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Inactivation of *Salmonella enteritidis* in Liquid Whole Egg using Combination Treatments of Pulsed Electric Field, High Pressure and Ultrasound

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The effects of pulsed electric field (PEF), hydraulic high pressure (HHP) and ultrasound alone and in combinations on inactivating *Salmonella enteritidis* in liquid whole egg were investigated. The optimum conditions were: PEF—30 pulses of 5·67 kV/mm (+ve peak) at 55 °C, HHP—2–2–4 min cyclic treatment at 138 MPa and 20 °C, and ultrasound—40 W at 55 °C for 5 min.The PEF was not effective due to high viscosity of the egg. The use of HHP in multiple cycles was better than single treatments of equivalent time. Increasing the exposure time to ultrasound resulted in greater microbial reductions. Increasing the temperature increased the effectiveness of ultrasound. Treatment combinations only exhibited additive effects. No synergy was observed. A combination of high pressure and ultrasound treatments resulted in the greatest microbial reduction of 3·2 log cycles and, with the addition of mild heat, provides an alternative to thermal processing where high temperature has caused protein coagulation.
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1. Introduction

Pasteurisation of egg products became mandatory in the United States in 1966 (Cunningham, 1995). Current regulations in the United States require that liquid whole egg is heated to at least 60 °C for a minimum of 3·5 min. Most researchers conclude that the functional performance of egg white is impaired when heated for several minutes above 57 °C (Ma *et al.*, 1997). Pasteurisation of liquid whole egg is limited to lower pasteurisation temperatures and longer holding times due to the coagulation of its proteins at higher temperatures. Subsequently, outbreaks involving *Salmonella enteritidis* have resulted due to incomplete pasteurisation at lower temperatures (Tauxe, 1991; Tood, 1996). Studies using combinations of non-thermal technologies have attempted to target microorganisms in foods by increasing the microorganism sensitivity to the non-thermal process or to cause death to sublethally injured cells with the alteration of parameters such as pH and temperature. The emerging interest in using non-thermal technologies such as pulsed electric fields (PEF), ultrasound, and hydraulic high pressure (HHP) to inactivate microorganisms in foods has led to many studies (such as Barbosa-Canovas *et al.*, 1999; Ho & Mittal, 2000; Hurst *et al.*, 1995; Lopez-Malo *et al.*, 1999) to investigate their efficacy and mechanisms of inactivation. However, very few studies (Lee *et al.*, 2003) have attempted to apply these treatments in combination.

Hermawan *et al.* (2004) reported a maximum 1 log reduction of *S. enteritidis* in liquid whole egg which was homogenised and filtered through four layers of cheesecloth to decrease apparent viscosity. The PEF conditions included field strength E_f of 25 kV/cm, 38 °C process temperature, 2·12 μs pulse width τ_p, 250 μs treatment time, a pulse frequency of 200 pulses/s, and at a flow rate of 1·5 m*l*/s. The medium entered the treatment chamber at 20 °C. However, using similar high-energy system where rapid heating and relatively slower chilling were used, Martin-Belloso *et al.* (1997) reported 5–6 log reductions of *Escherichia coli* in liquid whole egg; and Jeantet *et al.* (1999) and Calderon-Miranda *et al.* (1999) obtained 3·5 log reduction of *S. enteritidis* in liquid whole

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egg. Treatment temperature was not clearly reported and a proper control was not used.

The HHP subjects foods with pressures in the range of 100–1000 MPa with processing temperatures from below 0 °C to 100 °C where significant reductions in microbial populations can be achieved. Ponce *et al.* (1998b) applied 300–450 MPa with treatment times of 5–15 min at temperatures of −15, 2, and 20 °C to liquid whole egg inoculated with *Listeria innocua* at a pH of 8·0. The greatest inactivation (>5 log reductions) occurred at 450 MPa for 15 min and 20 °C. *Listeria innocua* inactivation at 400 MPa followed first-order kinetics for 0–20 min, exhibiting decimal reduction times D of 7·35 min at 2 °C and 8·23 min at 20 °C. The highest reduction of *E. coli* in liquid whole egg was obtained at 50 °C and it was more resistant to pressure at 20 °C and −15 °C than at 50 °C and 2 °C (Ponce *et al.*, 1998a). Cycling was more effective than continuous treatments at lower pressures (350 MPa). *Escherichia coli* inactivation at 400 MPa for treatment times between 0 and 60 min yielded values for D of 14·1 min at 2 °C and 9·5 min at 20 °C. Inoculation levels of 10^7–10^8 *S. enteritidis* cfu/m*l* (cfu denotes colony forming units) in liquid whole egg were subjected to 350 and 450 MPa, temperatures of 50, 20, 2 and −15 °C, with treatment times of 5, 10, and 15 min as well as cycles of 5-5, and 5-5-5 min treatments (Ponce *et al.*, 1999). Inactivation increased with pressure and exposure time, being minimal (1 log reduction) at the lowest pressure, temperature and time conditions, while the greatest inactivation (>8 log cycles) occurred at the severest treatment conditions (450 MPa, 50 °C, several cycles).

