Pulsed pressure treatment for inactivation of *Escherichia coli* and *Listeria innocua* in whole milk

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Abstract. *E. coli* and *L. innocua* in whole milk were subjected to continuous pressure treatments (300, 350, 400, 450, 500, 550 and 600 MPa) at ambient temperature for 5, 10, 15 and 20 min. These treatments underlined that at moderate pressure values (300, 350 and 400 MPa), increasing the pressurization time from 5 to 20 min did not improve cell death to a great extent. Therefore, pulsed pressure treatments (at 300, 350 and 400 MPa) for 5 min (2.5 min × 2 pulses, 1 min × 5 pulses and 0.5 min × 10 pulses), 10 min (5 min × 2 pulses, 2 min × 5 pulses and 1 min × 10 pulses), 15 min (5 min × 3 pulses, 3 min × 5 pulses and 1.5 min × 10 pulses) and 20 min (10 min × 2 pulses, 5 min × 4 pulses, 4 min × 5 pulses and 2 min × 10 pulses) were applied. As already observed in continuous pressure experiments, in pulsed pressure treatments the inactivation level is improved with increasing pressure level and in addition with the number of applied pulses; however, the effect of pulse number is not additive. Results obtained in this study indicated that pulsed pressure treatments could be used to pasteurize the whole milk at lower pressure values than the continuous pressure treatments. Nevertheless, an optimization appears definitely necessary between the number of pulses and pressure levels to reach the desirable number of log-reduction of microorganisms.

1. Introduction

The application of high hydrostatic pressure (HHP) in food preservation generally ranges from 100 to 1000 MPa; however, when high pressure equipment are operated over a long time at more than 600 MPa pressurization, creep phenomenon can be seen and HHP equipment may be damaged [1]. Moreover, the cost of pressure equipment rises more than linearly with the maximum operating pressure, therefore it is wise to study the interaction of the variables (pressure, temperature, time etc.) in HHP processing and determine the minimum conditions to obtain desirable levels of microbial inactivation while maintaining a maximum degree of sensory and nutritional quality [2, 3].

HHP treatment can consist of a single exposure period (one pressure cycle; i.e., longer holding time) or the application of multi-pulsed pressure in shorter periods (equal total duration but shorter holding times for each pulse). The question that arises is whether one form is more effective than the other or whether they are equivalent. If differences between the outcomes of the two treatments are

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observed, are they results of differences in the inactivation mechanism, or just manifestation of small differences in the exposure time, caused by inevitable slight differences in the compression (come-up) and decompression (pressure release) times [4].

Many researchers tried to give some answers to these questions. López-Caballero et al. [5] demonstrated that step-pulsed pressurization (400 MPa at 7 °C in two 5-min pulses) produced no apparent advantages over continuous pressurization (400 MPa at 7 °C for 10 min) on the inactivation of microbial flora of the oysters. Aleman et al. [6] observed that step-pulsed pressurization is more effective than continuous pressurization in reducing the microbial load in pineapple. Studies on spores were also tried; Hayakawa et al. [1] achieved complete inactivation of 6 log initial load of Bacillus stearothermophilus spores by use of oscillatory pressure treatments (6 pulses of 5-min pressurization with 600 MPa at 70 °C). Rigaldie et al. [7, 8] have successfully applied pulsed HHP treatment for the inactivation of pharmaceupia strains including the spores of Bacillus subtilis.

The objective of this study was to observe the differences between continuous and pulsed pressure treatments for the inactivation of Escherichia coli and Listeria innocua in whole milk and further to explore the potential usability of pulsed pressure treatment.

2. Materials and methods

2.1. Preparation of bacterial species

The microorganisms used were E. coli ATCC 11775 and L. innocua ATCC 33090 (both from Périgueux, France - E.R.A.P laboratory). The strains were maintained on tryptic soy agar plus 0.6 % yeast extract (TSAYE) (Merck, Darmstadt, Germany) slants. For growth, a loopful of each organism was transferred to tubes of 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 for use in this study.

Ultrahigh-temperature (UHT) whole milk (pH 6.64) was obtained from a local market (Bordeaux, France). It was inoculated with pure cultures from the stationary phase to obtain about 10^7–10^8 colony forming units (CFU) mL^-1 milk. All samples were inoculated at least 2 h before treatment to allow cells to acclimatize to the new environment. One mL of un-inoculated milk was transferred onto TSAYE plates (Sterilin, Staffordshire, UK) and incubated at 37 °C for 48 h to make sure that the milk samples were sterile.

