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GENERELLE ASPEKTE

Multi-pulsed high hydrostatic pressure treatment for inactivation and injury of *Escherichia coli*

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Abstract *Escherichia coli* cells in peptone water were pressurized at 300 MPa at ambient temperature with no holding time (pulse series) and with a total holding duration of 300 s for single- (300 s × 1 pulse) and multi-pulsed (150 s × 2 pulses, 100 s × 3 pulses, 75 s × 4 pulses, 60 s × 5 pulses, 50 s × 6 pulses and 30 s × 10 pulses) high hydrostatic pressure (HHP) treatments. Multi-pulsed HHP treatment with no holding time indicated that as the pulse number increased the number of inactivated and injured cells also increased. Holding time had significant effect on the inactivation of *E. coli*. There was low inactivation difference between single- and multi-pulsed HHP treatments with holding time. *Escherichia coli* cells showed at least 1.6 $\log_{10}$ more reduction on selective medium than the non-selective medium indicating that more than 95 % of the survivors severely injured for both single- and multi-pulsed treatments with holding time. Although the inactivation difference was low between single- and multi-pulsed HHP treatments, storage at 4 °C revealed that there was less recovery from injury for multi-pulsed HHP treatment.

Keywords High hydrostatic pressure · Multi-pulsed treatment · Inactivation · Injury · *Escherichia coli*

This paper is dedicated in memory of Mr. Pierre Tyndiuk, technician of High Pressure Laboratory in University of Bordeaux.

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1 Introduction

Non-thermal inactivation methods have been emerged to inactivate microorganisms in food products with minimal alteration to fresh sensory quality and nutrient content (Noma et al. 2004). Among these methods, high hydrostatic pressure (HHP) is perhaps the most popular one. Over the last 20 years the research about HHP has been intensively explored (Metrick et al. 1989; Styles et al. 1991; Cheftel 1992; Hayakawa et al. 1994; El Moueffak et al. 1995; Palou et al. 1997; Garcia-Graells et al. 1998; Linton et al. 2001; Tay et al. 2003; Buzrul and Alpas 2004; Avsaroglu et al. 2006; Buzrul et al. 2007; Buzrul 2009; Heinz and Buckow 2010; Erkan et al. 2010) and several commercial HHP-treated food products are available on the market shelves in different countries.

The use of very high pressures is not commercially suitable since the complexity and cost of HHP equipment rise more than linearly with maximum operating pressure. In order to make this process

economically feasible, the pressure magnitude needs to be reduced to a level sufficient to deliver satisfactory inactivation (Donsì et al. 2007).

There are several ways to lower the peak pressure necessary to inactivate the microorganisms in foods such as use of mild heat in combination with pressure (Alpas et al. 1998; Patterson and Kilpatrick 1998) and use of antimicrobial agents in combination with pressure (Ponce et al. 1998; Garcia-Graells et al. 1999). Another approach is the use of multi-pulsed HHP instead of single pulse pressure treatment (Rigaldie et al. 2007; Buzrul et al. 2008a). The superiority of multi-pulsed HHP treatment over single-pulsed one was demonstrated in several studies (Alemán et al. 1996; Palou et al. 1998; Fioretto et al. 2005).

Although the effectiveness of multi-pulsed HHP treatment on the inactivation of microorganisms has been widely studied, the investigation of the effect of multi-pulsed HHP on microbial injury has been relatively less well studied (a notable example is the work of Ponce et al. 1999). Therefore, the aim of this study was to examine the effect of multi-pulsed HHP on inactivation and injury of *E. coli* inoculated in peptone water.

2 Materials and methods

2.1 Preparation of bacterial cells

The microorganism used was *E. coli* ATCC 11775 (from E.R.A.P. laboratory, Périgueux, France). Previous studies had shown that this strain was relatively resistant to HHP treatment (Buzrul et al. 2008b). *Escherichia coli* cells were maintained on tryptic soy agar supplemented with 0.6 % yeast extract (TSAYE) (Merck, Darmstadt, Germany) slants. This strain was cultivated in tryptic soy broth supplemented with 0.6 % yeast extract (TSBYE) (Merck, Darmstadt, Germany) at 37 °C for 15–21 h and transferred to fresh broth every 48 h. *Escherichia coli* cells in their early stationary phase were inoculated in peptone water (pH = 7.0) to obtain about 10^7 colony forming units (CFU) mL^{-1}.