Lee *et al.* (2003) tested the effects of various pressures on *Listeria seeligeri* and *E. coli* (10^7 and 10^8 cfu/m*l*, respectively),in liquid whole egg at 5 °C. Treatment times were 886 and 200 s for the applied pressures of 250 and 350 MPa, respectively. Reductions in *Listeria* were not detected after both treatment conditions while >2 log reductions were achieved for *E. coli*. Treatments at 400 MPa for 5 min yielded *E. coli* inactivation of 5·5 log cycles in liquid whole egg, whereas no *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Listeria monocytogenes* were detected (Yuste *et al.*, 2003). Increasing the pressure had a significant effect on *S. enteritidis* inactivation in liquid whole egg (Isiker *et al.*, 2003). Increasing the treatment time had no effect at 250 and 350 MPa but did have an effect when increasing the time from 5 to 10 min at 450 MPa.Total inactivation was achieved after 10 min at 450 MPa and 20 °C.

Ultrasound waves have frequencies higher than 20 kHz. The indirect treatment was delivered by placing 1 m*l* of liquid whole egg inoculated with *S. typhimurium* in a water bath where ultrasound was applied (Wrigley & Llorca, 1992). Counts were reduced up to 3 log cycles at 50 °C. The ultrasound intensity had a greater effect on the reduction than an increase in wavelength within the testing frequencies of 35 and 40 kHz.Lee *et al.* (2003) used 24·6 W of 20 kHz of ultrasound on 10 m*l* samples of liquid whole egg inoculated with *E. coli* at 5 °C. After 5 min of treatment, a 1 log reduction was achieved. When 42 W of power was used, a 2 log reduction was observed, however, with respect to the energy input per m*l*, the reductions were equivalent.

The research objectives for this study were: (i) to investigate the effects of process parameters using PEF, ultrasound, and HHP on inactivating *S. enteritidis* in liquid whole egg; (ii) to identify the process conditions to maximise *S. enteritidis* reduction for each treatment; and (iii) to observe synergistic effects, if any, using double combinations of processes at optimal conditions.

2. Materials and methods

2.1. *Growth curve of Salmonella enteritidis*

The growth curve was determined for *S. enteritidis* to obtain Salmonellae in its stationary phase of growth for use in all treatments. The strain of *S. enteritidis* used was CB 919 Lux AB obtained from the Canadian Research Institute for Food Safety, Guelph, Ontario, Canada. A sterile 500 m*l* flask containing 250 m*l* of tryptic soya broth (TSB) was autoclaved at 121 °C for 15 min on a liquid cycle and allowed to cool to room temperature. Using a sterile loop, a single colony was placed in the flask and incubated overnight at 37 °C with a rotational shaking speed of 200 min^{-1}. From this new culture, a sterile loop was used to streak some *S. enteritidis* containing broth over a fresh tryptic soya agar (TSA) plate such that colonies could form and was incubated at 37 °C overnight. This served as a stock plate for experiments and a fresh one was made every 1–2 weeks.

A single colony of the strain was selected from the stock plate and placed in 250 m*l* of sterile TSB and incubated at 37 °C and rotational shaking speed of 200 min^{-1}. The broth turbidity was measured by taking 1 m*l* of broth from the culture at 1 h intervals for the first 5 h and then 0·5 h intervals thereafter until turbidity measurements stabilised. It required placing 1 m*l* of broth into 2 m*l* plastic cuvettes to measure the absorbance at a wavelength of 600 nm using a spectrophotometer (Pharmacia Biotech UltroSpec 1000E, Cambridge, UK).

2.2. *Culturing and suspension of Salmonella enteritidis into liquid whole egg*

The eggshells were sanitised by dipping and rubbing with 70% ethanol before breaking. The wet shells were

allowed to air dry for a few minutes. The eggs were removed from their shells and placed in a blender (Osterizer 6650, Mexico) that had been washed and sterilised using 70% ethanol. About 18–20 eggs were used to yield approximately 750 m*l* of liquid whole egg. The eggs were then blended at medium speed for 1 min using the blender.

A single colony was picked from the stock plate and placed in 250 m*l* of sterile TSB and incubated at 37 °C at a rotational shaking speed of 200 min^{-1} for 12 h. From this culture, 5 m*l* was placed in a 15 m*l* centrifuge tube and centrifuged (Beckman J2-MC; Rotor JA20·1) at a speed of 6000 min^{-1} for 10 min^{-1} at 4 °C. The supernatant was removed with the use of a pipette. To remove any residual broth, the cell pellet was washed twice using 1 m*l* of 0·1% peptone solution. From the blended liquid whole egg, 10 m*l* was placed into the cell pellet-containing centrifuge tube and mixed using a vortex for 30 s. The high cell density liquid whole egg was then slowly poured back into the beaker containing 500 m*l* liquid whole egg that was being stirred for 15 min. For PEF treatments, the necessary volume required was placed in 100 m*l* autoclaved glass beakers. For ultrasound treatments, samples were placed in test tubes with an inner diameter of 15 mm. For HHP treatments, samples were placed in sterile sample cups (50 m*l*). All prepared samples were stored on ice to be immediately transferred to the applicable devices for treatments.

