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The Effect of non-Thermal Processing Technologies on Microbial Inactivation: An Investigation into sub-Lethal Injury of Escherichia Coli and Pseudomonas Fluorescens

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1 **The effect of non-thermal processing technologies**
2 **on microbial inactivation: An investigation into**
3 **sub-lethal injury of *Escherichia coli* and**
4 ***Pseudomonas fluorescens***

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24 **Abstract**

25 In recent years, there has been an increased interest in food processing technologies that
26 could lessen the thermal impact on food products. In the present study, thermosonication
27 (TS) and pulsed electric fields (PEF), applied individually or in combination (TS/PEF),
28 were investigated to determine their effects on inactivation and sub-lethal injury of
29 *Pseudomonas fluorescens* and *Escherichia coli*. TS was applied at a low (L) and high
30 (H) wave amplitude (L; 18.6 μ m, H; 27.9 μ m, respectively), while PEF was applied at a
31 low and high electrical field strength (L; 29kVcm⁻¹, H; 32kVcm⁻¹, respectively). In
32 addition, the inhibitory effects of TS/PEF combined were assessed. For *P. fluorescens*,
33 when applied individually, TS and PEF resulted in \leq 9% and \leq 47% inactivation,
34 respectively, with 8% sub-lethal injury following PEF treatment. However, TS/PEF
35 treatment caused \leq 48% inactivation and \leq 34% sub-lethal injury, respectively. For *E.*
36 *coli*, TS caused \leq 6% inactivation, and \leq 2% sub-lethal injury, while PEF treatment alone
37 caused inactivation and sub-lethal injury of 86% and 29%, respectively. TS/PEF caused
38 a maximum of 66% inactivation, while sub-lethally injuring approximately 26% of the
39 of *E. coli* population. The present study confirms the ability of TS and PEF to inactivate
40 microorganisms, but shows that some bacteria were not killed, but sub-lethally injured.

41

42 **Keywords:** Thermosonication, PEF, *Escherichia coli*, *Pseudomonas fluorescens*, sub-
43 lethal injury.

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50 **1. Introduction**

51 Foods are not sterile substances and microbial spoilage in food is a reality that is
52 perhaps unavoidable. According to Raso *et al.* (2005), the two main contributors to food
53 spoilage are microorganisms and enzymes in food. There are ample ways of delaying
54 this spoilage process, with the most common method of microbial inactivation being by
55 thermal treatment, or pasteurisation (Raso *et al.*, 2005). Although thermal treatment may
56 effectively kill microorganisms, it can also have damaging effects on the food/beverage.
57 Thus, in recent years there has been considerable interest in food preservation by non-
58 thermal technologies. Some examples of non-thermal technologies include ultrasound
59 (US), high voltage pulsed electric fields (PEF), high intensity light pulses (HILP) and
60 ultraviolet light (UV) (Caminiti *et al.*, 2011). Ultrasound and PEF are two methods of
61 particular interest to the present study.

62 Ultrasound is a novel technology that produces sonic waves with frequencies of
63 16-20 kHz; this is above the upper limit of human hearing (Condón *et al.*, 2005).
64 Ultrasound operates on the mechanism of liquids coming into contact with sonic waves.
65 As these sonic waves penetrate into a liquid medium they create compression and
66 expansion cycles. The expansion cycle creates negative pressure in the liquid. Minute
67 bubbles can be formed when this negative pressure is minimal enough to surpass
68 intermolecular forces. These bubbles expand and contract throughout compression and
69 expansion cycles in a process known as cavitation (Condón *et al.*, 2005). The size of the
70 bubble fluctuates when the ultrasound wave comes into contact with a liquid, and with
71 each new cycle the size of the bubble increases. After alternating cycles of compression
72 and expansion, the sonic energy is no longer able to maintain the vapour phase inside of
73 the bubble and it implodes. The mechanism of microbial inactivation following
74 treatment with ultrasound is that when these bubble implode, it causes the surrounding
75 molecules to collide somewhat powerfully into one another, creating areas of extremely

76 high temperatures of up to 5500°C (Condón *et al.*, 2005). In addition, when these
77 bubbles implode they release shock waves that damage cell membranes, and also may
78 produce free radicals that could potentially contribute to microbial inactivation
79 (Piyasena *et al.*, 2003). It has been suggested that a mild application of heat when used
80 in conjunction with ultrasonication may lead to an increase in the microbial inactivation
81 capacity of US; a process known as thermosonication (TS). Ultrasound can also be
82 combined with pressure, referred to as manosonication, or pressure and heat
83 simultaneously, known as manothermosonication (Piyasena *et al.*, 2003).

84 The second non-thermal method relevant to this study is PEF. Microbial
85 inactivation due to PEF treatment is believed to be caused by disruption of the cell
86 membrane; a process known as ‘electroporation’ (Hamilton and Sale, 1967), which
87 results from recurring application of short pulses of high intensity electric fields
88 (Barbosa-Cánovas & Sepúlveda, 2005). Electroporation is, essentially the formation of
89 pores in the bacterial membrane, which results in the leakage of intercellular material
90 out of the cell due to an increase in permeability. The degree of microbial inactivation is
91 impacted, among other factors, by the strength of the electrical field applied, the pulse
92 duration and the dimensions of the microbe, including the shape (Barbosa-Cánovas &
93 Sepúlveda, 2005). The characteristic feature of PEF is that low heat conditions are
94 applied, which makes it highly desirable for heat-sensitive foods and beverages
95 (Barbosa-Cánovas & Sepúlveda, 2005).

96 Studies have shown that US (Condón *et al.*, 2005) and PEF (Barbosa-Cánovas &
97 Sepúlveda, 2005) can cause microbial inactivation. However, some microorganisms
98 believed to be “killed” may only be sub-lethally injured. Microbial injury can be
99 defined as a microorganism that has suffered some form of stress but that has the
100 potential to regain viability and to form a colony under the right conditions (Wu, 2008).
101 Injured cells pose quite a threat to food integrity as they are unpredictable and have the

102 potential to become viable under favourable environmental conditions (Wu, 2008).
103 There has been some controversy as to whether non-thermal technologies such as PEF
104 and US have an “all or nothing” effect, or whether some microbes may simply be sub-
105 lethally injured with the potential to become viable under optimal conditions (Jaeger *et*
106 *al.*, 2009). It is believed that after treatments, by either thermal or non-thermal
107 technologies, there may be one population of microbes which are dead, another
108 population that are viable, and a third population that are sub-lethally injured (Wu,
109 2008). It is of the utmost importance to be able to distinguish between viable cells and
110 impaired cells in order to gain complete food safety (Wu, 2008).

111 Some examples of spoilage microorganisms commonly found in beverages such
112 as milk, smoothies and fruit juices include *Salmonella* (Ross *et al.*, 2003), *Listeria*
113 *innocua* (Black *et al.*, 2005), *Pseudomonas fluorescens* (Barsotti & Cheftel, 1999) and
114 *Escherichia coli* (Walkling-Ribeiro *et al.*, 2008). In the present study, the main focus
115 was on *P. fluorescens* and *E. coli*. *P. fluorescens* is a Gram-negative microorganism, and
116 is regarded as one of the most common psychrotrophic bacteria dominating raw or
117 pasteurised milk at the time of spoilage (Sillankorva *et al.*, 2008). *E. coli* is also a Gram
118 negative facultative aerobe that is known to contaminate milk and cause spoilage
119 (Awuah *et al.*, 2005). This species has numerous pathogenic varieties which can inhabit
120 the intestinal tract of humans and animals (Dobrindt, 2005).

