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

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Milk Sourcing and Preparation

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

2.2 Treatment of Milk with MTS/PEF

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

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

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

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

co-linear treatment chambers with a refrigerated cooling module integrated; these cooling sections were present following the first and third chambers. Each chamber held two co-linear stainless steel electrodes separated by a 5.0 mm gap, with the electrode diameter being 3.0 mm, which resulted in a total treatment volume of 0.106 cm^3. PEF treatment was applied using monopolar square-wave pulses (10 µs pulse width, 320 Hz frequency). The EFS was 32kV/cm and the total treatment time was determined to be 127 µs. The product temperature was recorded using thermocouples (Testo 925, type-K probe, Testo AG, Lenzkirch, Germany) both before and after the treatment module, and was never allowed to exceed 37ºC or 55ºC depending on the treatment being applied. Following treatment with MTS/PEF, milk was collected in a sterile glass bottle and stored at 4°C until required.

2.3 Thermal Pasteurisation of Milk

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

2.4 Microbiological Analysis

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

(a) Yeasts and moulds: on potato dextrose agar (PDA; Oxoid; Basingstoke, U.K.), incubated at 30°C for 48 h.

(b) Staphylococcus aureus: on Baird Parker agar (BPA; Merck, Darmstadt, Germany), incubated at 37°C for 48 h.

(c) *Enterobacteriaceae*: serial decimal dilutions (1 ml) of milk samples were pour-plated in violet red bile lactose dextrose agar (VRBLD; Oxoid, Basingstoke, U.K.) with an over-layer, and incubated at 37°C for 24 h.

(d) Lactic acid bacteria: on DeMan, Rogosa and Sharpe agar (MRS; Oxoid, Basingstoke, U.K.), incubated at 30°C for 48 h.

(e) Pseudomonads: on *Pseudomonas* specific agar (Oxoid, Basingstoke, U.K.) supplemented with cetrimide, fucidin and cephaloridine (CFC supplement, Oxoid, Basingstoke, U.K.), incubated at 30°C for 48 h.

(f) Total psychrotrophic count: serially-diluted milk samples in sterile quarter-strength Ringer's solution (1 ml) were pour-plated in PCA with an over-layer, and incubated at 4°C for a period of 10 days. The quantity of psychrotrophic bacteria was monitored on days 0, 7, 14 and 21 only.

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

2.5 Statistical analysis

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

3. Results

Firstly, temperatures of milk before and after MTS and PEF treatments were noted during each experiment and the average calculated (n=3). For milk treated at 37ºC, milk entered the MTS chamber at 37ºC (± 0ºC) and left the MTS system at 36.5ºC (± 0.5º C). The milk then cooled to 25.7º C (± 0.5º C) before entering the PEF system, and the average temperature post-PEF processing was 32.1ºC (± 3ºC). In the case of milk treated at 55ºC, milk entered the MTS system at this temperature (± 0ºC), and left the MTS chamber at 57ºC (± 0.4ºC). The milk subsequently cooled, before entering the PEF system at a temperature of 40.5ºC (± 0.4º C). Temperatures post-PEF treatments were found to be on average 32.1ºC (± 5.1ºC).