2.3. *Inactivation of Salmonella enteritidis in liquid whole egg using pulsed electrical field*

The details on the PEF system used are given by Ho *et al.* (1995). The power supplied to the system originated from 110 V of alternating current that it is stepped up by a transformer and rectified to high-voltage direct current. The high voltage supplied to the capacitive circuit was discharged through 40 kΩ resistors and the treatment chamber at a pulse frequency of 0·5 Hz controlled by the pulse generator and a thyratron switch. The pulse shape was instant-charge reversal and its width was about 2 μs.

The treatment chamber has a stainless-steel parallel-electrode configuration with diameter of 10·7 cm and was housed in Delrin (insulation) shells. The details are given by Wu *et al.* (2005). The first shell housed one of the circular electrodes. An extension of its circumferential edge provided a threaded surface for the second shell. A spacer, also made of Delrin, was placed between the electrodes to provide a gap of 30 mm. The resultant treatment chamber volume was 22·5 m*l*. The treatment chamber design also allowed for maintenance of the process temperature with the circulation of hot water in spaces incorporated adjacent to the electrodes. This was needed to increase the sample temperature as the application of low-energy pulses did not increase the temperature. To insert samples for treatment, a small hole was drilled through the top of the first shell and spacing such that a syringe can be used to place the sample. The electrodes were connected to the circuit and the electrical potentials were measured using a high-voltage probe (Tektronix P6015A, Beaverton, OR) and an oscilloscope (Tektronix, Model No. TDS 340).

The liquid whole egg was first heated with a water bath (Fisher Scientific Inc., Model IC-2100) to the target temperature and inserted into the chamber that was preheated by circulating heated water with a peristastic pump (Cole Parmer 7553-80) and maintained at the process temperature with the use of a proportional controller (Omega CN9000A). The use of low-energy instant-charge-reversal pulses with 2 μs pulse widths used allowed up to 50 pulses without increase in medium temperature.

Using a vacuum pump (Emerson Model SA55JXGTD-4144, St. Louis, MO; 11–1600 Pa absolute), 250 m*l* of 25% liquid chlorine bleach (Hero™ Brand, Toronto, Canada) was run through the chamber to sterilise the chamber interior. Using the same method, rinsed the chamber using sterile deionised water to remove any residual bleach. The preheating of the liquid whole egg required raising the temperature slowly (5°C/min) since any rapid heat inputs to the liquid whole egg would cause coagulation of its proteins. A thermocouple probe (Omega Type J-K-T; Omega Engineering Inc., Stamford, CT) was used to monitor the rising temperature. The liquid whole egg was then drawn from the beaker using a 30 m*l* syringe (Becton Dickinson, Luer-Lok). With the bottom port of the treatment chamber closed, the syringe was placed in the small hole to insert the sample. The top port remained open to allow for the displacement of air as the sample was inserted. Excess medium was removed using cotton buds and the top port was closed. Output voltage level to 26 kV (resultant E_f of 5·67 kV/mm (+ve peak) with 30 mm electrode gap) was used.

From this treated sample, 1 m*l* was taken and placed in 9 m*l* of 0·1% peptone solution that was stored on ice so that the sample would rapidly cool to a temperature where microbial death due to the temperature (after the treatment) would not contribute to the overall log reductions.

2.4. *Inactivation of Salmonella enteritidis in liquid whole egg by high pressure*

A French press (P1603013600 American Instrument Company, Savage, MD), designed to apply up to

138 MPa by direct compression of a piston onto the medium, was used. A strain gauge measured the applied force exerted on the platform. The initial temperature of the treatment cell, piston, and seat was set by placing these for 5 min in a water bath that had been cooled to the desired temperature. The treatment cell and the piston were thoroughly wiped with 70% ethanol to inactivate microbes. Sterile deionised water was then flushed through the orifice to rid of any excess ethanol.

The untreated sample was then poured into the cylinder and the piston was slowly pushed up until the sample was about 1·9 cm from the edge of the cylinder. A variable knob manually controlled the pressure applied by observing on a pressure gauge. Once the treatment was completed, the seat was removed to open the chamber. Immediately temperature was recorded using the thermocouple probe. A 1 m*l* sample was transferred to a test tube containing 9 m*l* of 0·1% peptone solution that was stored on ice. For cyclic treatments, the process was repeated.

2.5. *Inactivation of Salmonella enteritidis in liquid whole egg by ultrasound*

For ultrasound treatments, a Virsonic Digital 475 sonicator (Virtis 274480, Gardiner, NY) was used, which was capable of delivering up to 475 W of ultrasound power at a frequency of 20 kHz through a titanium disrupter horn. A horn with a tip diameter of 3·2 mm (microtip) was used, delivering 40 W of ultrasound power. The test tube was held in the water bath by hand (such that the entire portion of the liquid whole egg was submerged) and agitated with circular motions. A thermocouple probe was used to monitor the rising temperature.