2.2 HHP treatment

The cell suspensions were aseptically transferred to sterile plastic vials (Nunc, Roskilde, Denmark) in 6 mL portions in duplicate. Air bubbles were avoided. Duplicate vials for each microorganism were vacuum sealed in sterile plastic bags (Fischer Scientific, PA, USA) and kept at 4 °C for up to 1 h prior to pressurization. Pressurization of samples was carried out using a computer controlled high pressure unit with 3 L sample compartment, capable of operating at up to 800 MPa and designed by NFM-Technologies (Le Creusot, France) and FRAMATOME (Paris, France), marketed by CLEXTRAL (Firminy, France). The pressure transmitting fluid was selected as ethylene glycol. Although the maximum compression and release rates were about 400 MPa/min, the come-up and pressure release rates were both set to 300 MPa/min due to safety considerations. Pressure and temperature were measured using sensors inside and outside the high pressure vessel and all data were stored in the computer system. *Escherichia coli* cells were pressurized in duplicate at 300 MPa at ambient temperature (1) without any holding time (compression to the target pressure followed by immediate decompression to atmospheric pressure) up to 10 pulses and (2) for a total holding duration of 300 s for both single- (300 s × 1 pulse) and multi-pulsed (150 s × 2 pulses, 100 s × 3 pulses, 75 s × 4 pulses, 60 s × 5 pulses, 50 s × 6 pulses and 30 s × 10 pulses) HHP treatments. The pressure holding time reported in this study did not include the process come-up or depressurization times. Temperature increases during pressurization due to adiabatic heat were predetermined using a K-type thermocouple. Compression heating during pressure was taken into consideration in the experiments so that temperature of the pressure transmitting fluid during HHP treatment was controlled near ambient temperature.

Immediately after pressure treatment, the vials were transferred to ice–water mixture. Untreated and pressurized cell suspensions from each vial were serially diluted in 0.1 % peptone water (Biokar, Beauvais, France). The non-selective and the selective agar media were TSAYE and Violet Red Bile Agar (Biokar, Beauvais, France), respectively. From the selected dilutions, 0.1 mL portions were surface plated in duplicate giving four plates (Sterilin, Staffordshire, UK) per dilution on prepoured non-selective and selective agar medium. With samples containing less than 25 CFU mL^{-1} in a 1:10 dilution, 1 mL of undiluted cell suspension was plated in three plates (0.3, 0.3 and 0.4 mL). The plates were incubated at 37 °C for 48–72 h and plates containing 25–250 CFU mL^{-1} were selected for enumeration. For each test, the survival ratio [$S(t) = N(t)/N_0$, where $N(t)$ and N_0 are the number of survivors after an exposure time t and initial number of microorganisms, respectively] and the level of inactivation [$\log_{10}S(t)$] were evaluated. All experiments were repeated once more and the averages were determined.

2.3 Storage of HHP-treated *E. coli* cells in peptone water

Samples of inoculated peptone water were pressurized in duplicate at 300 MPa at ambient temperature for both single- (300 s × 1 pulse) and multi-pulsed (60 s × 5 pulses and 30 s × 10 pulses) HHP treatments and held at 4, 20 and 37 °C for 3 days. At 0, 24, 48 and 72 h of storage, selected dilutions of each sample were surface plated on prepoured non-selective and selective medium. The total of four plates (two samples × two plates) were incubated at 37 °C for 48–72 h and plates containing 25–250 CFU mL^{-1} were selected for enumeration.

2.4 Statistical analyses of the data

Analysis of variance (ANOVA) as implemented in SPSS 10.0 for Windows (SPSS, Inc, Chicago, USA) was used to test effects of number of pulses on the logarithmic survival ratio. Tukey, Duncan and Student–Newman–Keuls post-hoc tests were used as paired comparisons between sample means. Level of significance was set to 0.05.

