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Rachel Halpin
Technological University Dublin, rachel.halpin@tudublin.ie

L. Duffy
School of Biomolecular and Biomedical Sciences, University College Dublin,

O. Cregenzán-Alberti,
School of Biomolecular and Biomedical Sciences, University College Dublin,

See next page for additional authors

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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*

Halpin, R.M.[1]*, Duffy, L., Cregenzán-Alberti, O.[1], Lyng, J.G.[1] and Noci, F[2].

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

[2] Galway Mayo Institute of Technology, Galway, Ireland.

***** Corresponding author: Dr. Rachel Halpin.

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

Tel No.: 0035317167711

E-mail: rachel.halpin@ucd.ie

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and Methods

2.1. Bacterial isolates and growth conditions

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

2.2. Treatment with Thermosonication

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

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

2.3. Treatment with Pulsed Electric Field

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

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

2.4. TS/PEF processing (combined treatment)

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

2.5. Thermal Treatment

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

2.6. Enumeration of viable and injured cells

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

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

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

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

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

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

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

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

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

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

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

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

In addition to the plating technique described in section 2.6, sub-lethal injury was also assessed using an optical density based method. Bacterial growth assays were carried out in sterile 96 well plates (Sarstedt, Numbrecht, Germany). Aliquots (100 µl) of BHI broth were pipetted into appropriate wells of the 96 well plate. Bacterial suspensions collected from the initial working culture (as a control) and the processed samples were pipetted in 50 µl aliquots into the appropriate wells. The plate was then incubated at 37°C for 18 h in a Multiskan Ascent plate reader (Thermo Electron Corporation, Vantaa, Finland). Optical density (OD) measurements were taken at hourly intervals (wavelength of 590 nm), and growth curves were plotted from the OD values using Microsoft Excel™ (Microsoft Corporation, 2007). In addition, a standard curve of CFU/ml versus OD_{590} was prepared for *E. coli* (data not shown). It was determined that an OD_{590} value of 0.2 corresponded to *c.* 6.1×10^8 CFU/ml.

2.7. Statistical Analysis

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

3. Results and Discussion

The average initial concentration of microorganisms in each working culture was determined to be c. 8.6×10^8 and 6.07×10^8 for *P. fluorescens* and *E. coli*, respectively.

3.1. Effect of TS processing on microbial viability

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

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

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

3.2 Effect of PEF processing on microbial viability

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

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

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

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

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

The levels of sub-lethally injured *E. coli* cells following treatment with PEF are also shown in Figure 3(b). The highest level of SLI was observed at the lowest energy input, concurring that the levels of SLI were reduced with increasing electric field intensity. This indicates that of the 32.3% and 86.1% of the population of *E. coli* killed 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 32$kVcm^{-1}$ 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

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

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

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

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

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

3.4. *Bacterial growth monitoring using optical density*

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

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

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

4. Conclusion

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

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

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

5. Acknowledgements

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List of Figures:

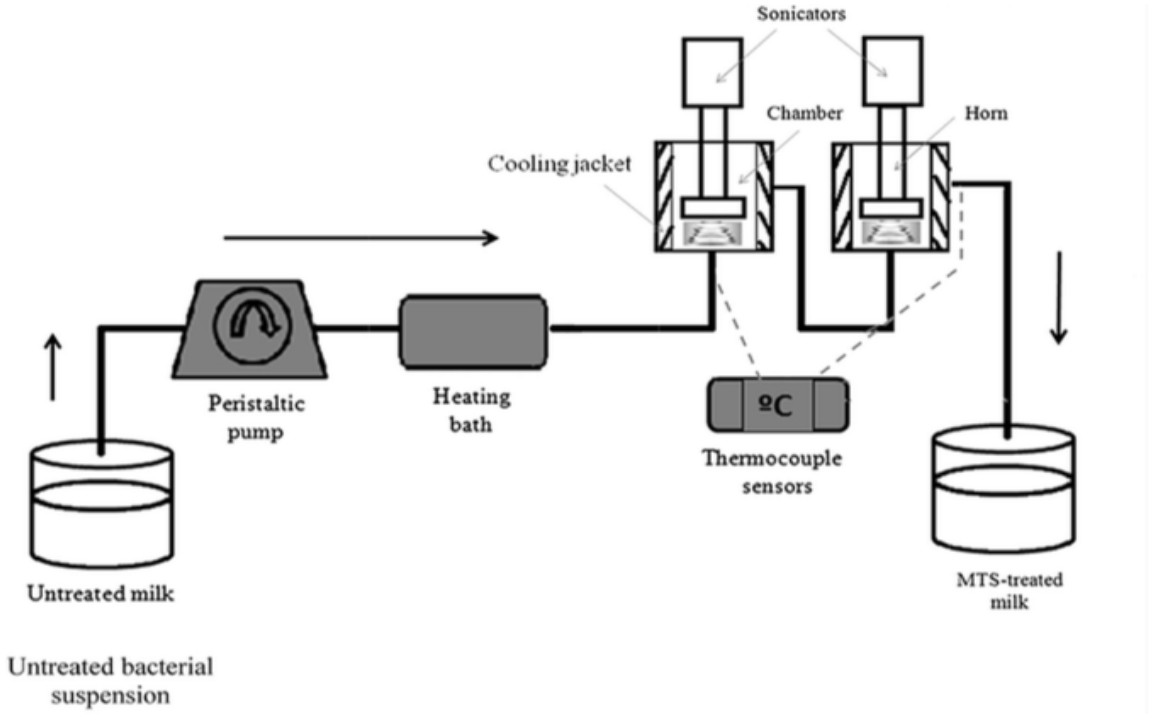
Figure 1: Schematic diagrams of laboratory scale (a) thermosonication and (b) pulsed electric field treatment chamber systems used in this study.