After the liquid whole egg had been heated to the target temperature, the test tube was placed in a copper coil that circulated water to serve as a heat exchanger to remove heat generated during the ultrasound treatments. The copper coil had an inner diameter of 4·9 mm. Two sets of coils were made both with diameters of 150 mm. The heights of the coils were 5 and 10 cm which were approximately the resultant heights of the 12·5 and 25 m*l* of liquid whole egg, respectively. The heat was removed by circulating 2·5 *l*/min water at 17°C through the copper coils using a refrigerated bath (Haake D1, W. Germany). Untreated samples were subjected to the same heat conditions as the ultrasound treated samples by raising the temperature from ice temperature to the process temperature and maintained for the same length of time as the treated samples. The sonicating disruptor horn was immersed approximately 2·5 cm below the surface of the liquid whole egg. After selecting the power intensity, the device was turned on. A thermocouple probe was inserted into the test tube to monitor temperatures at all depths of the medium during the treatments.

For combination treatments of pulsed electrical field, ultrasound, and high pressure, the same procedure for each treatment was followed.

2.6. *Enumeration of viable Salmonella enteritidis*

Untreated and treated samples of 1 m*l* each were placed in a sterile test tube containing 9 m*l* of 0·1% peptone solution. Each sample was then mixed using a vortex for 10 s. Subsequent serial dilutions were prepared such that 100 μ*l* can be plated on TSA using a spiral plater (Spiral Biotech Autoplate 4000, Exotech Inc., Gaithersburg, MD) to yield plate counts between 25 and 250 colonies. The plates were incubated for 15 h at 37 °C.

2.7. *Statistical analysis*

Factorial design with at least three replications was used for all experimental groups. An analysis of variance (ANOVA) was conducted using SAS (version 8·2, Unix based; SAS, 2001) to quantify any effects of experimental parameters such as process temperature and time. The general linear model (GLM) procedure was used for this analysis. A Duncan's multiple range test was also conducted to rank the means of each parameter.

3. Results and discussion

3.1. *Growth curve of Salmonella enteritidis*

Using TSB as a nutrient media, and an incubation temperature of 37 °C, the absorbance at 600 nm was plotted against the incubation time to determine the growth curve of *S. enteritidis* (*Fig. 1*). The absorbance readings began to stabilise after approximately 12 h of incubation at 37 °C. The cell density at this incubation time was $3{\cdot}37 \times 10^9$ cfu/m*l*. Other studies, using *S. enteritidis* in their stationary phases, have also reported cell densities of 10^9 cfu/m*l* (Ponce *et al*., 1999; Teo *et al*., 2001). Since this phase of growth was used for all experiments, the inactivation of *S. enteritidis* achieved in this study reflects the effectiveness of each treatment using the most resistant growth phase forms of the pathogen.

3.2. *Inactivation of Salmonella enteritidis in liquid whole egg using pulsed electrical field*

No inactivation occurred when 30 pulses of 5·67 kV/mm at 20 °C were applied. The treatment temperature was then raised to 50 and 55 °C. Control treatments were taken to determine thermal effects on microbial reductions by subjecting the samples to the same conditions without the application of the pulses. The numbers of pulses applied at 50 °C were 30, 40, and 50. The result of increasing the number of pulses at 50 °C showed no inactivation due to PEF (Table 1). Statistical analysis showed that there was no significant difference between PEF treated and control samples. At 55 °C, a higher reduction was obtained; however, the microbial reductions could be mainly attributed to thermal effects.

Even at such a E_f and temperatures, reasons for the lack of success of PEF in this study are thought to be mainly due to the high viscosity of the liquid whole egg, while the nature of the applied pulses and medium high electrical conductivity are also thought to be secondary PEF-inhibiting factors. Since egg has a relatively high electrical conductivity of 0·6 S/m (Gongora-Nieto *et al.*, 2003), it has a low resistivity that reduces the pulse width, which increases the current and the treatment time (Ho *et al.*, 1995; Barbosa-Canovas *et al.*, 1999). Therefore, the use of the instant-charge reversal pulses in inactivating *S. enteritidis* have not provided a sufficient electrical potential gradient for the required length of time to cause ion migration to accumulate at the cell membrane.

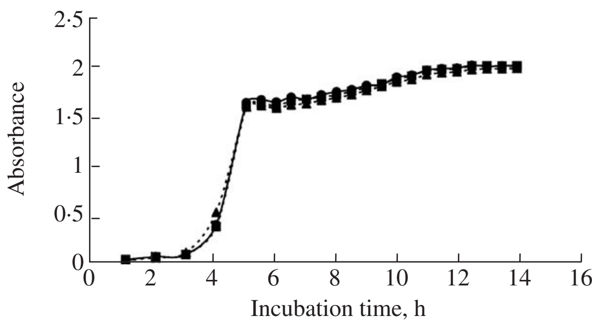

Fig. 1. Growth curve of Salmonella enteritidis when cultured in trypic soy broth at an incubation temperature of 37 °C; three curves are shown for three replications

Higher microbial reductions using PEF treatment by Martin-Belloso *et al.* (1997), Calderon-Miranda *et al.* (1999) and Gongora-Nieto *et al.* (2003), seem to be mainly due to thermal effect as rapid heating and relatively slow cooling were applied. It was further clarified by Hermawan *et al.* (2004), where very low inactivation was obtained for *S. enteritidis* in filtered liquid whole egg. Also without comparing with a suitable control, only 1 log reduction was achieved when applying a value for E_f of 2·5 kV/mm with a pulse frequency of 250 Hz.The small reduction may be only due to repeated heating and cooling. These conditions are harsher than the conditions applied by Martin-Belloso *et al.* (1997), yet they had achieved only a small reduction. A similar E_f was used for both treatments but the total treatment time corresponding to 1 log reduction for Hermawan *et al.* (2004) was more that 13 times greater. Jeantet *et al.* (1999) has observed inactivation of *S. enteritidis* suspended in diaultrafiltered (twice ultrafiltered) egg white. The success in inactivating *S. enteritidis* could also be due to the reduced viscosity that resulted from the diaultrafiltration.