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

429 **5. Conclusion**

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

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

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

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

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

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

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

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

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

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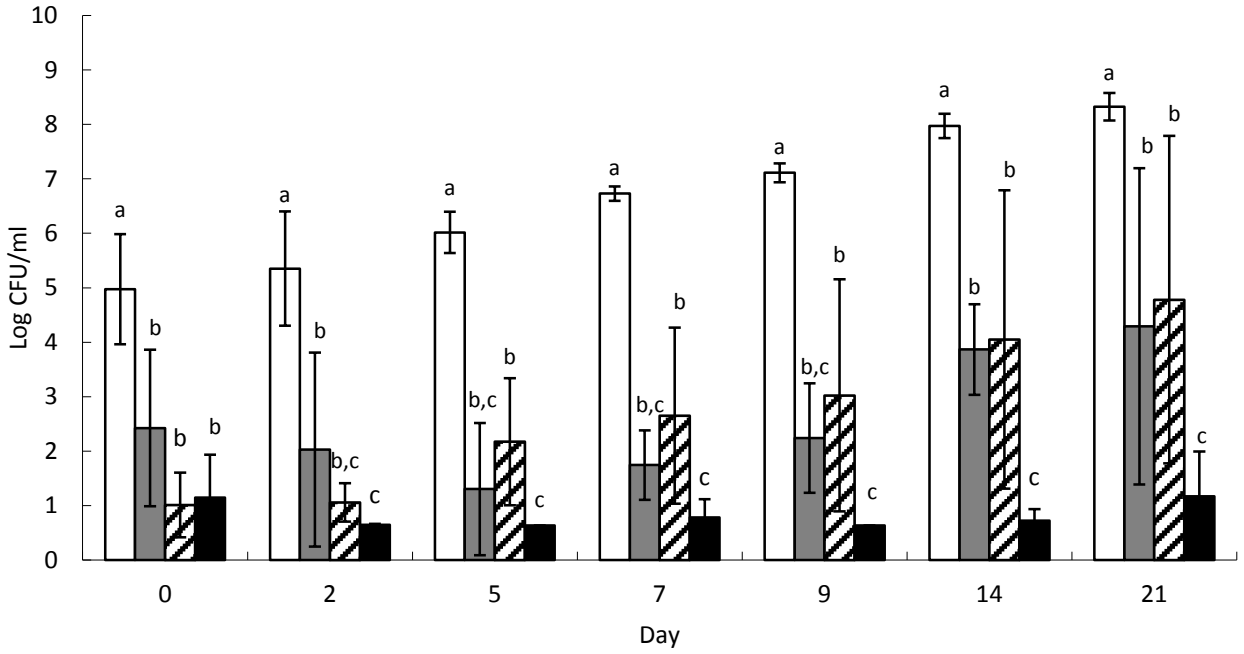

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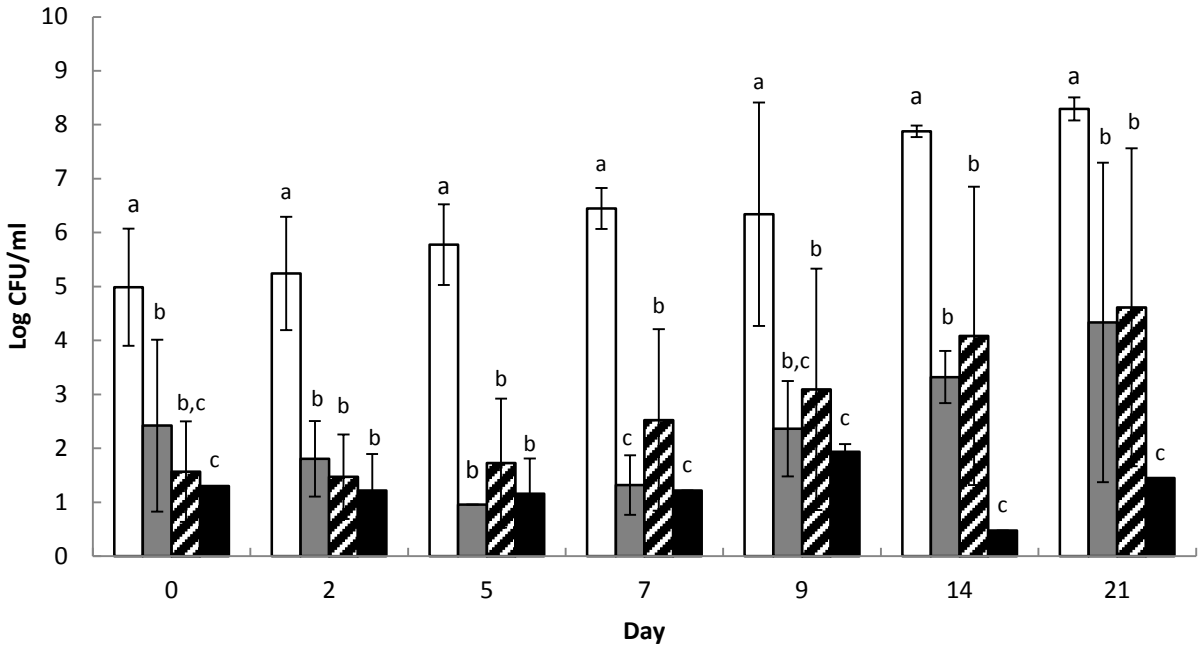

