgely, C., Versteeg, C., Coventry, M.J., & Wan, J.
464 (2008) Evaluation of pulsed electric field and minimal heat treatments for inactivation
465 of pseudomonads and enhancement of milk shelf-life. *Innovative Food Science &
466 Technologies*, 9 (2), 211-216.

- 467 Cserhalmi, Z., Sass-Kiss, A., Toth-Markus, M., & Lechner, N. (2006) Study of pulsed
468 electric field treated citrus juices. *Innovative Food Science & Emerging Technologies*,
469 7, 49-54.
- 470 Devlieghere, F., Vermeiren, L., & Debevere, J. (2004) New preservation technologies:
471 Possibilities and limitations. *International Dairy Journal*, 14 (4), 273-285.
- 472 Dolatowski, Z.J., Stadnik, J., & Stasiak, D. (2007) Applications of ultrasound in food
473 technology. *Acta Sci. Pol., Technol. Aliment.*, 6 (3), 89-99.
- 474 Engin, B., & Karagul-Yuceer, Y. (2012) Effects of ultraviolet light and ultrasound on
475 microbial quality and aroma-active components of milk. *Journal of the Science of*
476 *Food and Agriculture*, 92, 1245-1252.
- 477 Hamilton W.A., & Sale, A.J.H. (1967) Effects of high electric fields on microorganisms. II.
478 Mechanism of action of the lethal effect. *Biochimica et Biophysica Acta*, 87, 102-107
- 479 Herceg, Z., Jambrak, A.R., Lelas, V., & Thagard, S.M. (2012) The effect of high intensity
480 ultrasound treatment on the amount of *Staphylococcus aureus* and *Escherichia coli* in
481 milk. *Food Technology and Biotechnology*, 50 (1), 46-52.
- 482 Juraga, E., Salamon, B.S., Herceg, Z., & Jambrak, A.R. (2011) Application of high intensity
483 ultrasound treatment on *Enterobacteriae* count in milk. *Mljekarstvo*, 61 (2), 125-134.
- 484 Manas, P., & Pagan, R. (2005) Microbial inactivation by new technologies of food
485 preservation. *Journal of Applied Microbiology*, 98 (6), 1387-1399.
- 486 Noci, F., Walkling-Ribeiro, M., Cronin, D.A., Morgan, D.J., & Lyng, J.G. (2009) Effect of
487 thermosonication, pulsed electric field and their combination on inactivation of
488 *Listeria innocua* in milk. *International Dairy Journal*, 19, 30-35.

- 489 Odriozola-Serrano, I., Bendicho-Porta, S., & Martin-Belloso, O. (2006) Comparative study
490 on shelf life of whole milk processed by high-intensity pulsed electric field or heat
491 treatment. *Journal of Dairy Science*, 89, 905-911.
- 492 Pereda, J., Ferragut, V., Quevedo, J.M., Guamis, B., & Trujillo, A.J. (2009) Heat damage
493 evaluation in ultra-high pressure homogenized milk. *Food Hydrocolloids*, 23 (7),
494 1974-1979.
- 495 Piyasena, P., Mohareb, E., & McKellar, R.C. (2003) Inactivation of microbes using
496 ultrasound: a review. *International Journal of Food Microbiology*, 87 (3), 207-216.
- 497 Salvia-Trujillo, L., Morales-de la Pena, M., Rojas-Grau, M.A., & Martin-Belloso, O. (2011)
498 Microbial and enzymatic stability of fruit juice-milk based beverages treated by high
499 intensity pulsed electric fields or heat during refrigerated storage. *Food Control*, 22
500 (10), 1639-1646.
- 501 Sampedro, F., Geveke, D.J., Fan, X., Rodrigo, D., & Zhang, Q.H. (2009) Shelf-life study of
502 an orange juice-milk based beverage after PEF and thermal processing. *Journal of*
503 *Food Science*, 74 (2), S107-S112.
- 504 Sepulveda, D.R., Gongora-Nieto, M.M., Guerrero, J.A., & Barbosa-Canovas, G.V. (2009)
505 Shelf life of whole milk processed by pulsed electric fields in combination with PEF-
506 generated heat. *LWT- Food Science and Technology*, 42 (3), 735-739.
- 507 Shamsi, K., Versteeg, C., Sherkat, F., & Wan, J. (2008) Alkaline phosphatase and microbial
508 inactivation by pulsed electric field in bovine milk. *Innovative Food Science &*
509 *Emerging Technologies*, 9, 217-223.
- 510 Villamiel, M., & de Jong, P. (2000) Inactivation of *Pseudomonas fluorescens* and
511 *Streptococcus thermophilus* in Trypticase® Soy Broth and total bacteria in milk by