121 The objective of the present study was to investigate the effect of TS, the effect
122 of PEF and the effect of TS and PEF in combination (i.e. TS/PEF) on microbial
123 inactivation. In addition, the levels of microbial inactivation caused by these non-
124 thermal technologies were compared to those resulting from conventional
125 pasteurisation. A second objective was to determine the levels of sub-lethal injury of *P.*
126 *fluorescens* and *E. coli* following these treatments.

127

128 **2. Materials and Methods**

129 2.1. *Bacterial isolates and growth conditions*

130 Experiments were conducted using *E. coli* K12 (DSM 1607) and *P. fluorescens* (NCTC
131 10038) to determine the effects of the chosen non-thermal technologies on (i) microbial
132 inactivation and (ii) sub-lethal injury of these microorganisms. The *E. coli* culture was
133 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ,
134 Braun-schweig, Germany) and *P. fluorescens* was obtained from the National Collection
135 of Type Cultures (NCTC; Public Health Laboratory Service, London, U.K.). Both *E.*
136 *coli* and *P. fluorescens* strains were initially grown on tryptone soya agar (TSA; Oxoid,
137 Basingstoke, Hampshire, UK). Following this, a single colony from the relevant agar
138 plate was used to inoculate 1L of brain heart infusion broth (BHI; Oxoid, Basingstoke,
139 Hampshire, UK). Cultures of *E. coli* or *P. fluorescens* were incubated for 18 h at 37°C
140 or 30°C, respectively. Bacterial cells were sedimented by centrifugation at $6153 \times g$ for
141 10 min, and pellets were resuspended in Ringer's solution (Oxoid, Basingstoke,
142 Hampshire, UK). Bacterial suspensions were left to stand at room temperature for at
143 least 20 min prior to being subjected to any thermal/ non-thermal treatments.

144

145 2.2. *Treatment with Thermosonication*

146 A peristaltic pump (Masterflex ® L/S ®, Model No. 77250-62, Cole-Parmer
147 Instrumental Company, IL, USA) was used to pass the bacterial suspension through the
148 TS system at a fixed flow of 160 ml/min. In order to preheat the samples before
149 sonication, the suspension was pumped through a coil immersed in a heated water bath
150 until the temperature at the inlet of the sonication chamber reached 55°C. The
151 suspension was then sonicated using two ultrasonic processors (Model No. UIP 1000hd,
152 Hielscher, Germany). These sonicators were connected in a row, and had an operational
153 frequency of 20 kHz (Figure 1a). Two sonotrodes (Model No. BS2d40, Hielscher)

154 which had a 40 mm frontal face diameter were used. Also, boosters were used to
155 increase the amplitude (Model No. B2-1.8, Hielscher). Sonication was applied at two
156 energy inputs, resulting from varying the amplitude: low (TS-L; 19 μm) or high (TS-H;
157 28 μm), with the average residence time being *c.* 2.1 min. The temperature within the
158 chamber was maintained at 55°C, and overheating of the bacterial suspension during
159 sonication was prevented by water cooling of the treatment chamber. Temperature was
160 monitored using T-type thermocouples and a data logger (Model No. SQ2020, Grant
161 Instruments, Cambridge, UK). A sample of bacterial suspension post-treatment with TS
162 was collected and stored on ice until serial dilutions were prepared (within 1 h)..

163

164 *2.3. Treatment with Pulsed Electric Field*

165 As described earlier for treatment with TS, the bacterial suspension was pumped into
166 the PEF treatment chamber at a fixed flow rate of 160 ml/min. A lab scale customized
167 system (ELCRACK HVP 5, DIL, German Institute of Food Technologies,
168 Quackenbruck, Germany) was used. The treatment module consisted of three co-linear
169 treatment chambers with integrated refrigerated cooling modules. Each chamber held
170 two co-linear stainless steel electrodes separated by a 5 mm gap, with the electrode
171 diameter being 3 mm; which resulted in a total treatment volume of 0.106 cm^3 . The
172 system was monitored using a digital oscilloscope (TDS 2012, Tektronix, Beaverton,
173 OR, USA). The product temperature was recorded with thermocouples (Testo 925, type-
174 K probe, Testo AG, Lenzkirch, Germany) at three locations; before and after the
175 treatment module and immediately before being collected (Figure 1 b). Two power
176 levels were applied by varying the electrical field strength; low (PEF-L; 29 kVcm^{-1}) and
177 high (PEF-H; 32 kVcm^{-1}). The PEF system was operated at a constant frequency of 320
178 Hz, and a pulse width of 10 μs . It was ensured that the temperature of the bacterial
179 suspension at the inlet of the PEF system was kept below 40°C . A sample of bacterial

180 suspension post-treatment was taken and stored on ice until required.

181

182 *2.4. TS/PEF processing (combined treatment)*

183 After the bacterial suspension was preheated to 55°C, it was pumped into the sonicators
184 and treated with L or H (19 µm or 28 µm, respectively) energy inputs. The suspension
185 was then immediately passed into the PEF system where again it was treated with either
186 low (29 kVcm⁻¹) or high (32 kVcm⁻¹) electrical field strength. Treatments where TS and
187 PEF were combined (i.e. TS/PEF) were referred to as LL (TS=19 µm, PEF=29 kVcm⁻¹),
188 LH (TS=19 µm, PEF= 32kVcm⁻¹), HL (TS=28 µm, PEF= 29kVcm⁻¹) or HH (TS=28
189 µm, PEF=32 kVcm⁻¹). The bacterial suspension was passed through both systems and a
190 sample was collected and stored on ice until required.

191

192 *2.5. Thermal Treatment*

193 A tubular heat exchanger (Model No. FT74T, Armfield, Ringwood, UK) was used for
194 pasteurisation of the bacterial suspension. The suspension was heated at 72°C for 20 s.
195 An attached cooling system ensured the temperature of the liquid was below 10°C after
196 treatment. A sample was taken and stored on ice until required.

197

198 *2.6. Enumeration of viable and injured cells*

199 Firstly, a sample of the untreated bacterial suspension was collected and the number of
200 colony forming units per ml (CFU/ml) were determined, for the initial working culture
201 (i.e. CFU/ml of initial sample, denoted as 'A' in Equation (3)). This was achieved by
202 preparing decimal dilutions in 9 ml volumes of Ringer's solution. Aliquots (100 µl) of
203 these dilutions were plated on TSA plates (in duplicate), and incubated at the
204 appropriate temperature; 37°C for *E. coli*, 30°C for *P. fluorescens*. To determine the
205 microbial kill due to non-thermal processing by individual or combined methods (i.e.

206 TS and/or PEF), a survival fraction study was performed. The number of surviving cells
207 post-processing was determined, and denoted by 'B' in Equation (3), which was
208 subsequently used to determine the percentage of microbial inactivation:

209

$$210 \quad \text{CFU/ml of initial culture} = A \quad (1)$$

$$211 \quad \text{CFU/ml of processed sample} = B \quad (2)$$

$$212 \quad \% \text{ Inactivation} = (1 - (B/A)) \times 100 \quad (3)$$

213

214 To define the levels of sub-lethal injury (if any), appropriate dilutions of the
215 processed bacterial suspensions were plated onto TSA agar plates containing sodium
216 chloride (TSA+SC; SC: Oxoid, Basingstoke, Hampshire, UK) and incubated for 72 h.
217 These TSA+SC plates were supplemented with 3% NaCl (referred to as the 'selective
218 media') in accordance with the method described by Perni *et al.* (2007). In order to
219 determine the percentage sub-lethal injury (SLI), Equation (6) was used, according to
220 the method of Uyttendaele *et al.* (2008) and Zhao *et al.* (2013). Samples of the initial
221 culture were plated on regular TSA plates without any sodium chloride added (referred
222 to as the 'non-selective' media); denoted by 'C' in Equation (6). The CFU/ml
223 determined from both selective and non-selective media were compared, in order to
224 determine the SLI (refer to Equation (6)).