2.2. HHP treatment

The cell suspensions were dispensed in 6 mL-portions in sterile plastic vials (Nunc, Roskilde, Denmark) in duplicate. Air bubbles were avoided. The vials were vacuum sealed in sterile plastic bags (Fischer Scientific, PA) and kept at 4 °C prior to pressurization that did not exceed 1 h. 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 ethylene glycol. Although the maximum pressure increase and release rate of this equipment was 375 MPa/min, the pressure increase and pressure release time were both set to 300 MPa/min for 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. E. coli and L. innocua in whole milk were subjected to continuous pressure treatments (300, 350, 400, 450, 500, 550 and 600 MPa) at ambient temperature (about 22 °C) for 5, 10, 15 and 20 min. Pulsed pressure treatments were applied at 300, 350 and 400 MPa for a total duration of 5 min (2.5 min × 2 pulses, 1 min × 5 pulses, and 0.5 min × 10 pulses), 10 min (5 min × 2 pulses, 2 min × 5 pulses and 1 min × 10 pulses), 15 min (5 min × 3 pulses, 3 min × 5 pulses and 1.5 min × 10 pulses) and 20 min (10 min × 2 pulses, 5 min × 4 pulses, 4 min × 5 pulses and 2 min × 10 pulses). The pressure holding time 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 inside the
pressure vessel. Compression heating during pressure was taken into consideration in the experiment so that temperature of the pressure transmitting fluid during pressure was controlled at near ambient temperature (~ 22 °C) [the temperature increase of ethylene glycol (pressure transmitting fluid) and whole milk were about 4.0 °C/100 MPa and 3.4 °C/100 MPa, respectively]. Immediately after pressure treatment, the vials were transferred to ice-water mixture. Cells suspensions from each vial was serially diluted with 0.1 % peptone water (Biokar, Beauvais, France) and each dilution (from each sample) were surface plated in duplicate giving four plates per dilution on prepoured TSAYE. 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 72 to 96 h prior to counting colonies to allow injured cells to form visible colonies. The entire experiment was repeated once more and the averages were determined.

3. Results and discussion

3.1. Continuous HHP treatment

Figure 1 shows the continuous HHP treatments between 300 and 600 MPa for E. coli and L. innocua in whole milk. Results indicated that, at a constant moderate pressure (300, 350 and 400 MPa), increasing pressurization time from 5 to 20 min did not increase cell death to a great extent. Inactivation of both microorganisms were about one log₁₀ at 400 MPa for 20 min. Another observation was: as the pressure increases the inactivation increases. In fact, 7 log₁₀ reduction (complete inactivation) for both microorganisms is only possible at 600 MPa for 15 min and at 550 MPa at 20 min.

![Figure 1](image1.png)

**Figure 1.** Inactivation of E. coli (a) and L. innocua (b) in whole milk at 300 (●), 350 (○), 400 (▼), 450 (∇), 500 (■), 550 (□) and 600(♦) MPa for 5, 10, 15 and 20 min.

3.2. Effect of pressure level in pulsed HHP treatment for a constant duration and pulse number

Figure 2 shows 20 min pulsed HHP treatment (4 min × 5 pulses) at 300, 350 and 400 MPa. As already observed in continuous pressure treatment, in pulsed HHP treatment inactivation increases with increasing pressure level; however inactivation differences between pressure levels were more pronounced in pulsed HHP treatment. Moreover, 4 log₁₀ reduction is possible at 400 MPa for 4 min × 5 pulses while in continuous HHP treatment only one log₁₀ reduction was observed at 400 MPa for 20 min as mentioned before. Note that even at 300 MPa inactivation is more than one log₁₀ for both microorganisms.
3.3. Effect of pulse number for a duration constant of 5 min for each pulse

Figure 3 shows the pulsed HHP treatment at 400 MPa for 5 min × 1 pulse (continuous 5 min), 5 min × 2 pulses, 5 min × 3 pulses and 5 min × 4 pulses. Figure indicates as the pulse number increases inactivation is improved. Again a comparison can be made with continuous HHP treatment and can be observed that increasing time 5 min (5 min × 1 pulse) to 20 min (5 min × 4 pulses) increases cell death significantly ($P < 0.05$) in pulsed HHP treatment. It was also observed that the effect of pulse number is not additive.
3.4. Effect of pulse holding time
Choosing a total holding time of 20 min at 400 MPa, different combinations of pulse holding time and number of pulses were applied (20 min \(\times\) 1 pulse, 10 min \(\times\) 2 pulses, 5 min \(\times\) 4 pulses, 4 min \(\times\) 5 pulses and 2 min \(\times\) 10 pulses). Figure 4 shows the results of these treatments. As the number of pulses increases (up to 10 pulses) and the pulse holding time decreases inactivation increases. More than 4 log₁₀ reduction is possible at 400 MPa for 2 min \(\times\) 10 pulses for both \textit{E. coli} and \textit{L. innocua}. In the literature, Donsì \textit{et al.} \cite{9} observed that, for the inactivation of \textit{Saccharomyces cerevisiae} in orange juice, pulsed HHP treatment is more effective than isostatic (continuous) HHP treatment if the pulse holding time is longer than 60 sec.

![Figure 4.](image)

**Figure 4.** Inactivation of \textit{E. coli} and \textit{L. innocua} in whole milk at 400 MPa for 20 min \(\times\) 1 pulse (continuous 20 min), 10 min \(\times\) 2 pulses, 5 min \(\times\) 4 pulses, 4 min \(\times\) 5 pulses and 2 min \(\times\) 10 pulses.

4. Conclusions
Results obtained in this study indicated that pulsed HHP treatments could be used to pasteurize the whole milk at lower pressure values than the continuous pressure treatments. Note that, the total duration of a pulsed HHP treatment at 300 MPa for 2 min \(\times\) 10 pulses is 40 min (20 min of holding time + 10 min compression time + 10 min decompression time) which is too long for an industrial application. Therefore an optimization appears necessary between the number of pulses and pressure levels to reach the desirable number of log-reduction of microorganisms compatible with an industrial application.

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**Acknowledgments**

This research work was developed in the scope of a thesis in co-tutelle (S B) between the University Bordeaux 1 “Sciences and Technologies” and Middle East Technical University (METU). The French Embassy in Ankara, Turkey is deeply acknowledged for this support. Authors would like to thank Mr. Pierre Tyndiuk for his help during pressurization experiments.