512 continuous-flow ultrasonic treatment and conventional heating. *Journal of Food*
513 *Engineering*, 45 (3), 171-179.

514 Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J.G., & Morgan, D.J. (2008)
515 Inactivation of *Escherichia coli* in a tropical fruit smoothie by a combination of heat
516 and pulsed electric fields. *Journal of Food Science*, 73 (8), M395-M399.

517 Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J.G., & Morgan, D.J. (2009a) Shelf life
518 and sensory evaluation of orange juice after exposure to thermosonication and pulsed
519 electric fields. *Food and Bioproducts Processing*, 87 (2), 102-107.

520 Walkling-Ribeiro, M., Noci, F., Riener, J., Cronin, D.A., Lyng, J.G., & Morgan, D.J. (2009b)
521 The impact of thermosonication and pulsed electric fields on *Staphylococcus aureus*
522 inactivation and selected quality parameters in orange juice. *Food and Bioprocess*
523 *Technology*, 2 (4), 422-430.

524 Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J.G., & Morgan, D.J. (2010) Shelf life
525 and sensory attributes of a fruit smoothie-type beverage processed with moderate heat
526 and pulsed electric fields. *LWT- Food Science and Technology*, 43 (7), 1067-1073.

527 Walkling-Ribeiro, M., Rodriguez-Gonzalez, O., Jayaram, S., & Griffiths, M.W. (2011)
528 Microbial inactivation and shelf life comparison of 'cold' hurdle processing with
529 pulsed electric fields and microfiltration, and conventional thermal pasteurisation in
530 skim milk. *International Journal of Food Microbiology*, 144 (3), 379-386.

531 Yeom, H.W., Streaker, C.B., Zhang, Q.H., & Min, D.B. (2000) Effects of pulsed electric
532 fields on the quality of orange juice and comparison with heat pasteurization. *Journal*
533 *of Agricultural and Food Chemistry*, 48 (10), 4597-4605.

534

535 **List of Figures**

536 **Figure 1:** TVC levels of various milk samples over a 21-day period; Raw milk (white bar),
537 MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and thermally
538 pasteurised (black bar). Data= mean \pm S.D. (n=3).

539 **Figure 2:** Yeast and mould levels of various milk samples over a 21-day period; Raw milk
540 (white bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and
541 thermally pasteurised (black bar). Data= mean \pm S.D. (n=3).

542 **Figure 3:** *S. aureus* content of various milk samples over a 21-day period; Raw milk (white
543 bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and thermally
544 pasteurised (black bar). Data= mean \pm S.D. (n=3).

545 **Figure 4:** *Enterobacteriaceae* content of various milk samples over a 21-day period; Raw
546 milk (white bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and
547 thermally pasteurised (black bar). Data= mean \pm S.D. (n=3).