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 ±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 ±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. Treatmens 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 ±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 OD_{590} 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 ±S.D., n=2).



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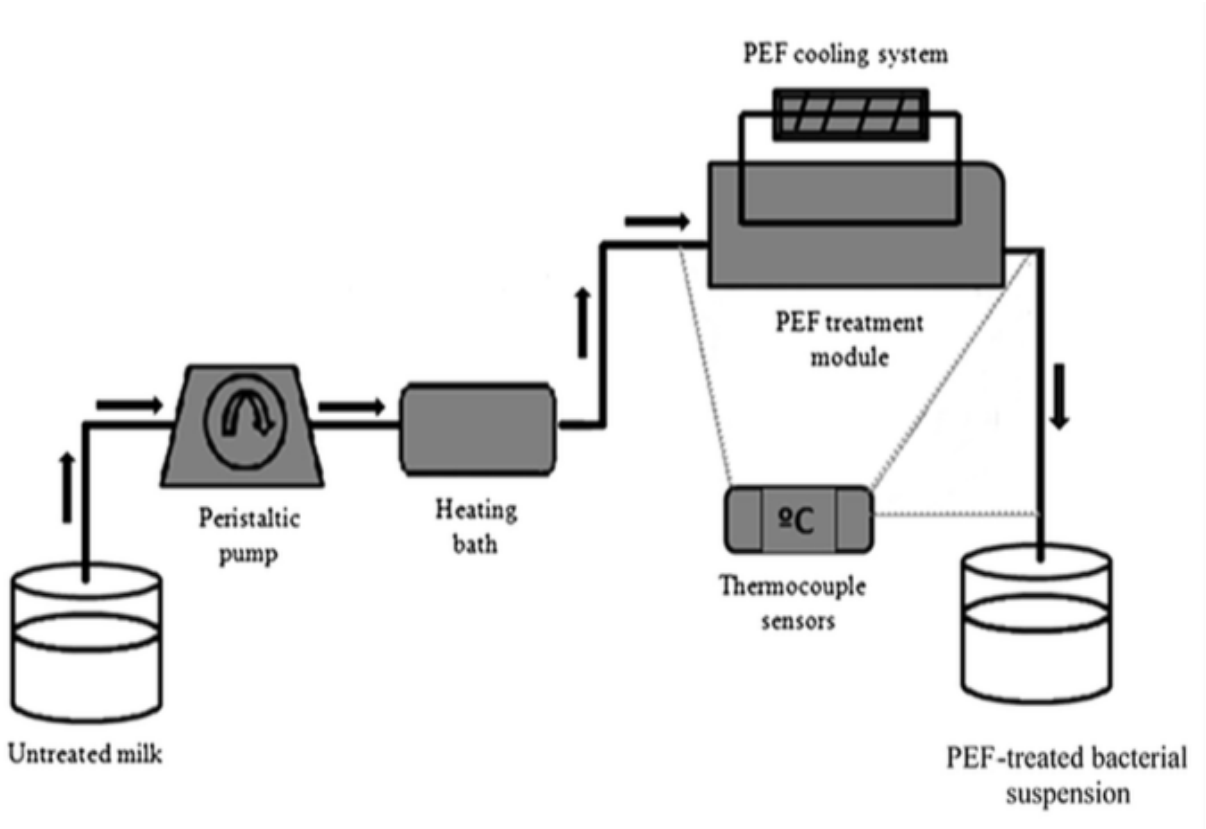