225

$$226 \quad \text{CFU/ml of initial culture} = C \quad (4)$$

$$227 \quad \text{CFU/ml of sub-lethally injured cells} = D \quad (5)$$

$$228 \quad \% \text{ SLI} = (1 - (D/C)) \times 100 \quad (6)$$

229

230 The CFU/ml determined from both selective and non-selective media were compared, in
231 order to determine the SLI (refer to Equation(6)). Processed samples were plated onto

232 four agar plates in total (2×TSA and 2×TSA+SC) which were incubated for 24 h and 72
233 h, respectively, to determine microbial inactivation and SLI.

234

235 2.6.1. Bacterial growth monitoring using optical density (*E. coli* only)

236 In addition to the plating technique described in section 2.6, sub-lethal injury was also
237 assessed using an optical density based method. Bacterial growth assays were carried
238 out in sterile 96 well plates (Sarstedt, Numbrecht, Germany). Aliquots (100 µl) of BHI
239 broth were pipetted into appropriate wells of the 96 well plate. Bacterial suspensions
240 collected from the initial working culture (as a control) and the processed samples were
241 pipetted in 50 µl aliquots into the appropriate wells. The plate was then incubated at
242 37°C for 18 h in a Multiskan Ascent plate reader (Thermo Electron Corporation, Vantaa,
243 Finland). Optical density (OD) measurements were taken at hourly intervals
244 (wavelength of 590 nm), and growth curves were plotted from the OD values using
245 Microsoft Excel™ (Microsoft Corporation, 2007). In addition, a standard curve of
246 CFU/ml versus OD₅₉₀ was prepared for *E. coli* (data not shown). It was determined that
247 an OD₅₉₀ value of 0.2 corresponded to *c.* 6.1×10⁸ CFU/ml.

248

249 2.7. Statistical Analysis

250 Results were expressed as the mean ± standard deviation (S.D). Differences between
251 treatments were determined using the least significant difference (L.S.D) function of
252 SAS version 9.1 (SAS Institute, Cary, NC). Data was considered significantly different
253 if *P*<0.05.

254

255 3. Results and Discussion

256 The average initial concentration of microorganisms in each working culture was
257 determined to be *c.* 8.6×10⁸ and 6.07×10⁸ for *P. fluorescens* and *E. coli*, respectively.

258

259 3.1. Effect of TS processing on microbial viability

260 The results for inactivation and SLI of *P. fluorescens* following treatment with
261 TS and thermal pasteurisation are shown in Figure 2(a). Only a small percentage of
262 inactivation was observed following treatment with TS; 9.2% and 6.4% inactivation at
263 TS-L (19 μm) and TS-H (28 μm) power settings, respectively. No significant
264 differences in inactivation levels due to TS were observed between these power settings
265 ($P>0.05$), while pasteurisation resulted in complete inactivation of *P. fluorescens*.

266 In terms of SLI, no injured cells were detected following treatment with TS at
267 either power setting. Therefore, it can be suggested that the population inactivated by
268 TS remained 'dead', and the population that was viable stayed this way. It has been
269 reported previously that when treatment time with ultrasonication (temperature $39 \pm$
270 0.3°C) is increased, the destruction of bacteria such as *Pseudomonas aeruginosa* is also
271 increased (Scherba *et al.*, 1991). There is a limited amount of literature regarding the
272 effects of non-thermal technologies on the viability of *P. fluorescens* following
273 treatment with TS. Thus, very few published studies can be directly compared to the
274 present study. For example, the study by Scherba *et al.* (1991) discussed the reduction in
275 viability of *P. fluorescens* due to treatment with ultrasound some time ago. In addition, a
276 study by Villamiel and de Jong (2000) examined the inactivation of *P. fluorescens* by
277 ultrasound. However, in recent years the focus of research on inactivation of
278 *Pseudomonas* by ultrasound technology has shifted towards destruction of this
279 microorganism in biofilms (Xu *et al.*, 2012) and disinfection of instruments used for
280 medical procedures (Jatzwauk *et al.*, 2001). A search for literature specifically
281 discussing inactivation of *Pseudomonas* by non-thermal technologies does not yield
282 many results, with the main publication found being a study by Shamsi, Versteeg,
283 Sherkat and Wan (1997) which evaluated inactivation by PEF. For future studies

284 employing ultrasonication, certain parameters (e.g. residence time) could be increased
285 to examine whether a greater level of inactivation of *P. fluorescens* may be achievable.

286 The effects of TS on the viability of *E. coli* are presented in Figure 2(b). Low
287 levels of inactivation were recorded at both power settings; 1.1% (TS-L) and 6.3% (TS-
288 H). Minor (yet significant, $P<0.05$) differences in inactivation were recorded at different
289 power outputs. It could be suggested that *E. coli* has a higher resistance to TS
290 processing, as less inactivation was observed for this microorganism than for *P.*
291 *fluorescens* following treatment with TS. No SLI was observed at the high energy input
292 (28 μm), but 1.5% was observed at the low energy input (19 μm) (Figure 2(b)).
293 However, these results for SLI of *E. coli* following TS-H and TS-L were not
294 significantly different from each other ($P>0.05$).

295 In a review by Scherba *et al.* (1991) it was reported that when analysed in an
296 aqueous medium using a frequency of 24 kHz, the intensity of TS did not affect the
297 level of inactivation of *E. coli*, and that results remained similar for all intensities used.
298 This observation is in contrast to the results shown in Figure 2(b), as there was a
299 significant difference observed between low and high power outputs ($P<0.05$). This
300 author also reported that significant reductions in viable populations were achieved with
301 an increase in residence time (Scherba *et al.*, 1991). Limaye and Coakley (1998)
302 suggested that the initial temperature of the bacterial suspension can have significant
303 effects on the survival of *E. coli*. It was reported that heating to an initial temperature of
304 32°C resulted in a 99% reduction of *E. coli*, whereas heating at 17°C resulted in a 62%
305 reduction. In the present investigation, greater inactivation levels may have been
306 obtained if longer residence times or greater power settings had been used.

307 From the results of the present study, it is difficult to visualise a future for this
308 technology used alone for microbial inactivation, as under the experimental conditions
309 used in the present study, relatively low inactivation levels were achieved for *E. coli* and

310 *P. fluorescens* following treatment with TS. However, a synergistic effect could have the
311 potential to be more successful in terms of microbial inactivation than TS used alone,
312 and may offer a solution to the partial success of treatment with ultrasound (Condón *et*
313 *al.*, 2005). An investigation carried out by Noci, Walking-Ribeiro, Cronin, Morgan and
314 Lyng (2009) suggested that thermosonication may be more useful as a hurdle within a
315 system, instead of a stand-alone method for microbial inactivation in foods and
316 beverages.

317 *3.2 Effect of PEF processing on microbial viability*

318 The levels of microbial inactivation following PEF processing (Figures 3(a) and 3(b))
319 were found to be substantially greater than those resulting from treatment with TS. In
320 the case of *P. fluorescens*, a 26.4% inactivation was reported at the low power output
321 (28 kVcm⁻¹), and a significantly greater ($P<0.05$) inactivation of 47.1% was recorded at
322 high energy input (32 kVcm⁻¹). However, treatment of *P. fluorescens* with PEF was still
323 significantly less effective than pasteurisation ($P<0.05$). The level of SLI of *P.*
324 *fluorescens* following treatment with PEF is illustrated in Figure 3(a). A larger
325 proportion of sub-lethally injured bacteria was observed at higher electric field intensity
326 (7.6% for PEF-H, in contrast with 2.3% for PEF-L; $P<0.05$).

