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Inactivation of *Escherichia coli* K12 in phosphate buffer saline and orange juice by high hydrostatic pressure processing combined with freezing

Sami Bulut*[1,2] and Kimon A. G. Karatzas[1]

[1] Department of Food & Nutritional Sciences, School of Chemistry, Food & Pharmacy, University of Reading, Reading, RG6 6AD, UK

[2]* Corresponding author's current address: Trakya University, Faculty of Engineering, Department of Food Engineering, 22030, Edirne, TURKEY, e-mail: samibulut@trakya.edu.tr

Abstract

Synergistic action of high hydrostatic pressure (HHP) and freezing on inactivation of *Escherichia coli* K12 in phosphate buffered saline (PBS) was investigated by employing response surface methodology. Samples containing *E. coli* K12 were stored at 4, −24 and −80 °C overnight before they were pressurized. A maximum of 1.83 log reduction of CFU·ml^{-1} was obtained following a 9-min treatment at 400 MPa and 4±1 °C in samples stored at 4 °C whereas, 5.63 and 6.83 log reductions were obtained in samples frozen at −24 and −80 °C, respectively. Major disruption of *E. coli* cells observed by scanning electron microscopy and increased amounts of DNA and RNA measured in pressure treated frozen PBS samples indicated that the main mechanism of inactivation in frozen samples was due to cell rapture. The validity of enhanced microbial inactivation by freezing before HHP for a real food system was tested by using orange juice. Pressurization (250 MPa, 15 min) of frozen (−80 °C) orange juice resulted in 4.88, 4.15 and 4.61 log CFU·ml^{-1} reductions in number of *E. coli* for the samples having pH 3.2, 4.5 and 5.8, respectively. In the absence of freezing, the same treatment caused only up to 0.42 log reduction in samples having pH 4.5 and 5.8.

Keywords: High hydrostatic pressure processing, *Escherichia coli,* cell rapture, frozen state, orange juice.

1. INTRODUCTION

High hydrostatic pressure processing (HHP) is a non-thermal method for inactivating microorganisms in food matrices and other biomaterials (Luscher, Balasa, Fröhling, Ananta, & Knorr, 2004) that are sensitive to heat. As one of the most promising non-thermal processing technologies, HHP is already used in the food industry around the world. However, the usage of this method is limited due to high equipment cost. Therefore, the scientific community has been focusing on reducing the intensity of processing by minimising pressures or times. This is normally achieved through the use of hurdle technology by combining other stresses with HHP aiming to reduce operational costs (Huang, Wu, Lu, Shyu, & Wang, 2017).

In recent years, few studies have focused on a possible synergistic action of HHP and freezing on microbial inactivation when foods or model food systems are frozen prior to pressurization at ambient or subzero temperatures. Luscher, et al. (2004) achieved reductions of a 2.5 and 2.9 log cycles of CFU·ml^{-1} for *Listeria innocua* BGA 3532 at 400 MPa and subzero temperatures (PBS, pH 7.0) with zero holding times at −45 °C. The high rate of microbial inactivation was explained by the mechanical stress associated with the phase transition of ice I to other ice polymorphs (ice II or ice III) during pressurization.

Working with *Bacillus subtilis* vegetative cells in ACES (pH 8.5), Shen, Urrutia Benet, Brul, and Knorr (2005) showed that a 20 s treatment at 250 and 350 MPa at −25 °C on frozen cell suspensions resulted in more than 4 log reduction of CFU·ml^{-1}. The authors suggested that solid–solid phase transitions were mainly responsible for the observed drop in cell viability.

Bulut (2014b) showed that a 5 min pressure treatment at 300 MPa and 0 °C resulted in 1.8 log cycles of CFU·ml^{-1} reduction in numbers of *E. coli* strain ATCC 25922 in milk. Freezing the samples at -21°C overnight prior to pressurization under the same conditions increased the log reduction to 5.2 log CFU·ml^{-1}. Another study conducted on minced meat inoculated with *E. coli* strain ATCC 25922 resulted in 3.0 log reduction after a 5 min pressure treatment of frozen samples (at −21 °C) at 300 MPa and −5 °C, whereas only a reduction of 1.5 log unit was obtained in pressure treated unfrozen samples (Bulut, 2014).