3.3. *Inactivation of Salmonella enteritidis in liquid whole egg using high pressure*

Applying 138 MPa with an initial vessel temperature of 20 °C, the samples were subjected to a single treatment of 8 min, and multiple cycles of treatments for a total treatment times of 4 and 8 min.The treatment

Table 1
Inactivation of *Salmonella enteritidis* in liquid whole egg using pulsed electrical field (PEF) at an electric field E_f of 5·67 kV/mm and temperatures of 50 and 55 °C

Number of pulses	*Replications*	*Mean heat control log_{10} reduction*	*Mean PEF treated log_{10} reduction*	*Mean log_{10} reduction due to PEF only*	*Standard deviation*
Temperature of 50 °C					
30	3	0·04	0·05	0·0[a]	0·02
40	3	0·06	0·06	0·0[a]	0·00
50	3	0·07	0·06	0·0[a]	0·00
Temperature of 55 °C					
30	12	0·79	0·82	0·02[a]	0·02
40	3	0·83	0·87	0·05[a]	0·05
50	3	0·92	0·90	0·00[a]	0·03

Different letters in a column represent significant difference (probability $P < 0{\cdot}05$).

cycles used were 4-4, 2-2-4, and 1-1-2 min (*Fig. 2*). Treatments, comprised of multiple cycles of shorter treatment times, yielded greater inactivation compared to single treatments of equivalent treatment times. A single treatment of 8 min only yielded a 0·43 log reduction while treatments of 4-4 and 2-2-4 min resulted in 1·27 and 2·20 log reductions, respectively. The same trend was observed when comparing a single treatment of 4 min to the 2-2 and 1-1-2 min treatments. No coagulation of liquid whole egg was observed as temperature was around 20°C and low pressure was applied.

The increased inactivation when using multiple cycles was in agreement with Ponce *et al.* (1999) where liquid whole egg was inoculated with 10^7 cfu/m*l* of *S. enteritidis* and treated at 350 MPa and 20 °C. Single treatments for 5, 10, and 15 min resulted in 1.44, 1·73, and 1·90 log reductions, respectively. Cyclic treatments of 5-5 and 5-5-5 min yielded log reductions of 1·93 and 6·30, respectively. Lower inactivation was achieved in this study since a lower pressure of 138 MPa was used, however, the same trend is observed. The inactivation of *S. enteritidis* using cyclic treatments of HHP was not as effective at lower temperatures compared to those at 20 °C (*Fig. 2*, Table 2). This was also in agreement with Ponce *et al.* (1999).

When plotting single treatments of 8 min along with the log reductions achieved from the first cycle of the cyclic treatments near 20 °C, an inactivation curve at 138 MPa is revealed (*Fig. 3*). The log reductions per treatment time of *S. enteritidis* reduced with increasing the treatment time. A relatively large portion of the total inactivation occurred within first few minutes (*Fig. 3*). Thus, initial pressurisation has a significant effect on microbial destruction. This is also illustrated by Ramaswamy *et al.* (2003) where *E. coli* was pressurised in apple juice at 150–400 MPa for 0–80 min at 25 °C.

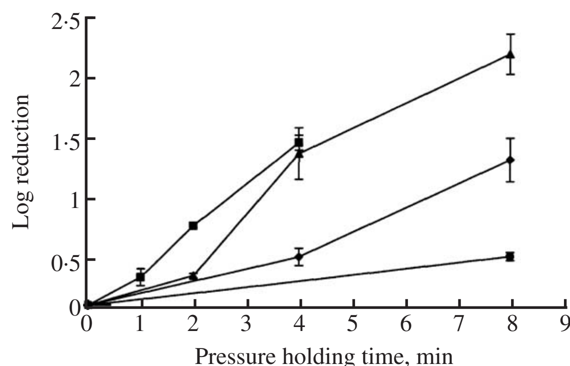

Fig. 2. Effect of cyclic treatments at a pressure of 138 MPa on microbial inactivation around 20 °C: -●- 8 min, average of three observations; -◆-, 4-4 min, average of three observations; -▲-, 2-2-4 min, average of four observations; and -■-, 1-1-2 min, average of three observations

Table 2
Effect of cyclic hydraulic high pressure (HHP) treatments (138 MPa) at an initial vessel temperature of 4 °C

Treatment conditions	*Average final temperature, °C*	*Mean log reduction*
8 min	$9{\cdot}3 \pm 0{\cdot}8^{a}$	$0{\cdot}20 \pm 0{\cdot}07^{b}$
4-4 min	$9{\cdot}5 \pm 1{\cdot}0^{a}$	$0{\cdot}36 \pm 0{\cdot}04^{ab}$
2-2-4 min	$8{\cdot}1 \pm 1{\cdot}0^{a}$	$0{\cdot}43 \pm 0{\cdot}31^{ab}$
1-1-2 min	$7{\cdot}4 \pm 0{\cdot}8^{a}$	$0{\cdot}59 \pm 0{\cdot}10^{a}$

Different letters in a column represent significant difference (probability $P < 0{\cdot}05$).