327 It was reported by Barbosa-Cánovas and Sepúlveda (2005) that the only factors
328 that have any significant impact on the functionality of PEF in microbial inactivation
329 are electric field intensity and residence time. The results in Figure 3(a) are in
330 agreement with this, as a significant ($P<0.05$) difference in microbial inactivation was
331 observed as electric field intensity increased from the PEF-L to PEF-H. It was also
332 suggested that in order for PEF to result in any microbial inactivation at all, a minimum
333 threshold of field intensity must be applied, otherwise the technology is not effective
334 (Barbosa-Cánovas & Sepúlveda, 2005).

335 It has been reported that studies conducted with mild temperature treatments are

336 more effective than those performed at room temperature (Barbosa-Cánovas &
337 Sepúlveda, 2005); this may have been a potential factor that led to the lower levels of *P.*
338 *fluorescens* inactivated, as the bacterial suspension was introduced into PEF at ambient
339 temperature. In the present study it was noted that the level of SLI increased with
340 electric field intensity ($P < 0.05$). Similar results were obtained by García, Gómez,
341 Manas *et al.* (2005) and Garcí, Gómez, Raso and Pagán (2005), where a higher
342 proportion of SLI of various species of bacteria was recovered as the field strength
343 increased. However, only a general assumption may be drawn as limited information
344 exists on *P. fluorescens* and how it behaves following PEF application.

345 Interestingly, *E. coli* was observed to have greater sensitivity to PEF, at all
346 electric field intensities when compared to *P. fluorescens* (Figure 3(b)). A substantial
347 reduction of 86.1% was noted following PEF-H treatment, which was significantly
348 higher than inactivation achieved at PEF-L, but not significantly different from thermal
349 pasteurisation at the 5% significance level. While treatment with PEF-L (29 kVcm^{-1})
350 was significantly ($P > 0.05$) less effective than pasteurisation, this non-thermal
351 processing method demonstrated an impressive level of microbial inactivation as a
352 stand-alone technology. An inactivation level of 32.3% was observed following PEF-L
353 treatment, while a reduction of 86.1% was recorded following treatment with PEF-H. A
354 less notable increase in inactivation was observed from low to high field intensity
355 application for *P. fluorescens* when compared to the results obtained for *E. coli*,
356 suggesting that *E. coli* is more susceptible to PEF processing.

357 The levels of sub-lethally injured *E. coli* cells following treatment with PEF are
358 also shown in Figure 3(b). The highest level of SLI was observed at the lowest energy
359 input, concurring that the levels of SLI were reduced with increasing electric field
360 intensity. This indicates that of the 32.3% and 86.1% of the population of *E. coli* killed
361 following treatment with PEF-L and PEF-H, respectively, 29.3% and 4% of those

362 bacteria were only sub-lethally injured, respectively.

363 The results presented in Figure 3 (b) correlate well with a similar study
364 conducted by Aronsson *et al.* (2004), where it was reported that the level of inactivation
365 of *E. coli* increased with increasing electric field intensity. The results of the present
366 study may be in agreement with the theory of Barbosa-Cánovas and Sepúlveda (2005),
367 i.e. that it is necessary for a critical electrical field strength to be applied in order for
368 treatment with PEF to be efficient. This is evident with the vast differences between the
369 percentage killed following treatment with PEF-L and PEF-H, suggesting that a lower
370 field intensity of 29 kVcm⁻¹ only achieved a certain degree of microbial inactivation,
371 while leaving a greater proportion of cells injured.

372 The results observed in the present study for inactivation of *E. coli* following
373 treatment with PEF may offer some value to the food processing industry, as *E. coli* is a
374 potential safety hazard in beverages such as milk. The possible use of PEF processing as
375 a hurdle technology to combat *E. coli* contamination may be worthy of consideration
376 (Awuah *et al.*, 2005). Further studies should be conducted to assess the inactivation
377 ability of PEF when used at a higher inlet temperature, as there have been some positive
378 reports of this effect (Barbosa-Cánovas & Sepúlveda, 2005).

379 In a study conducted by García *et al.* (2005) and García, Gómez, Raso *et al.*
380 (2005), the highest proportion of sub-lethally injured *E. coli* cells were recorded
381 following PEF treatment at 19 kVcm⁻¹, with the numbers decreasing at 25 kV cm⁻¹. It
382 was reported by García Gómez, Manas *et al.* (2005) and García, Gómez, Raso *et al.*
383 (2005) that due to the sensitivity of *E. coli* to PEF, the population of dead cells increased
384 with increasing electric field intensity, while the proportion of sub-lethally injured cells
385 decreased at higher electric field intensities. The results in Figure 3(b) are in agreement
386 with the findings of García, Gómez, Manas *et al.* (2005) and García, Gómez, Raso *et al.*
387 (2005). Although there were variations in proportions of sub-lethally injured *E. coli*

388 between electrical field intensities (i.e. 29 kVcm⁻¹ and 32 kVcm⁻¹), no significant
389 differences were determined ($P>0.05$). This is not surprising considering the difference
390 between the energy inputs was not that large.

391 Further investigation may be required in order to assess the application of PEF
392 processing when higher field intensity is applied, as favourable results have been
393 observed in previous studies (Alvarez *et al.*, 2003). From the results of the present study,
394 it can be concluded that PEF processing is not an all or nothing event (Wu, 2008) and
395 that there was some evidence of SLI following the application of PEF. Interestingly,
396 there was no significant difference found between inactivation levels of *E. coli*
397 following treatment with PEF at 32kVcm⁻¹ and pasteurisation ($P>0.05$).

398

399 *3.3 Effect of TS and PEF processing (combined) on microbial viability*

400 Four combinations of TS/PEF were used to treat both *E. coli* and *P. fluorescens*; referred
401 to as LL, LH, HL, HH. The results of microbial inactivation and SLI, of *P. fluorescens*
402 following these treatments are shown in Figure 4(a). No significant difference was
403 observed between the power combinations used ($P>0.05$) for results quantifying both
404 kill and SLI for this microorganism.

405 It has been suggested that a synergistic effect may be observed with the
406 application of TS and PEF combined (Noci *et al.*, 2009) and that the cell damage caused
407 by one technology may increase the effects of the second technology. Such reports of
408 synergistic effects may be of benefit to the food and beverage industry. For instance, the
409 microbial inactivation achieved by TS alone is incomparable to the total bacterial kill
410 following traditional pasteurisation, yet treatment with PEF alone appears to be quite
411 effective. A publication by Noci *et al.* (2009) suggested that if the first hurdle
412 mechanism provides a weaker effect in terms of its inactivation, then inevitably it is
413 leaving a greater number of microorganisms to be inactivated by the second hurdle. This

414 could be relevant in the present study, as due to the poor inactivation levels observed
415 following treatment with TS, it is therefore leaving a large quantity of microorganisms
416 to be inactivated by PEF.