3 Results and discussion

3.1 Effect of multi-pulsed HHP treatment with no holding time

Effects of multi-pulsed HHP treatment on *E. coli* cells without any holding time i.e., compression to the target pressure followed by immediate decompression to atmospheric pressure, was investigated. Figure 1a shows the pressure and temperature profiles of these treatments. The initial temperature of the pressure transmitting fluid was about 12 °C and upon compression to target pressure (300 MPa) it reached ~24 °C as seen in Fig. 1a. The maximum temperature reached during the 10 pulses of HHP treatment was about 28 °C. Figure 1b shows the inactivation levels of *E. coli* on non-selective and selective media during the multi-pulsed HHP treatment without holding time. As the pulse number increased the number of inactivated and injured cells also increased and after two pulses, level of inactivation differences between non-selective and selective media became significant ($p < 0.05$). When 10 pulses of HHP with no holding time was applied inactivation of *E. coli* was slightly more than one $\log_{10}$. Moreover no additive effect of pulses was observed just like the results of Donsì et al. (2007).

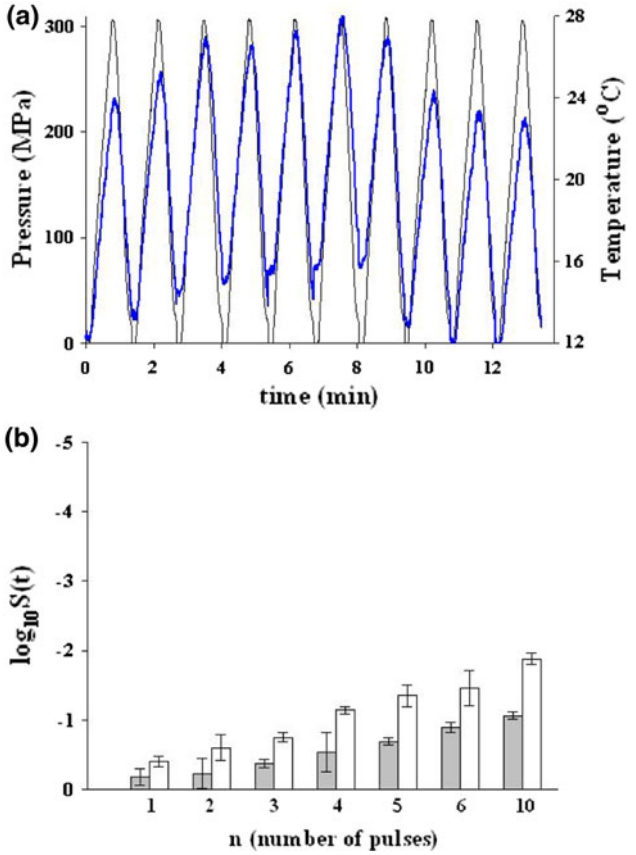

Fig. 1 **a** Pressure (*upper line*) and temperature (*lower line*) profiles of multi-pulsed HHP treatment (300 MPa) with no holding time (pulse number ranged from 1 to 10). **b** Levels of inactivation of *E. coli* in non-selective (*grey*) and selective (*white*) media. *Error bars* represent 95 % confidence intervals

Chapleau et al. (2006) demonstrated that compression and decompression rates had significant effect on the bacterial inactivation when the multi-pulsed HHP treatment with no holding time was applied. They observed that slower rates of compression and decompression caused higher reduction of *Salmonella* Typhimurium and *Listeria monocytogenes*. However, in this study constant compression and decompression rates were used.

3.2 Effect of the multi-pulsed HHP treatment with holding time

The effect of multi-pulsed HHP treatment up to ten pulses with a total holding time of 300 s was also studied. Figure 2a shows the pressure and temperature profile of 10 pulses of HHP treatment i.e., 300 MPa for 30 s × 10 pulses. The temperature of the pressure transmitting fluid stabilized at about 22 °C after the second pulse. Figure 2b shows the inactivation levels of *E. coli* during single- and multi-pulsed