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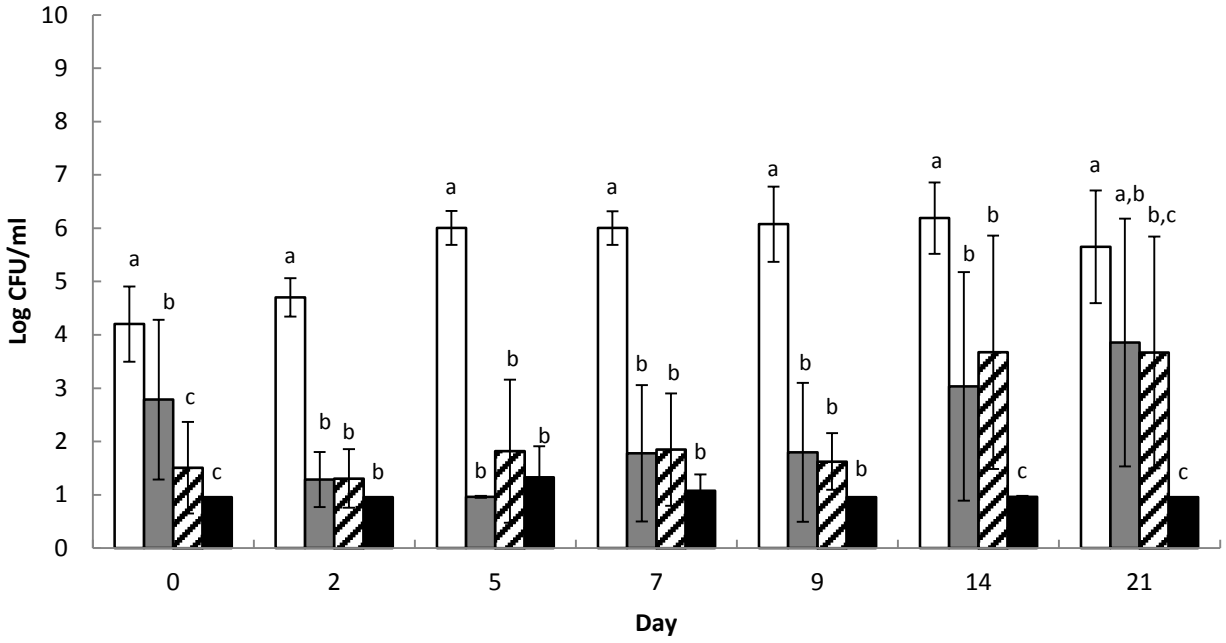

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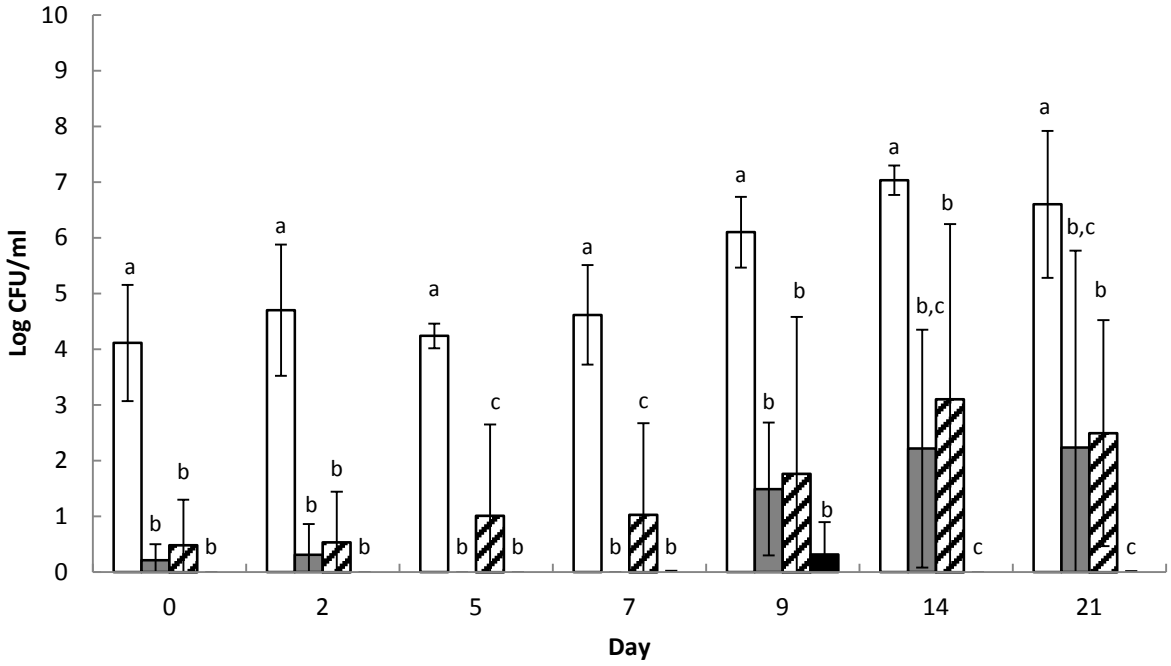