417 A possible explanation for the lower inactivation observed at higher TS power
418 settings may be attributed to the fact that sonication may increase the availability of
419 nutrients, and that nutrients may become more abundant at higher TS levels (Piyasena *et*
420 *al.*, 2003). In the present study the same residence times were used for low and high
421 amplitudes. This may have been a factor that led to the decline in inactivation at higher
422 amplitudes, in the treatments using (i) TS alone and (ii) TS/PEF combined. However, it
423 should be taken into consideration that different trends were observed between the
424 inactivation levels achieved using TS-L and TS-H for *E. coli* and *P. fluorescens*, as
425 significant differences ($P<0.05$) were observed between the low and high energy inputs
426 for inactivation of *P. fluorescens*, but not for *E. coli*. From the results presented in
427 Figure 4(a) it could be suggested that, with regard to the inactivation of *P. fluorescens*,
428 PEF operates at its optimum at higher electric field intensities. The levels of microbial
429 inactivation obtained following TS/PEF processing were significantly less than thermal
430 pasteurisation ($P<0.05$). This may be due to the ability of *Pseudomonas* to survive well
431 and to adapt to stressful environments. A study conducted by Lu *et al.* (2011) where *P.*
432 *aeruginosa* was subjected to cold stress reported that this microorganism was able to
433 survive well in environmental stress, with only a 3.1 log CFU/ml reduction observed
434 when stored at -18°C . Although a direct comparison cannot be made between the
435 results reported by Lu *et al.* (2011) for inactivation resulting from frozen storage and the
436 present study where inactivation following treatment with high voltage PEF was
437 described, it is possible that *P. fluorescens* may be capable of surviving adverse
438 conditions, similar to the way *P. aeruginosa* can withstand challenging conditions. Even
439 though the TS/PEF treatment inactivated a low level of microbes, in general it achieved

440 more inactivation than either technology used alone. Interestingly, for *P. fluorescens*, no
441 significant difference in SLI levels were detected between PEF alone and TS/PEF
442 ($P>0.05$).

443 The results for microbial inactivation of *E. coli* following TS/PEF treatments
444 combined are illustrated in Figure 4(b). Similar to *P. fluorescens*, there was no
445 significant difference observed for inactivation or SLI at any of the power combinations
446 for *E. coli* ($P>0.05$). From the results described here, it could be suggested that *E. coli*
447 is more sensitive to TS/PEF processing than *P. fluorescens*. Although considerable
448 levels of inactivation following treatment with TS/PEF were observed (71% at HH),
449 PEF-H treatment was found to achieve greater inactivation, with an average kill of
450 86.1% observed. The capability of PEF to inactivate *E. coli*, when used at low field
451 intensities, was increased when combined with TS. The inactivation increased from
452 32.3% at PEF-L, to between 62.6% and 71.5%, when TS/PEF were used in
453 combination.

454 The highest quantity of SLI was observed at low electric field intensities of PEF
455 (i.e. 29 kV cm⁻¹), LL (25.5%) and HL (24.9%). Inactivation due to thermal
456 pasteurisation was significantly greater ($P<0.05$) than inactivation following TS/PEF
457 combined processing of *E. coli*, although an impressive inactivation level was obtained
458 for the HH combination (71.5%).

459 It is possible that TS did not have a vast impact on the inactivation of *E. coli*
460 when used at high power combinations such as HH, as it has been suggested by
461 Piyasena *et al.* (2003) that the intensity of TS does not largely effect the amount of
462 inactivation of *E. coli*, and that the majority of inactivation may have been attributed to
463 PEF. However, it is plausible that TS weakened the cell membrane of the bacteria
464 (Barbosa-Cánovas & Sepúlveda, 2005) and that the weakened cell was compromised
465 thus becoming more susceptible to PEF. Despite the report by Piyasena *et al.* (2003), it

466 was observed that TS did, in fact, have beneficial effects on the performance of PEF at
467 low field intensities, increasing the percentage killed from 32.3% when PEF-L was
468 applied alone, to 62.6% (LL) and 64.8% at (LH).

469

470 3.4. Bacterial growth monitoring using optical density

471 It is evident from Figure 5(a) that the growth of *E. coli* was not vastly affected by
472 treatment with TS at either the high or low energy input (19 μm and 28 μm ,
473 respectively) when compared to control growth. However, *E. coli* cells treated with L
474 and H power outputs took slightly longer to enter the log phase (*c.* 1 h). It appears from
475 Figure 5(a) that very low levels of sub-lethally injured *E. coli* were present, which is in
476 agreement with the results presented in Figure 2(b).

477 Following treatment with PEF, it was evident that cells treated at PEF-L entered
478 the log phase more rapidly than *E. coli* treated at PEF-H, where it took approximately 7
479 h and 12 h, respectively, to enter the log phase (Figure 5b). These results suggesting SLI
480 correlate quite well to the findings presented in Figure 3(b).

481 The growth curves for *E. coli* following treatment with TS/PEF combined are
482 shown in Figure 5(c). The level of SLI is clearly evident, as it took each *E. coli* culture
483 (following treatment with TS/PEF) at least 12 h to enter the log phase. This
484 demonstrates SLI, and confirms the results presented in Figure 4(b). Thus, there
485 appeared to be agreement between the results obtained from the OD-based method
486 (Figures 5a-5c) and the crude plating technique (Figures 2b, 3b, 4b).

487

488 **4. Conclusion**

489 In conclusion, it was established that TS treatment alone was not an effective method
490 for the inactivation of *P. fluorescens* and *E. coli*. Also, this study has shown the potential
491 of PEF for effective inactivation of *E. coli*, with less favourable results obtained for *P.*

492 *fluorescens*. However, TS/PEF combined proved to be substantially more effective with
493 regard to microbial inactivation of *E. coli* than when applied to *P. fluorescens*.

494 SLI was observed following the majority of treatments, with substantial levels of
495 injury evident when TS/PEF were applied for both *P. fluorescens* and *E. coli*. A future
496 challenge may be to focus on eliminating this population of sub-lethally injured
497 bacteria.

498

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Figure 1: Schematic diagrams of laboratory scale (a) thermosonication and (b) pulsed electric field treatment chamber systems used in this study.

Figure 2: Levels of (a) *P. fluorescens* and (b) *E. coli* killed (■) and sub-lethally injured (□) following treatment with thermosonication at low (TS-L; 19 μm) and high (TS-H; 28 μm) energy inputs and thermal pasteurisation. (Data= mean \pm S.D., n=2). Values for 100% viability were 8.6×10^8 CFU/ml and 6.1×10^8 CFU/ml for *P. fluorescens* and *E. coli*, respectively.

Figure 3: Levels of (a) *P. fluorescens* and (b) *E. coli* killed (■) and sub-lethally injured (□) following treatment with pulsed electric fields (PEF) at low (PEF-L; 29 kVcm^{-1}) and high (PEF-H; 32 kVcm^{-1}) power intensities and thermal pasteurisation. (Data= mean \pm S.D., n=2). Values for 100% viability were 8.6×10^8 CFU/ml and 6.1×10^8 CFU/ml for *P. fluorescens* and *E. coli*, respectively.

Figure 4: Levels of (a) *P. fluorescens* and (b) *E. coli* killed (■) and sub-lethally injured (□) following combined treatment with thermosonication (TS) and pulsed electric fields (PEF) and thermal pasteurisation. Treatments of TS/PEF were as follows; LL (19 μm , 29 kVcm^{-1}), LH (19 μm , 32 kVcm^{-1}), HL (28 μm , 29 kVcm^{-1}) and HH (28 μm , 32 kVcm^{-1}). (Data= mean \pm S.D., n=2). Values for 100% viability were 8.6×10^8 CFU/ml and 6.1×10^8 CFU/ml for *P. fluorescens* and *E. coli*, respectively.

Figure 5: (a) Standard curve of OD₅₉₀ Vs. CFU/ml of *E. coli*, (b) effect of TS at 19 μm (TS-L; ■) and 28 μm (TS-H; ▲) on growth of *E. coli*, (c) effect of PEF at 29 kV cm^{-1} (PEF-L) (■) and 32 kV cm^{-1} (PEF-H; ▲) on growth of *E. coli*, and (d) effect of TS/PEF combined (LL; ■, LH; ▲, HL; □ and HH; Δ) on growth of *E. coli*. Control growth for *E. coli* (●) is included for comparison purposes. (Data= mean \pm S.D., n=2).