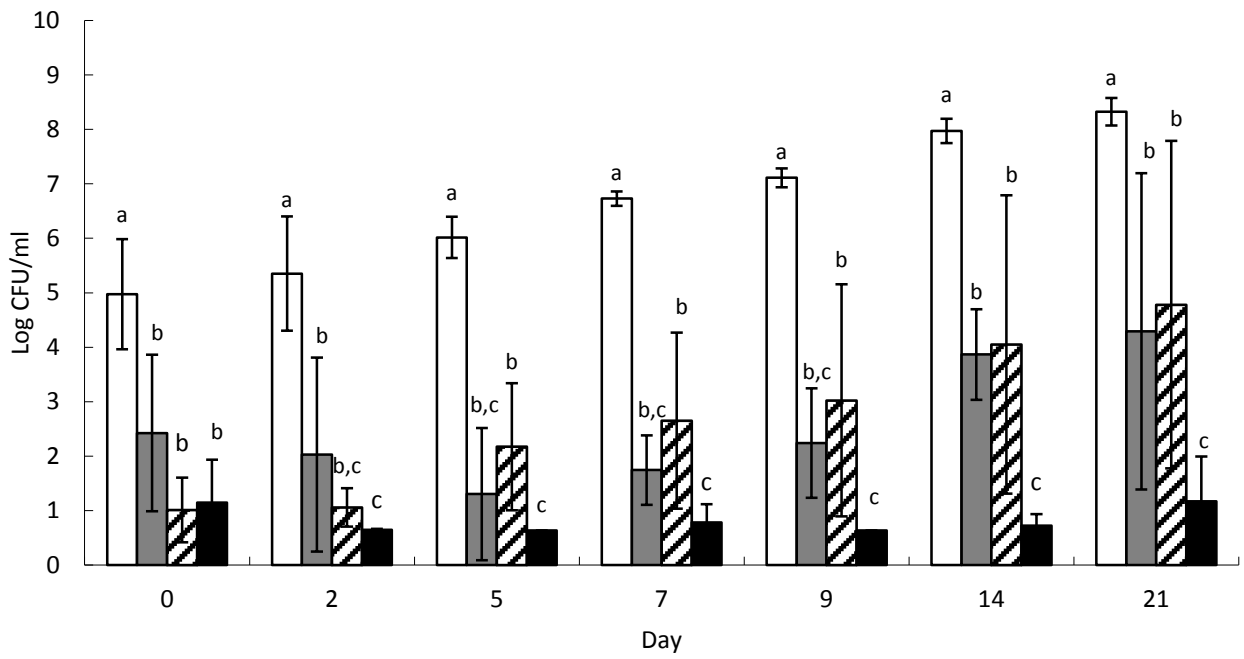
548 **Figure 5:** Lactic acid bacteria levels of various milk samples over a 21-day period; Raw milk
549 (white bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and
550 thermally pasteurised (black bar). Data= mean \pm S.D. (n=3).

551 **Figure 6:** Pseudomonads content of various milk samples over a 21-day period; Raw milk
552 (white bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and
553 thermally pasteurised (black bar). Data= mean \pm S.D. (n=3).

554 **Figure 7:** Psychrotrophic bacteria content of various milk samples over a 21-day period; Raw
555 milk (white bar), MTS/PEF at 37°C (grey bar), MTS/PEF at 55°C (striped bar) and
556 thermally pasteurised (black bar). Data= mean \pm S.D. (n=3).