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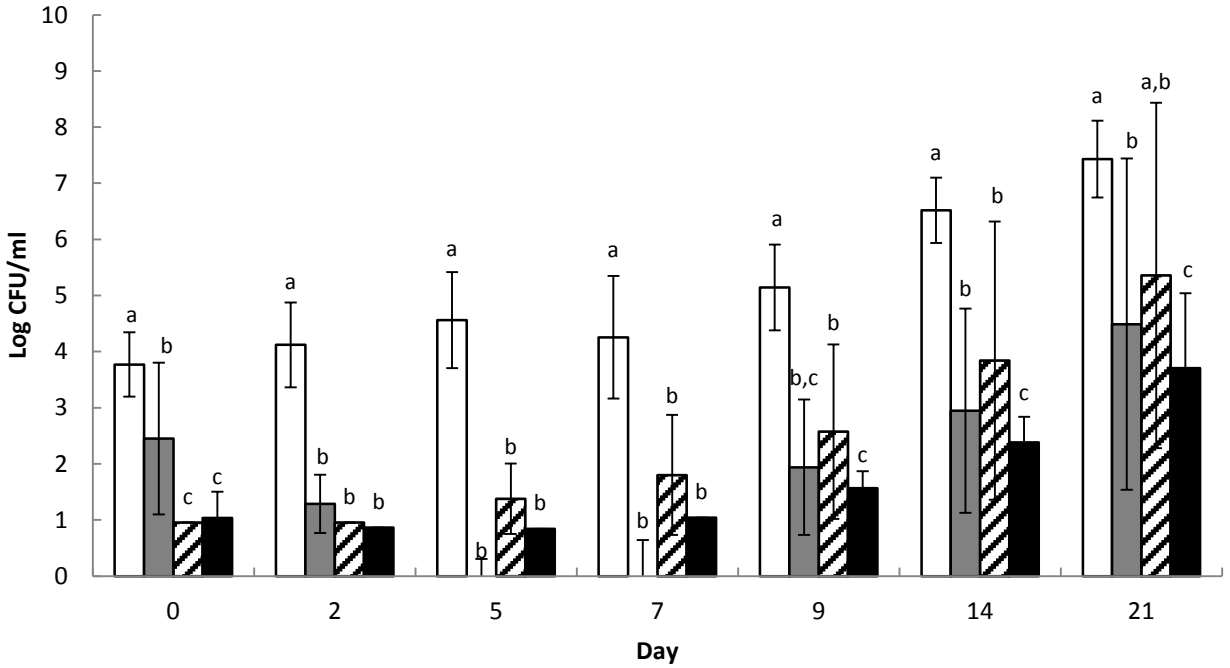