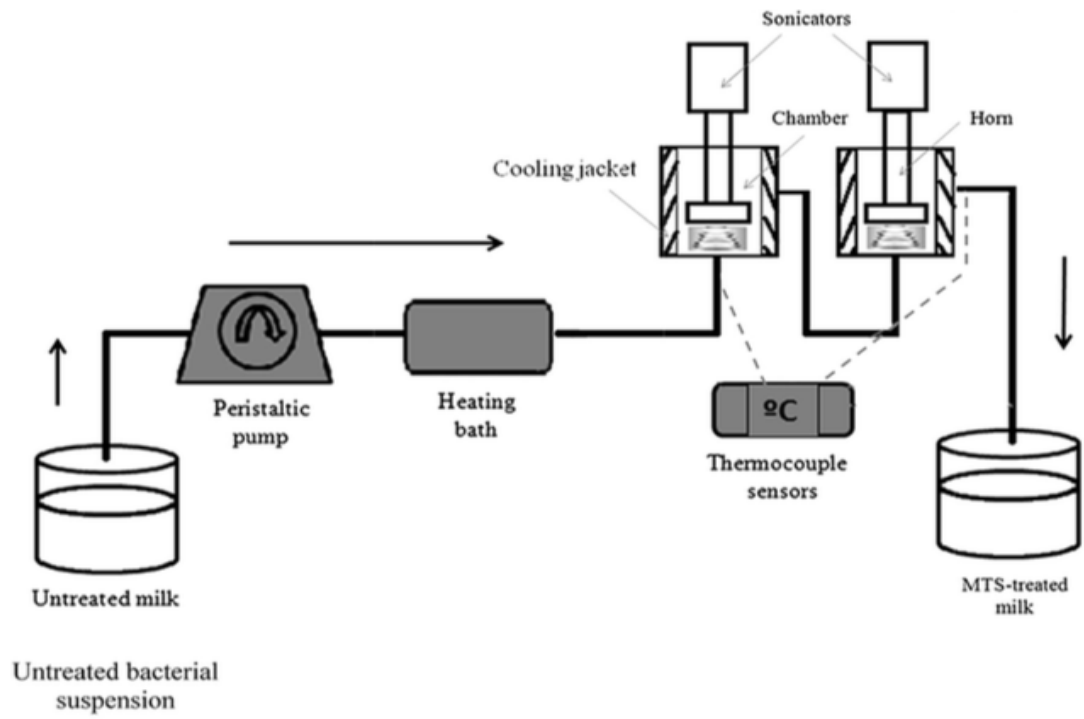


Figure 1 (a)

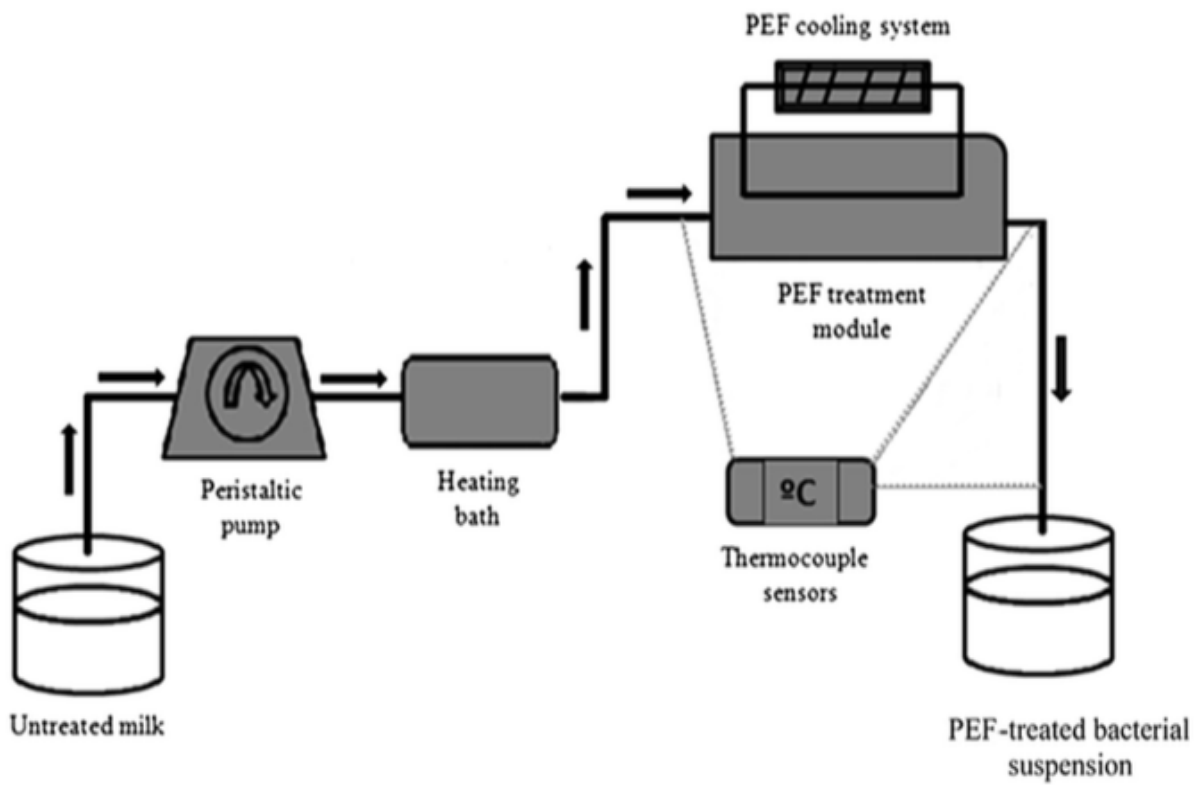


Figure 1 (b)

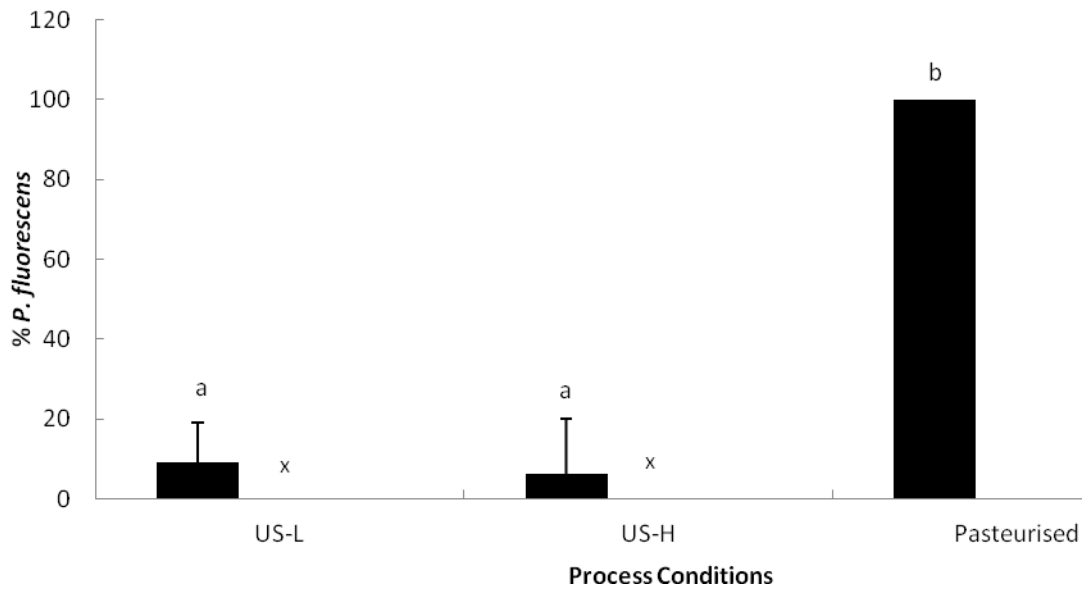


Figure 2 (a)