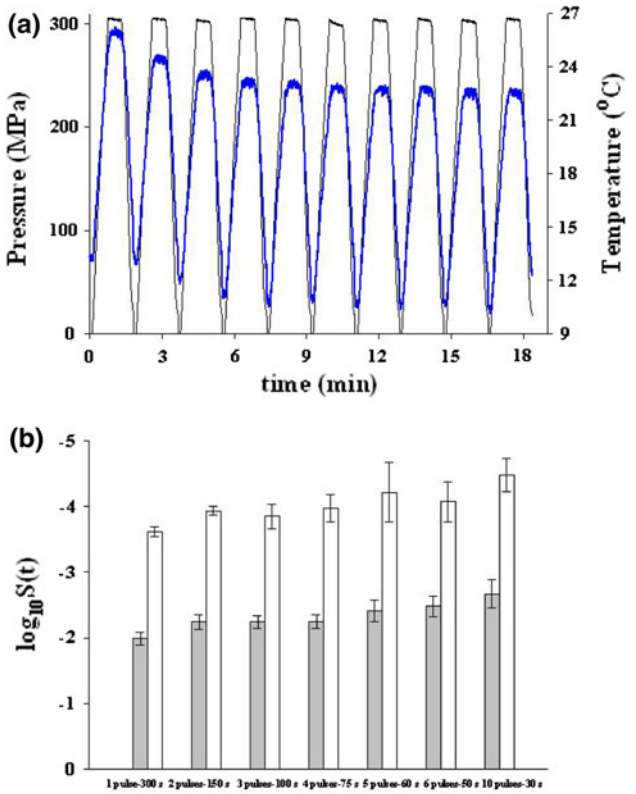

Fig. 2 **a** Pressure (*upper line*) and temperature (*lower line*) profiles of multi-pulsed HHP (300 MPa) treatment with holding time (30 s × 10 pulses). **b** Levels of inactivation of *E. coli* up to 10 pulses with holding time (total holding time = 300 s) in non-selective (*grey*) and selective (*white*) media. *Error bars* represent 95 % confidence intervals