557

558 **Figure 1:**

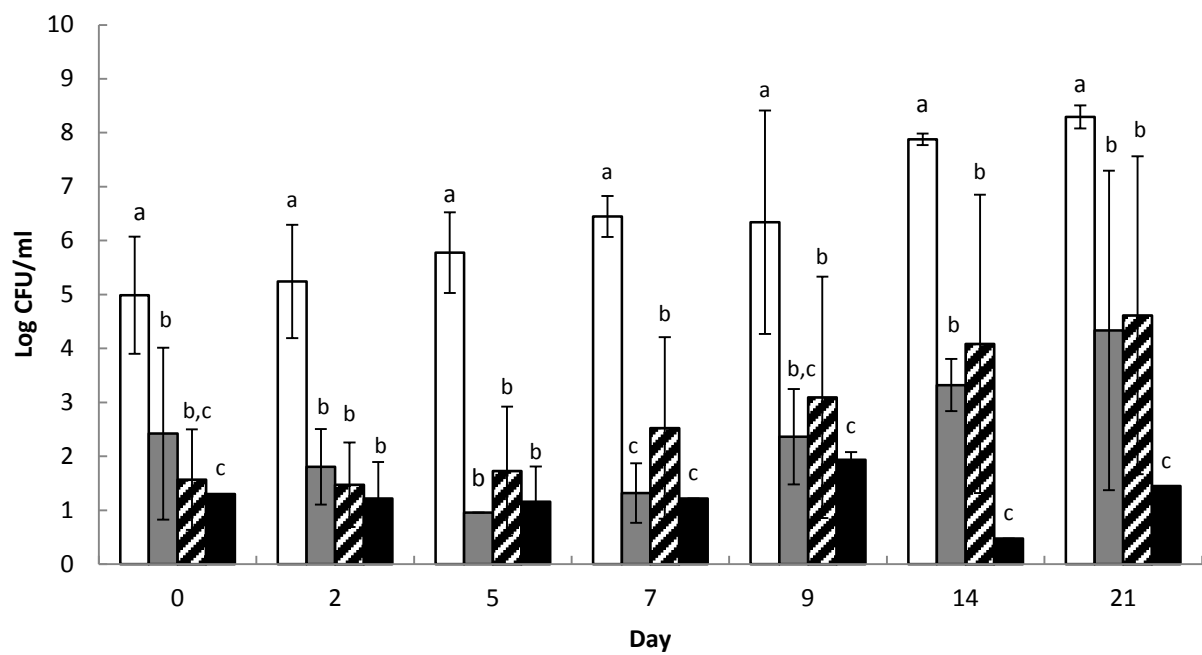


559

560

561

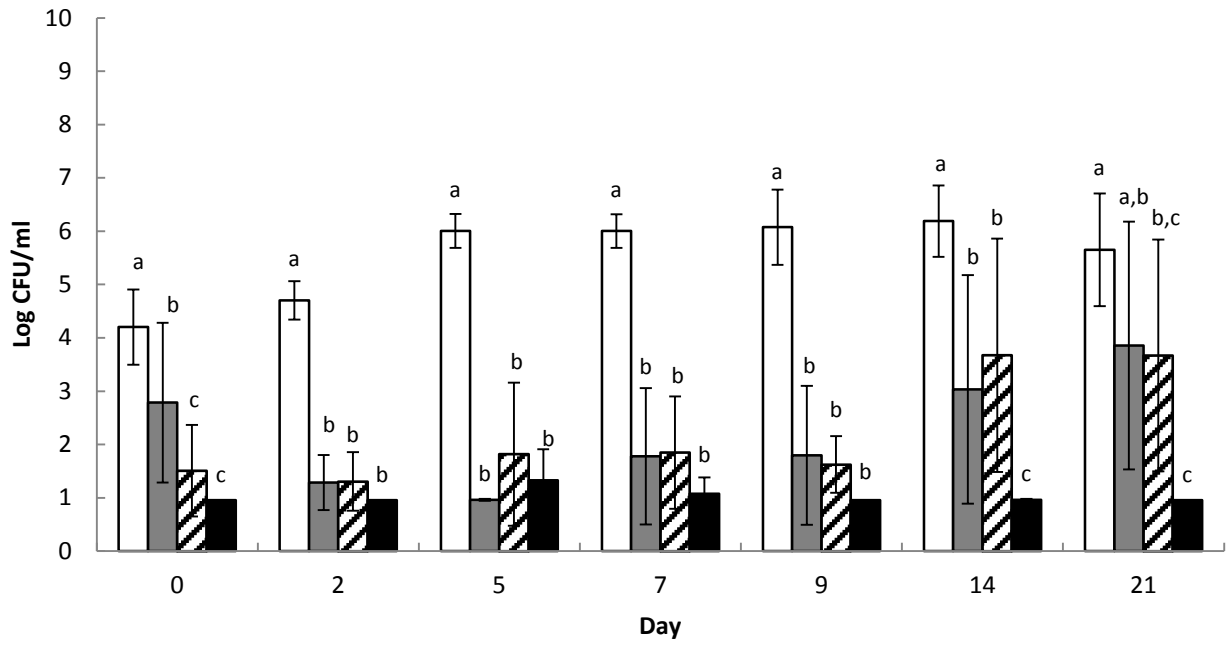