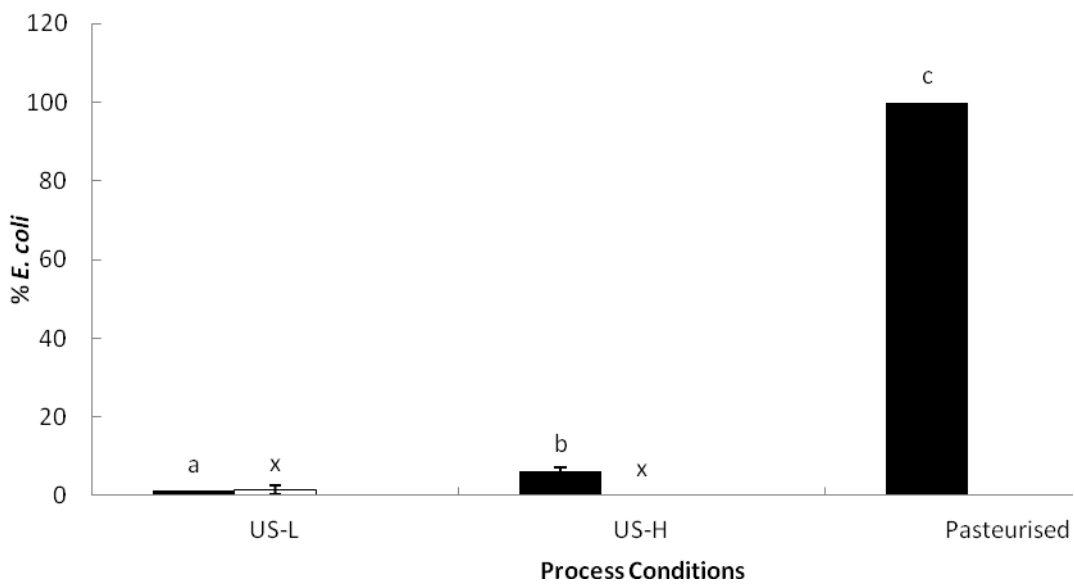


Figure 2 (b)

Footnote: Statistical differences (if any) between percentage killed are shown as a,b,c etc. while statistical differences (if any) between sub-lethal injury are shown as x,y,z etc.

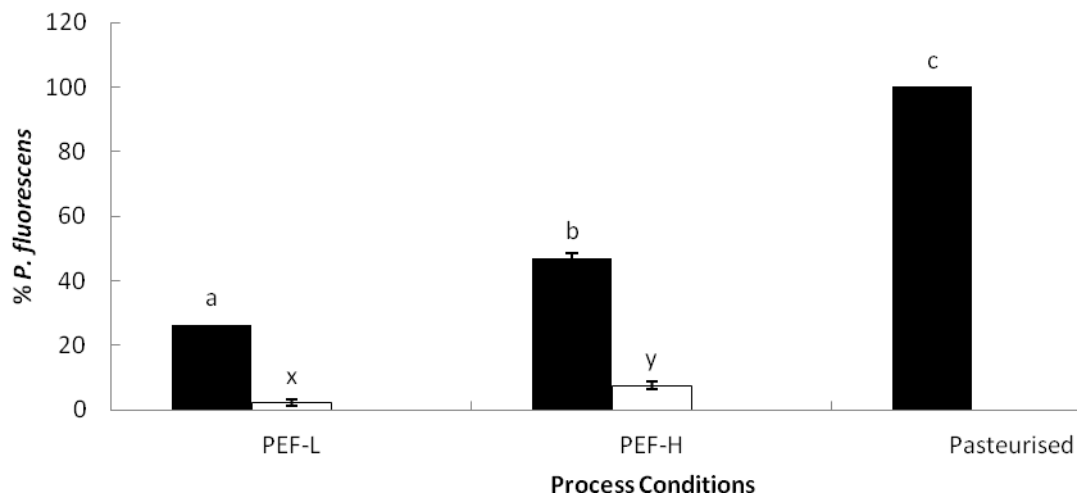


Figure 3 (a)

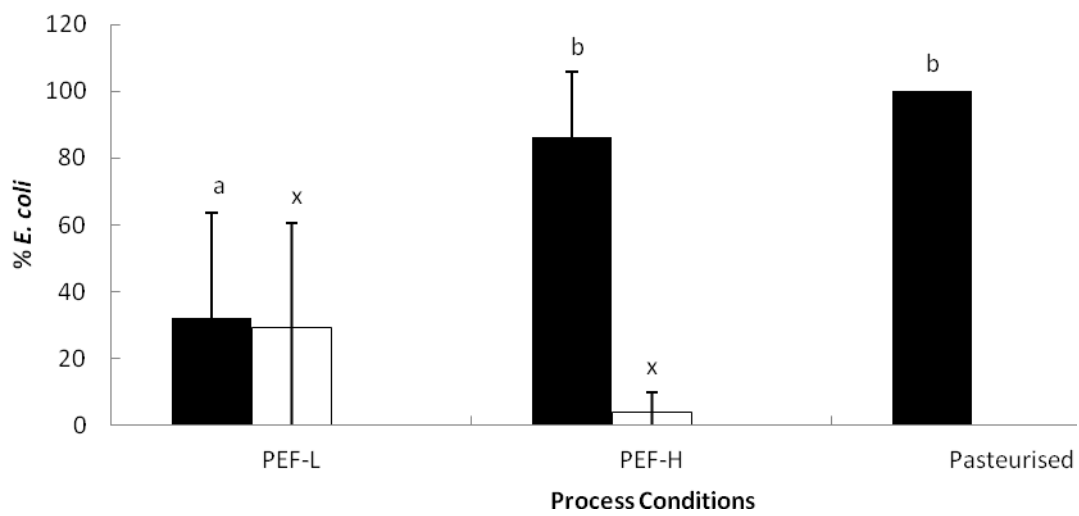


Figure 3 (b)

Footnote: Statistical differences (if any) between percentage killed are shown as a,b,c etc. while statistical differences (if any) between sub-lethal injury are shown as x,y,z etc.

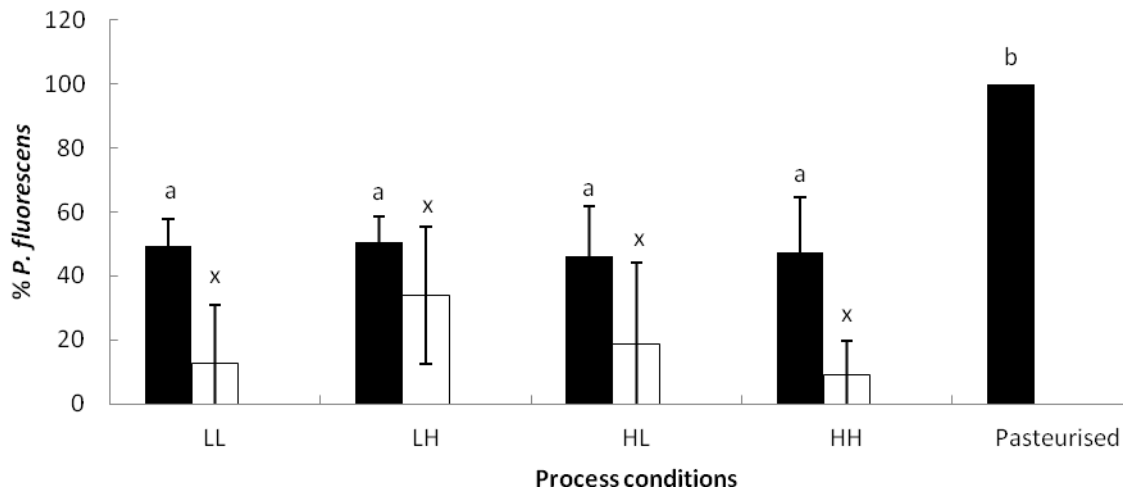


Figure 4 (a)