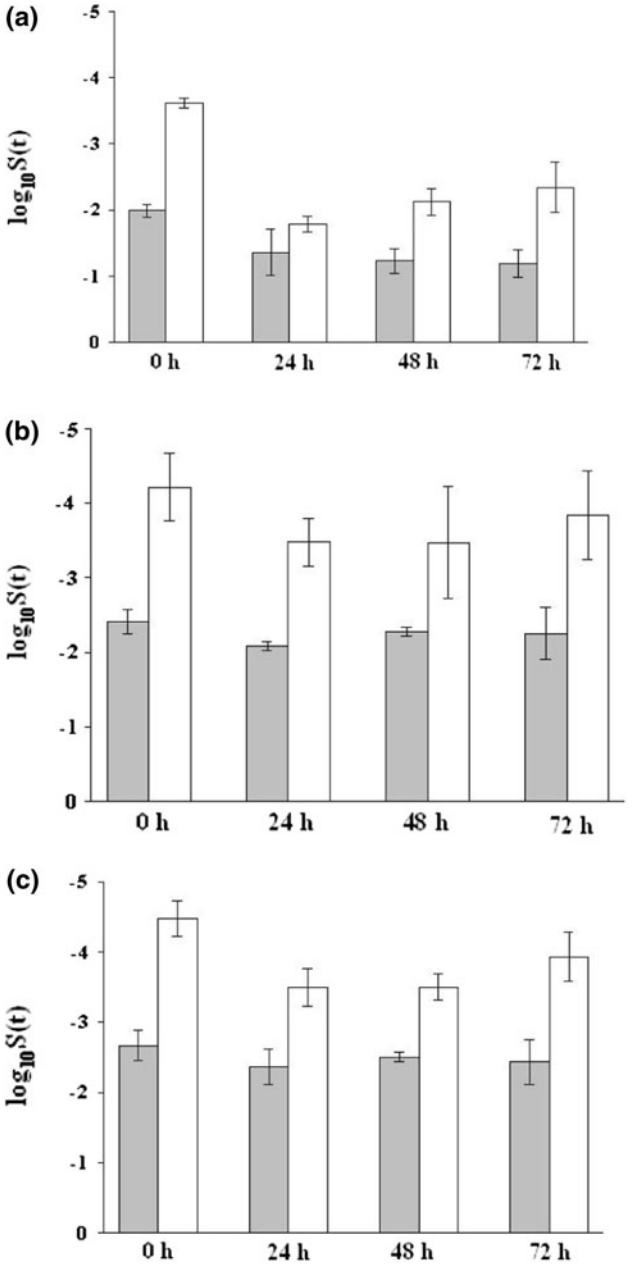

Fig. 3 **a** Inactivation levels of multi-pulsed HHP (300 MPa) treated *E. coli* in 0.1 % peptone water in non-selective (*grey*) and selective (*white*) media for 300 s × 1 pulse at 0 (just after HHP treatment), after 24, 48 and 72 h at 4 °C. *Error bars* represent 95 % confidence intervals. **b** Inactivation levels of multi-pulsed HHP (300 MPa) treated *E. coli* in 0.1 % peptone water in non-selective (*grey*) and selective (*white*) media for 60 s × 5 pulses at 0 (just after HHP treatment), after 24, 48 and 72 h at 4 °C. Error bars represent 95 % confidence intervals. **c** Inactivation levels of multi-pulsed HHP (300 MPa) treated *E. coli* in 0.1 % peptone water in non-selective (*grey*) and selective (*white*) media for 30 s × 10 pulses at 0 (just after HHP treatment), after 24, 48 and 72 h at 4 °C. *Error bars* represent 95 % confidence intervals

HHP treatment. Comparison of Figs. 1b and 2b shows an appreciable difference in the inactivation levels on *E. coli* with and without holding time treatments. Although inactivation differences between single- (300 s × 1 pulse) and ten-pulsed (30 s × 10 pulses) HHP treatments was significant ($p < 0.05$), the difference was only about 0.7 $\log_{10}$. *Escherichia coli* cells showed at least 1.6 $\log_{10}$ more reduction on selective medium than the non-selective medium indicating that more than 95 % of the survivors severely injured for all the treatments shown on Fig. 2b.

Chapleau et al. (2006) observed that higher compression and decompression rates in combination with holding time were more effective for the inactivation of *Salmonella* Typhimurium and *L. monocytogenes*. Donsì et al. (2007), however, found that lower compression rate was more effective for inactivation of *Saccharomyces cerevisiae* if several pulses (more than 3 pulses) were applied. Donsì et al.

(2007) also stated that the lethality of multi-pulsed HHP treatment depend on the combination of holding time and pulse number.

3.3 Effect of refrigerated storage on the recovery of *E. coli*

Figure 3 shows the inactivation levels of *E. coli* in peptone water for single- (300 s × 1 pulse) and multi-pulsed (60 s × 5 pulses and 30 s × 10 pulses) HHP treatments on both non-selective and selective media during storage at 4 °C after 0, 24, 48 and 72 h. Although the inactivation differences were low between single- and multi-pulsed HHP treatments (Fig. 2b), storage at 4 °C revealed that there was less recovery from injury for multi-pulsed treatments than the single-pulsed treatment.

Recovery was observed in 24 h when the single- (300 s × 1 pulse) and multi-pulsed (60 s × 5 pulses and 30 s × 10 pulses) HHP-treated samples were stored at 25 and 37 °C. Garcia-Graells et al. (1998) observed that HHP treatment caused sub-lethal injury to *E. coli* cells resulting in a reduced resistance to low pH which was also confirmed by Buzrul et al. (2008c). In literature, it was reported that refrigeration enhances survival of *E. coli* in acidic environments (Zhao and Doyle 1993; Miller and Kaspar 1994; Conner and Kotrola 1995). However, in this study most probably due to neutral pH of peptone water (pH = 7.0), storage at 25 and 37 °C favored the recovery of *E. coli* cells when compared with refrigeration temperature (4 °C).

4 Conclusions

Multi-pulsed HHP treatment with no holding time and with holding time indicated that holding time had significant effect on the inactivation of *E. coli*. Multi-pulsed HHP treatment with holding time revealed that more than 90 % of the survivors severely injured for both single- and multi-pulsed treatments with holding time. Although the inactivation difference was low between single- and multi-pulsed HHP treatments with holding time, storage at 4 °C revealed that there was less recovery from injury for multi-pulsed treatment.

This work has shown that multi-pulsed HHP treatment can be an alternative to extend the shelf life of refrigerated foods; however, more research should be performed in food matrices.

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