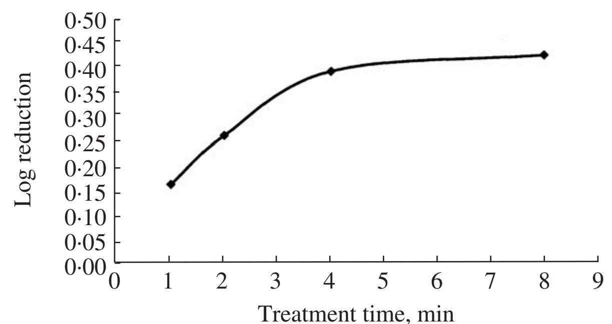

Fig. 3. Effect of treatment time on Salmonella enteritidis log reductions in liquid whole egg at a constant pressure of 138 MPa with the initial vessel temperature of 20 °C; average of three replications

High pressure acts by disrupting the structure of secondary or tertiary-bonded molecules, but covalently bonded molecules generally remain unaffected (MacFarlane, 1985). This results in the denaturation of large molecules such as proteins, while many components responsible for sensory and nutritional quality remain unaffected such as flavour compounds and vitamins (Mertens, 1995). Lee *et al.* (1999) reported that liquid whole egg began to coagulate at 400 MPa and 25 °C but remained stable at 350 MPa. Since the applied pressure was less than half of the lower limit, it can be concluded that cyclic treatments using a pressure of 138 MPa has not lead to any protein coagulation.

3.4. *Inactivation of Salmonella enteritidis in liquid whole egg using ultrasound*

When applying 1, 2, and 3 min treatments at 40 W ultrasound to 12·5 m*l* liquid whole egg (initial vessel temperature of 20 °C), there was an increase in the

inactivation of *S. enteritidis* (log reductions of 0·17 ± 0·02, 0·27 ± 0·03, and 0·28 ± 0·03, respectively for 1, 2 and 3 min; 3 replications). Increasing the treatment time improved the reductions in *S. enteritidis*. The treatment time was inversely related to the number of microbial survivors (Mett *et al*., 1988; Ordonez *et al*., 1984, 1987). The treatment time was significant (probability $P < 0·05$) in inactivating *S. enteritidis* except between 2 and 3 min.Further treatments of 3, 4, and 5 min at 50 °C and 40 W ultrasound were used to observe the effect of treatment time on log reductions (Table 3). The reduction of *S. enteritidis* observed in the heat control of equivalent treatment time was very small in comparison to the reductions due to the 40 W of ultrasound applied at 50 °C. Similar inactivation kinetics was also observed by others (Lopez-Malo *et al*., 1999). Increasing the length of exposure to ultrasound resulted in greater log reductions where inactivation was an exponential function of time (Mett *et al*., 1988).

The decimal reduction times D for yeast cells were reported as 739·0, 72·5, 18·3, 4·8 and 2·7 min for temperatures of 45, 47·5, 50, 52·5 and 55 °C respectively (Lopez-Malo *et al*., 1999). With the simultaneous application of 20 kHz ultrasound with an amplitude of 90 µm, these values for D were reduced to 22·3, 8·2, 5·6, 3·0 and 0·8 min at corresponding temperatures respectively. In this study, 1 log reduction was achieved after only 5 min of treatment compared to a similar reduction achieved after a 30 min treatment by Wrigley and Llorca (1992). The higher inactivation rate is likely due to the direct sonication of the liquid whole egg compared to the indirect application used by Wrigley and Llorca (1992).

The effect of raising the treatment temperature to 55 °C showed an increase in inactivation of *S. enteritidis* in liquid whole egg (Table 4). It could be due to increase in the vapour pressure and decrease in the tensile strengths of cavities formed when applying ultrasound (Hurst *et al*., 1995).

While maintaining the power input of 40 W, treatment time of 5 min and 55 °C, the effect of changing the volume to 25 from 12·5 m*l* on log reductions in *S. enteritidis* was determined. As expected, a decrease in the reduction of *S. enteritidis* was observed when increasing the treatment volume due to decrease in ultrasound energy applied per m*l* of liquid whole egg (Table 5).

3.5. *Combination treatments*

The conditions (PEF–ultrasound, ultrasound–PEF, HHP–PEF, PEF–HHP, and ultrasound–HHP, and HHP–ultrasound) that yielded the maximum log reductions of *S. enteritidis* for each single treatment were applied, except for ultrasound where 25 m*l* sample was taken, in combination treatments. The PEF conditions applied were 30 pulses with a field strength of 5·67 kV/mm and 55 °C while the ultrasound conditions were 40 W at 55 °C for 5 min with 25 m*l* volume. The optimal conditions for HHP were 2-2-4 min cyclic treatment at 138 MPa and 20 °C.