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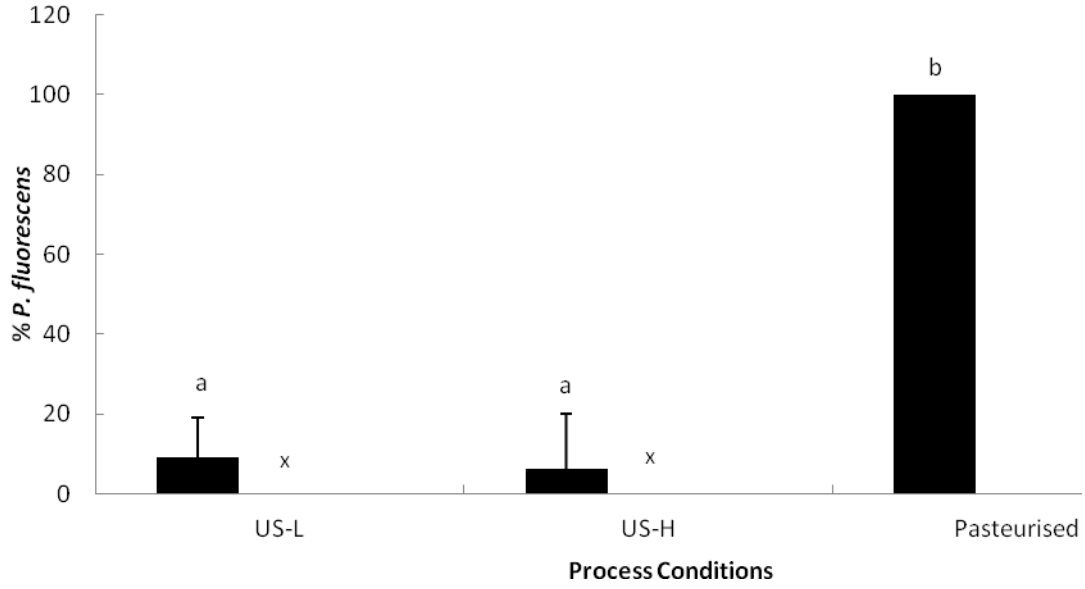