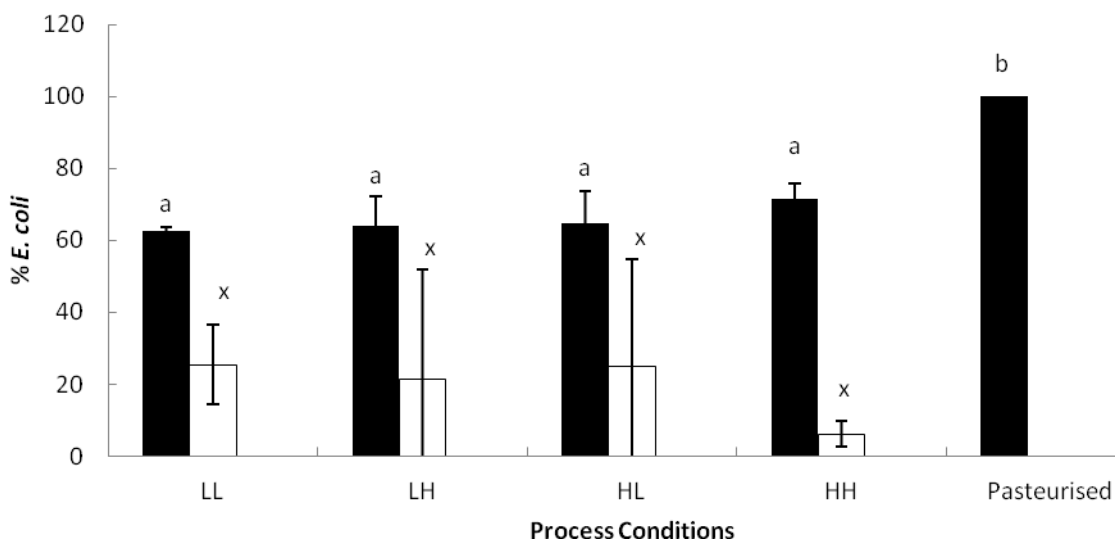


Figure 4 (b)

Footnote: Statistical differences (if any) between percentage killed are shown as a,b,c etc. while statistical differences (if any) between sub-lethal injury are shown as x,y,z etc.

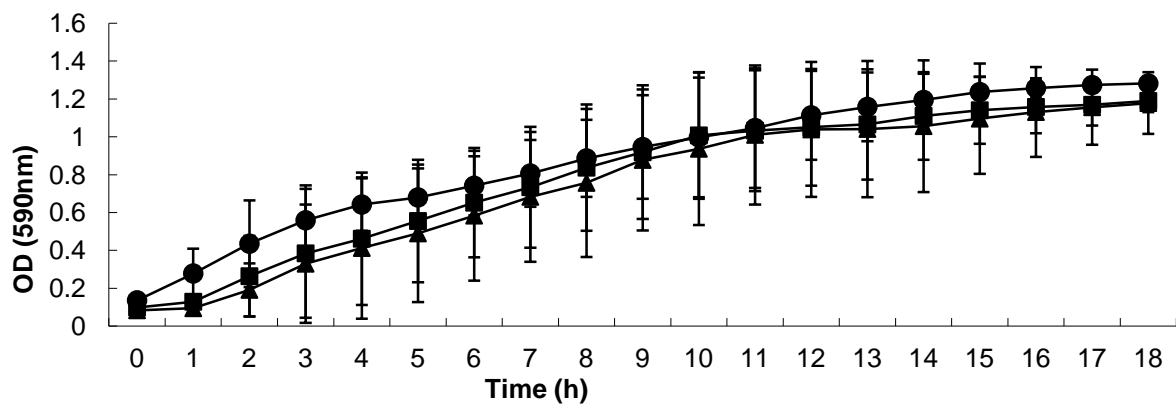


Figure 5 (a)

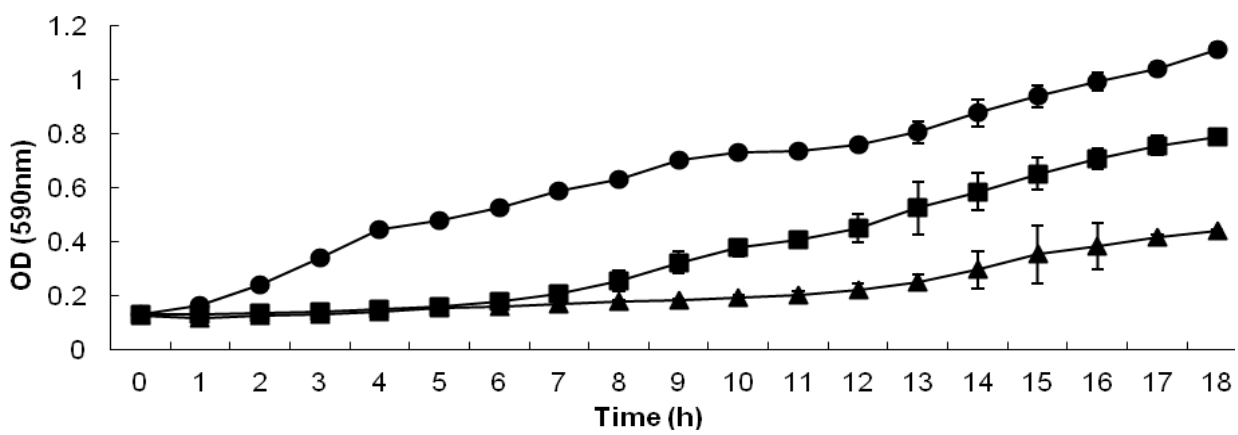


Figure 5 (b)

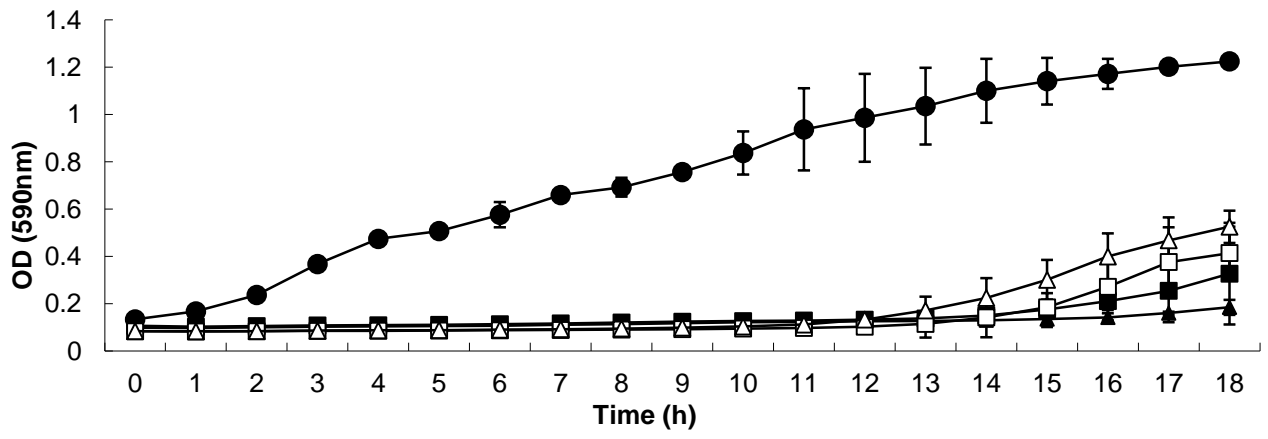


Figure 5 (c)