562 **Figure 2:**



563

564

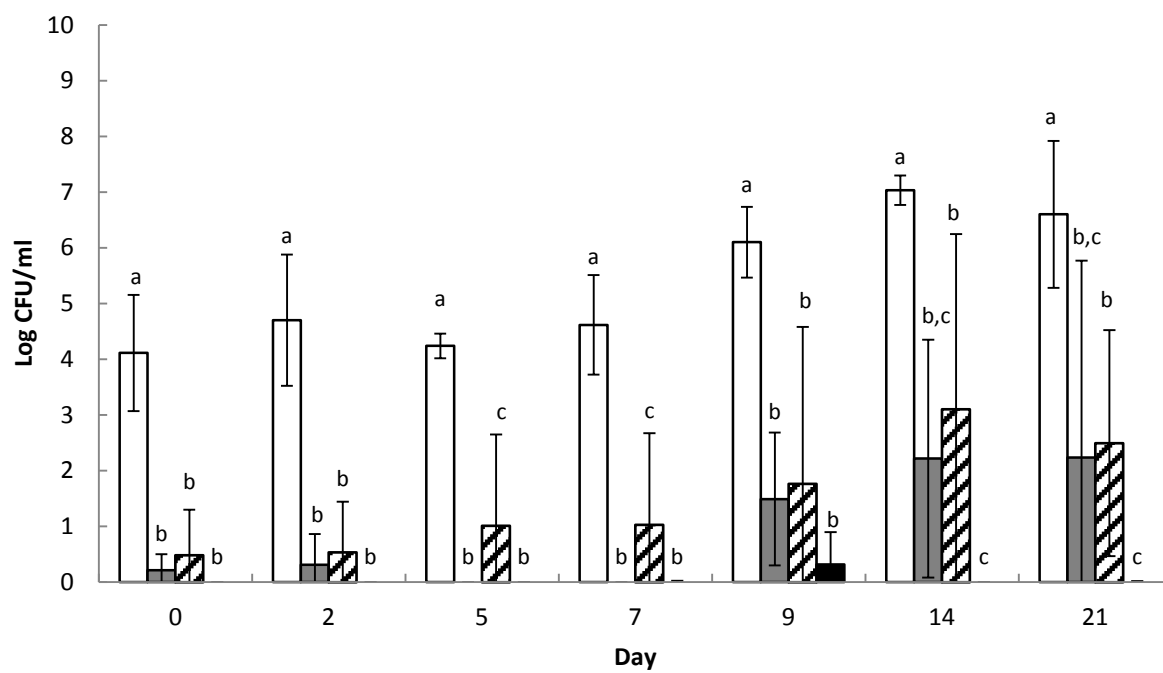
565 **Figure 3:**



566

567