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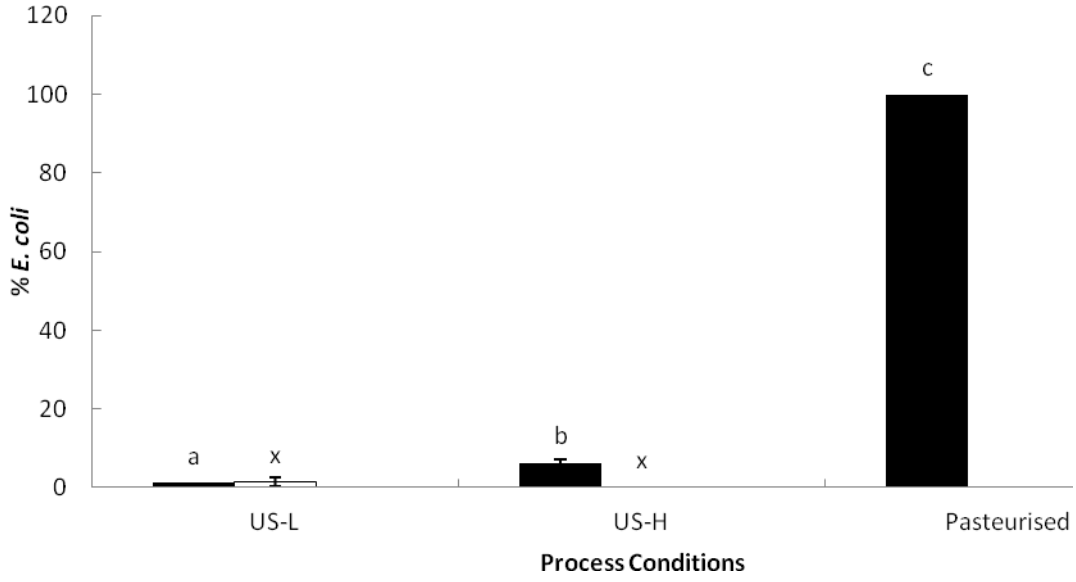
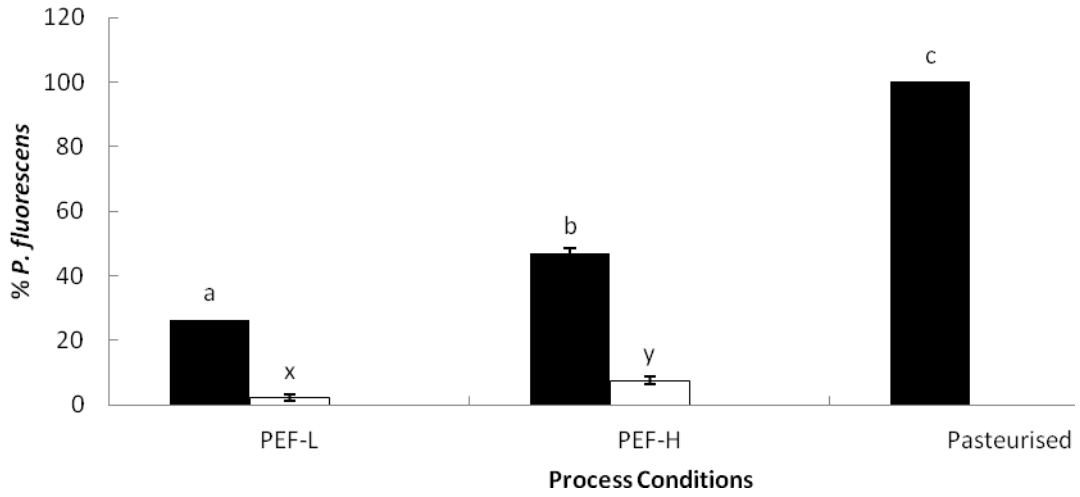


Figure 2 (b)

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.



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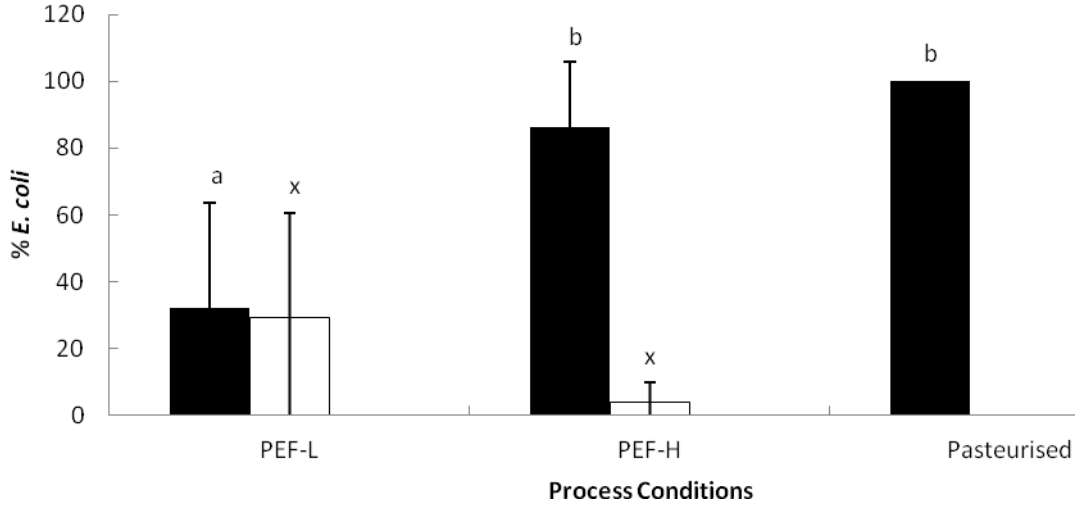