Table 3
Log reductions in *Salmonella enteritidis* in 12·5 m*l* of liquid whole egg using 40 W ultrasound at 50 °C for treatment times of 3, 4, and 5 min

Treatment time, min	*Mean heat control log_{10} reduction*	*Mean ultrasound treated log_{10} reduction*	*Mean log_{10} reduction due to ultrasound only*
3	0·04 ± 0·02	0·71 ± 0·01[c]	0·67 ± 0·01[c]
4	0·05 ± 0·01	0·87 ± 0·03[b]	0·82 ± 0·03[b]
5	0·06 ± 0·01	1·05 ± 0·04[a]	0·99 ± 0·04[a]

Replications = 3; different letters in a column represent significant difference (probability $P < 0·05$).

Table 4
Log reductions of *Salmonella enteritidis* in 12·5 m*l* of liquid whole egg using 5 min treatment time of 40 W ultrasound at 50 and 55 °C

Treatment temperature, °C	*Mean heat control log_{10} reduction*	*Mean ultrasound treated log_{10} reduction*	*Mean log_{10} reduction due to ultrasound only*
50	0·06 ± 0·01	1·05 ± 0·04[b]	0·99 ± 0·04[b]
55	1·06 ± 0·11	2·30 ± 0·09[a]	1·24 ± 0·10[a]

Replications = 3; different letters in a column represent significant difference (probability $P < 0·05$).

Table 5
Log reductions of *Salmonella enteritidis* in liquid whole egg at 55 °C with treatment times of 5 min and 40 W of ultrasound with treatment volumes of 12·5 and 25 m*l*

Treatment volume, ml	*Replications*	*Mean heat control log_{10} reduction*	*Mean ultrasound treated log_{10} reduction*	*Mean log_{10} reduction due to ultrasound only*
12·5	3	1·06 ± 0·11	2·30 ± 0·09[a]	1·24 ± 0·10[a]
25	11	0·97 ± 0·07	1·62 ± 0·10[b]	0·65 ± 0·08[b]

Different letters in a column represent a significant difference (probability $P < 0{\cdot}05$).

3.5.1. *Pulsed electrical field and ultrasound combination treatments*

Both combinations (PEF–ultrasound and ultrasound–PEF) resulted in less than the sum of the two treatments alone of 2·40 log cycles (*Fig. 4*). The log reductions for the PEF–ultrasound and ultrasound–PEF treatments were 2·30 and 2·25 respectively. The ANOVA revealed that there was no significant difference between the two treatments.

However, others have not observed any injury to microorganisms that had been subjected to PEF therefore concluding that PEF may be an 'all or nothing' event, *i.e*. it may not injure but inactivate the microorganism fully (Ho & Mittal, 2000; Simpson *et al*., 1999; Russel *et al*., 2000; Dutreux *et al*., 2000a, 2000b; Ulmer *et al*., 2002). Furthermore, these studies that have not observed any injury in PEF treated cells utilised media with pH near 7. Since liquid whole egg has a pH of approximately 6·8, it was concluded that no injury occurred. Thus, the combinations involving PEF and ultrasound were essentially a preheat treatment followed by a ultrasound treatment and vice versa. In both combination treatments (PEF–ultrasound, ultrasound–-PEF), the additive sums were statistically equivalent to the sum of the two treatments (PEF and ultrasound) alone (*Fig. 4*).

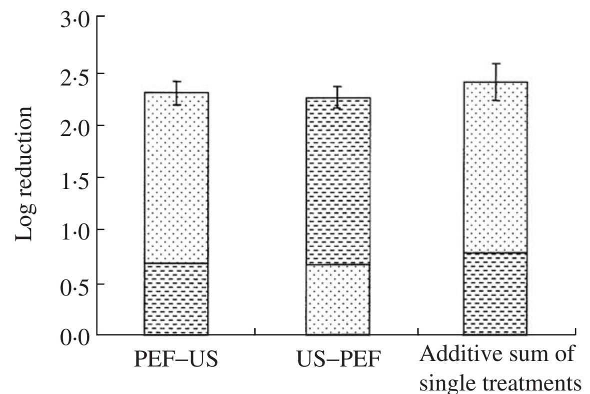

Fig. 4. Pulsed electrical field (PEF, ▢) and ultrasound (US, ▒) as treatment combinations PEF–US and US–PEF along with the additive sum of the two treatments alone; PEF–ultrasound implies that PEF was the first treatment which was followed by a ultrasound treatment; means of three replications are shown

3.5.2. *Ultrasound and high-pressure combination*

The consecutive combination treatments involving ultrasound and HHP resulted in significant reductions of *S. enteritidis*. Although no synergy was observed, up to a 3 log reduction was achieved (*Fig. 5*). The sum of the combination treatments was less than (probability $P < 0{\cdot}05$) the additive sum of the two individual treatments alone. This is due to a more resistant subpopulation remained after an initial treatment and cells of *S. enteritidis,* that have been easily killed, have already been inactivated by the initial treatment. Lee *et al*. (2003) used liquid whole egg with *E. coli* for ultrasound-HHP treatments. The ultrasound conditions applied were 34·6 W for 30 s and 150 s at 5 °C while the following HHP conditions applied were of 250 MPa for 886 s and 300 MPa for 200 s. An additive effect was also observed where the slight increase was due to the