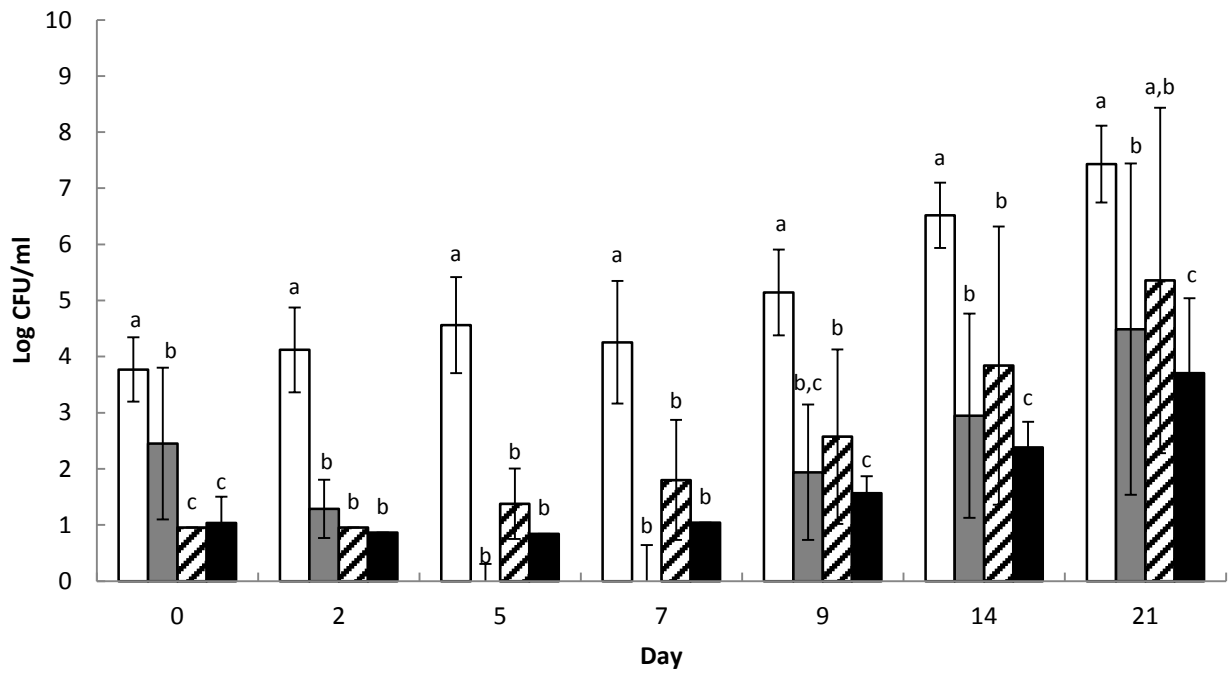
568 **Figure 4:**



569

570

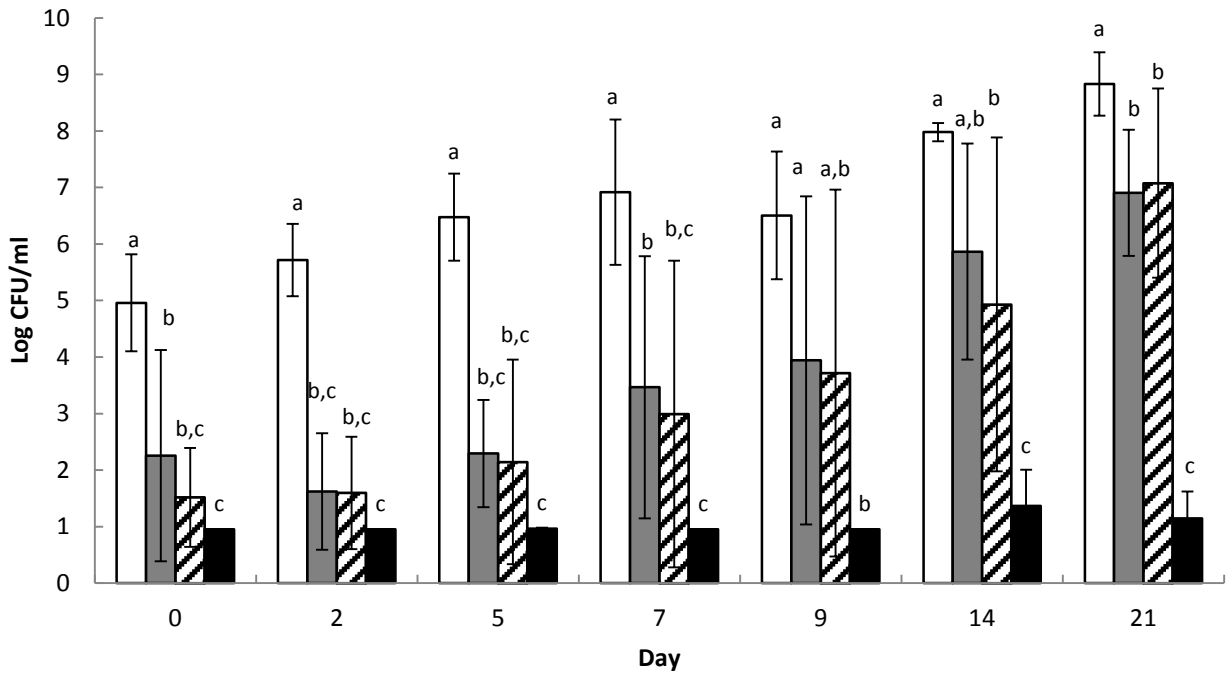
571 **Figure 5:**