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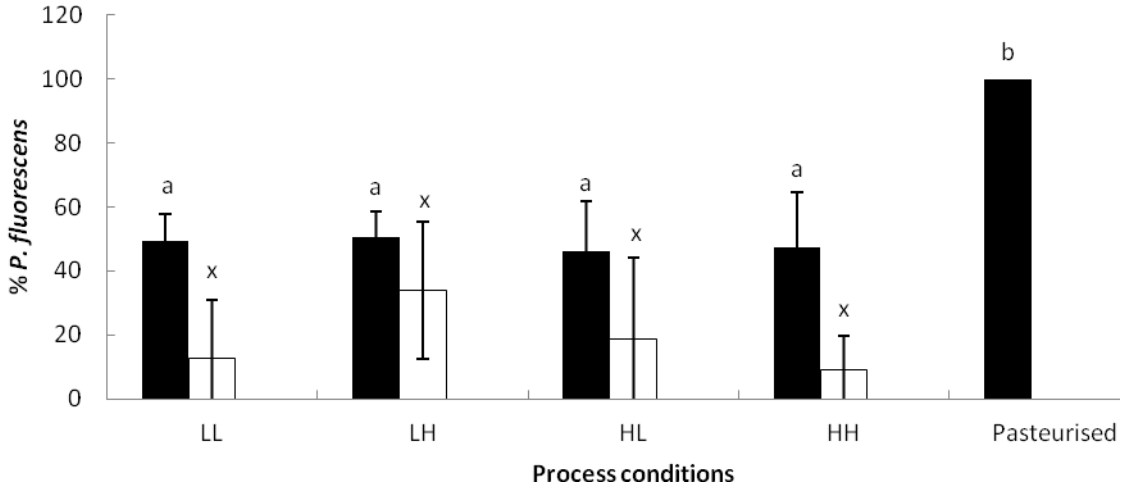


Figure 4 (a)

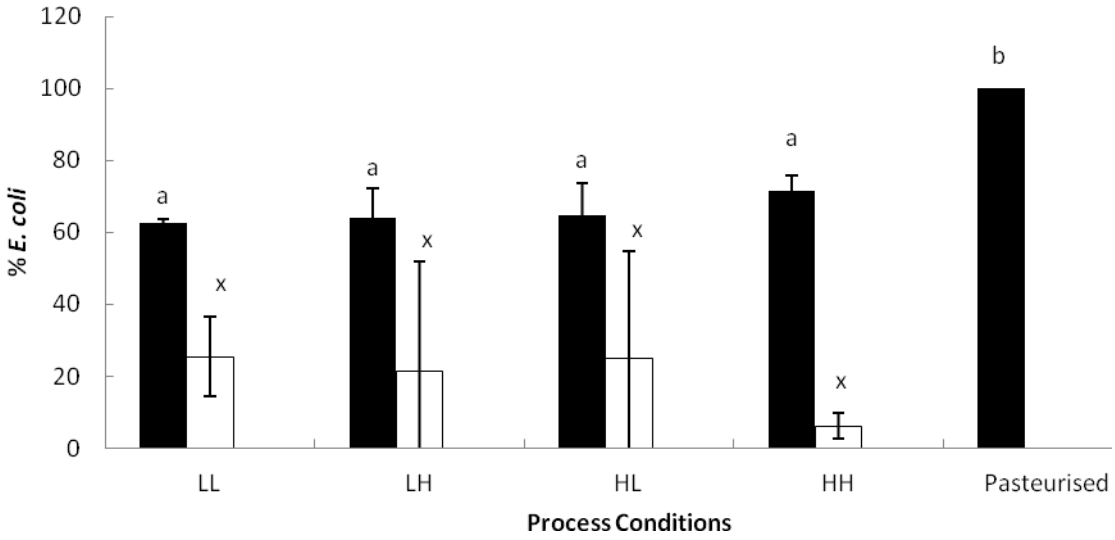
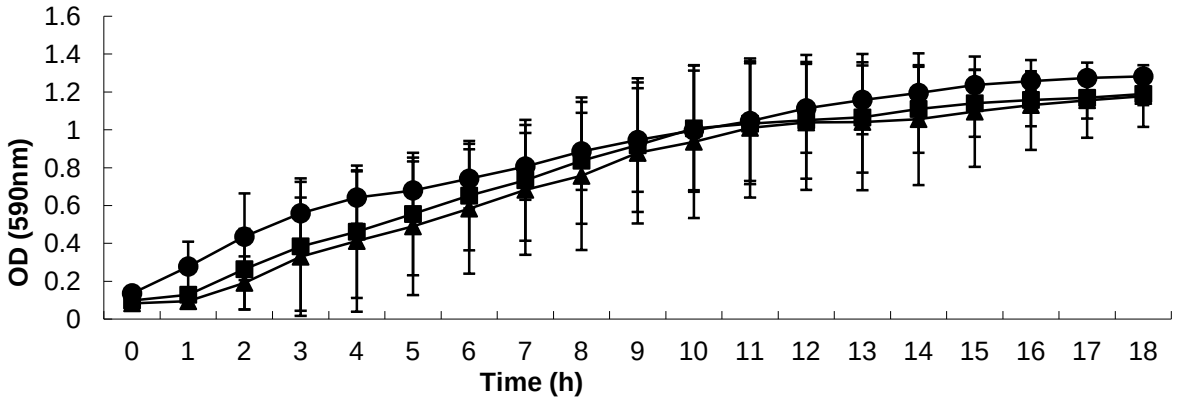


Figure 4 (b)

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.