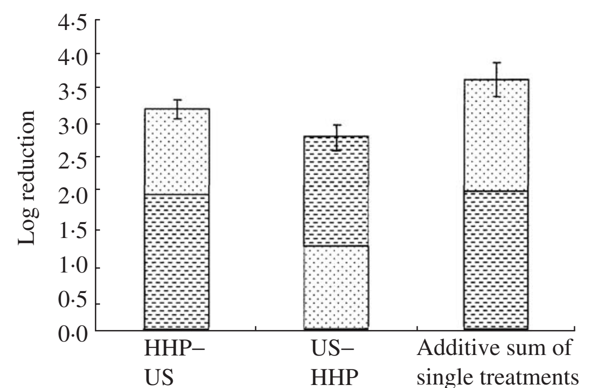

Fig. 5. Hydraulic high pressure (HHP, ▢) and ultrasound (US, ▒) as treatment combinations HHP–US and US–HHP shown along with the additive sum of the two treatments alone; HHP–ultrasound implies that HHP was the first treatment which was followed by a ultrasound treatment; means of three replications are shown

ultrasound pretreatment. A similar result was achieved in this study.

It appears that the HHP–ultrasound combination was more efficient than the ultrasound–HHP treatment since its additive sum is closer to the additive sum of the two treatments alone. Using the Duncan's multiple range tests, the HHP–ultrasound treatment was ranked better of the two treatments. A reason for the greater inactivation observed with ultrasound as a second treatment could be the synergistic effect of heat and ultrasound acting on the injured survivors from the HHP treatment.

3.5.3. *Pulsed electrical field and high-pressure combination*

Both treatment combinations (PEF–HHP and HHP–PEF) did not exhibit a synergistic effect in inactivating *S. enteritidis* (*Fig. 6*). It appears that HHP treatments that were followed by heat were more effective than a pretreatment of heat to HHP. The heat accelerated the inactivation of cells that have been injured by the HHP treatment.

The PEF–ultrasound, ultrasound–PEF, and HHP–PEF treatment combinations were equal to their respective additive sums while the HHP–ultrasound treatment was slightly below its additive sum. A common factor between these treatment combinations was that heat was used as second process. This is the reason for the reductions of *S. enteritidis* at or near their respective upper limits. The HHP treatments have exhibited diminishing inactivation rates with increased exposure times (Lee *et al*., 2001). Conversely, processes involving heat and ultrasound at elevated temperatures have shown linear inactivation rates. The synergistic effect of ultrasound and heat have shown to exhibit linear inactivation rates over increasing exposure times as evidenced in this study and by Lopez-Malo *et al*. (1999). Unlike HHP processes, a stabilisation effect is not as pronounced compared to heat and ultrasound at higher temperatures. With this in mind, PEF and ultrasound treatment combinations were equal to their additive sum since a non-stabilising mechanism was involved. The HHP–ultrasound treatment combination resulted in the greatest inactivation of *S. enteritidis* yielding a mean log reduction of 3·23 log cycles.

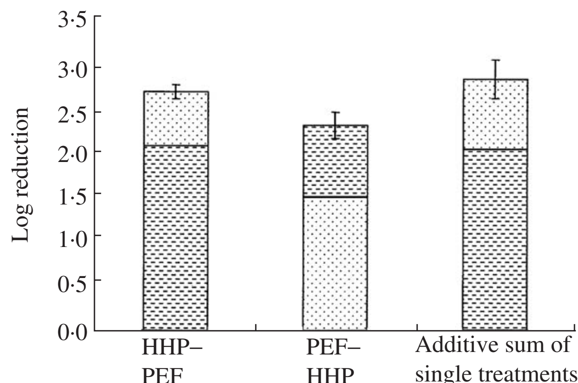

Fig. 6. Pulsed electyrical field (PEF, ▒) and hydraulic high pressure (HHP, ☐) as treatment combinations HHP–PEF and PEF–HHP shown along with the additive sum of the two treatments alone; PEF–HHP implies that PEF was the first treatment which was followed by ultrasound treatment; mean of three replications are shown

4. Conclusions

The pulsed electrical field (PEF) to inactivate *Solmonella enteritidis* in liquid whole egg was not effective due to its high viscosity. Increasing the number of pulses and the treatment temperature up to 55 °C did not improve the effectiveness of the PEF. The hydraulic high pressure (HHP) in multiple cycles at 138 MPa was better in inactivating *S. enteritidis* than the single treatments of equivalent time. The effect was much greater at 20 °C than at lower temperatures. Increasing the number of cycles had a marked effect in reducing *S. enteritidis*. The effectiveness values of the single pressure treatments diminished as a result of the treatment time.

Ultrasound (US) treatments with heat have shown a synergistic effect on inactivating *S. enteritidis*. Greater inactivation was achieved by increasing temperature and treatment time. Increase in treatment volume reduced the effectiveness of the treatment. The combination treatments exhibited only the additive effects, and no synergy was observed. Treatments involving heat that followed initial HHP treatments provide better inactivation of *S. enteritidis* compared to preheat treatments to HHP. Accordingly, the HHP–ultrasound treatment combination resulted in the greatest reduction of *S. enteritidis*.

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