572

573

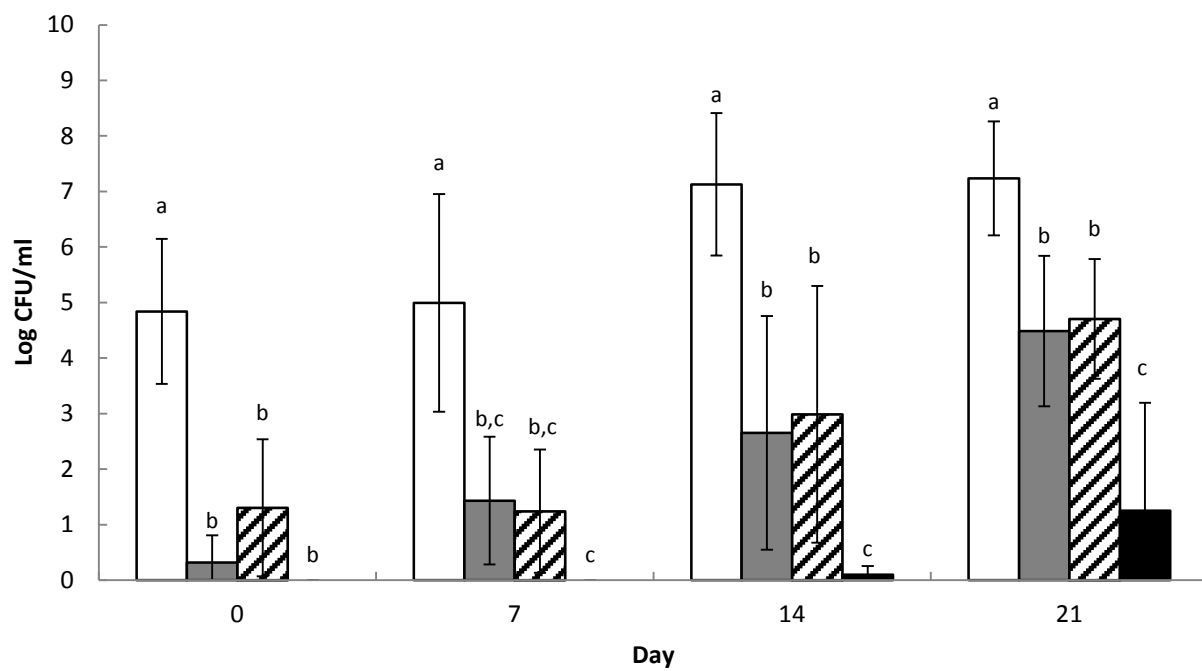
574 **Figure 6:**



575

576

577 **Figure 7:**



578

579