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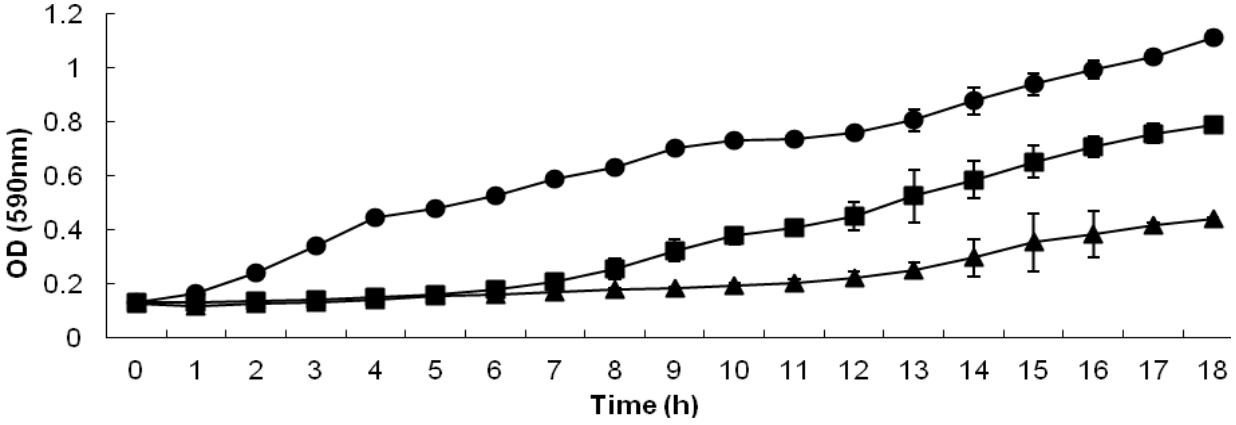

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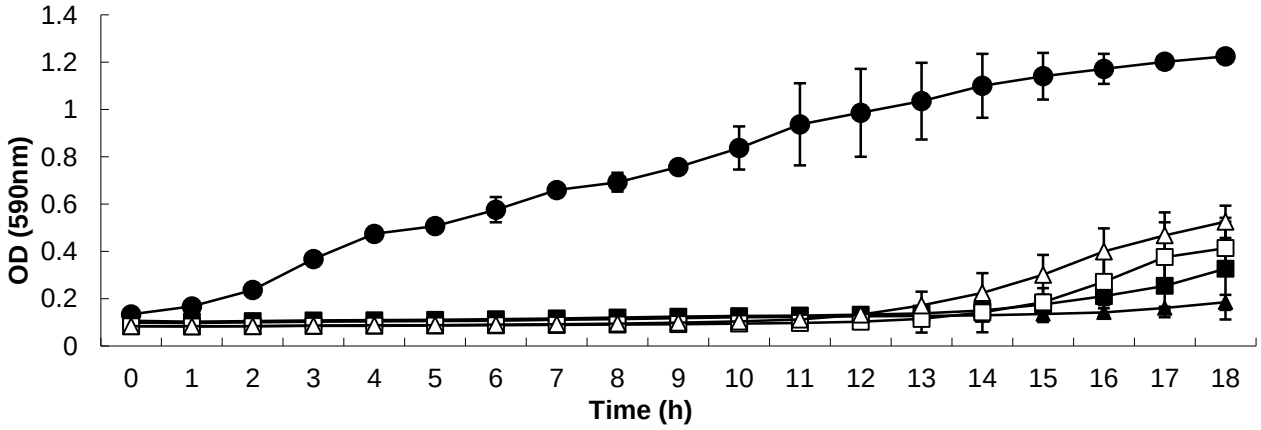
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0.6
0.4
0.2
0
OD (590nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time (h)

Figure 5 (c)