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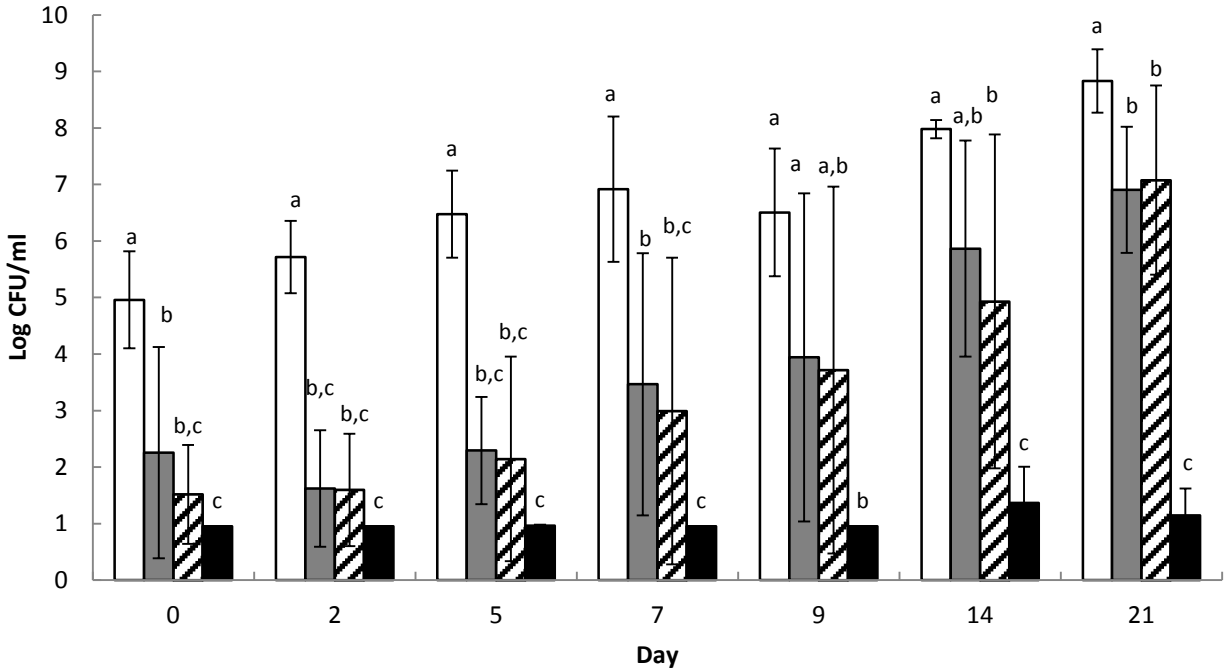

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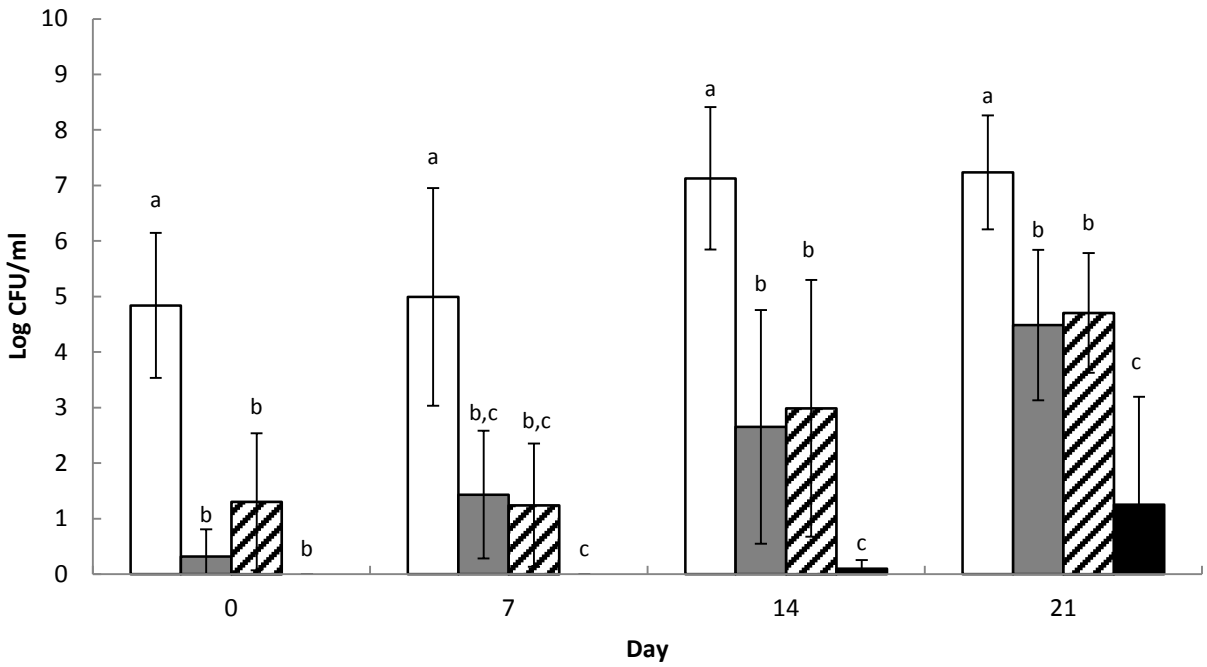

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