Su, Yu, Ramaswamy, Hu, Xu, and Zhu (2014) investigated HHP death kinetics of *Escherichia coli* strain ATCC 25922 in frozen and unfrozen nutrient broth. Pressure treatment at 400 MPa with zero holding time or a pressure treatment at 350 MPa for 1 min inactivated *E. coli* strain ATCC 25922 in frozen (−20 °C) nutrient broth by more than 6 log CFU·ml^{-1} cycles, whereas the inactivation in unfrozen nutrient broth was less than 1.0 log of CFU·ml^{-1}. The same research group, (Zhu, Wang, Ramaswamy,

& Yu, 2017) studied the effect of HHP on phase transition of frozen carrot juice and the inactivation kinetics of *E. coli* ATCC 25922. D values of *E. coli* in frozen carrot juice (at −20 °C) reported to be between 2.62 and 2.12 min in the range of 300-400 MPa, whereas unfrozen carrot juice had significantly higher D values of 28.5 and 5.32 min for the same pressure range. The authors concluded that the combination of frozen state, phase transition status and pressure level were likely to be responsible for the higher inactivation of *E. coli* in frozen carrot juice.

Shiba and Furushita (2017) created a thermal buffer zone around the plastic bags of bacterial suspensions in 1% skim milk by using two polytetrafluoroethylene plates. The bacterial suspensions held between the polytetrafluoroethylene plates were brought to −50 °C before a 20 min pressure treatment at 200 MPa and 4 °C, which resulted in a reduction of 8 log cycles of *E. coli* strain ATCC 10798. The authors suggested that a homogeneous temperature within the sample achieved by an initial temperature that was low enough to keep the sample temperature below the melting point during compression, and the completion of melting within the holding time were necessary to take the advantage of combined subzero temperature and high pressure during pressure-assisted thawing.

Ekonomou, Bulut, Karatzas, and Boziaris (2020) investigated the possibility of using a very low pressure in combination with liquid smoke and freezing to eliminate *Listeria monocytogenes* 10403S in trout samples. Treatment with liquid smoke followed by freezing prior to pressurization at 200 MPa for 15 min reduced the number of *L. monocytogenes* by more than 5-log CFU·ml^{-1}.

From the limited number of studies as summarized above, it is clear that microorganisms could be efficiently inactivated at relatively low pressures when the food material is frozen prior to pressure a treatment which could take place at subzero or above zero temperatures. The significant antimicrobial effect of this type of treatment should be further studied as it is able to easily eliminate spoilage and pathogenic organisms from foods while offering major advantages to the food industry. Subzero HHP processing is relatively new field and although there are some explanations for the increased microbial inactivation, there is no a complete understanding of the mode of action. A better understanding of the mechanism behind this could be utilized for reduction of the treatment intensity, which in turn could significantly reduce the cost of HHP equipment manufacturing and could lead to wider adoption of the technology by the food industry.

Escherichia coli is a rod-shaped Gram-negative facultative anaerobe bacterium that normally resides in the intestine of humans and other animals. A few strains are pathogenic some of which cause genitourinary infections and some are responsible for traveler's diarrhea (Neidhardt & Kushner, 2017).

Various strains of *E. coli* have been widely used as a test organisms in life sciences including in some of the studies on nonthermal technologies for fruit and vegetable juices (Bevilacqua, et al., 2018). Among the pathogenic strains, *E. coli* O157:H7 received a great deal of attention due to its link to food borne illness outbreaks.

FDA guideline for the fruit juice industry (FDA, 2004) states that a 5-log reduction must be targeted for the pertinent pathogen which is the most resistant microorganism of public health concern that may occur in the fruit juice. FDA also states that the pertinent pathogen may vary with the type of juice and the type of treatment used, though typically it could be *Salmonella* or *Escherichia coli* O157:H7 (FDA, 2001). FDA does not have a definitive suggestion as a test organism for HHP processing however, *E. coli* O157:H7 is the main microorganism of concern that should be studied for process development, challenge work and process validation (Institute of Food Technologists, 2000). However, it was shown that *E. coli* O157:H7 (ATCC 700728) in orange juice was more sensitive to pressure compared to *E. coli* K12 (Torres, González-M, Klotz, & Rodrigo, 2016), suggesting that *E. coli* K12 could be a more suitable strain for HHP studies.

In this study, we used *E. coli* K12 as a test organism. Samples were prepared by inoculation of *E. coli* K12 in phosphate buffered saline (PBS, pH 7.1) and then they were pressurised at either 4 °C (unfrozen), or at −24 °C and −80 ° C (frozen). Using PBS as a model liquid let us to investigate the bacterial morphology by scanning electron microscopy and determination of the amount of nucleic acids in PBS by NanoDrop spectroscopy, which helped us to better understand the mechanism of bacterial inactivation by HHP under frozen state. In order to test the effectiveness of HHP in frozen state in a real liquid food with different pH, orange juice was inoculated with the same strain of *E. coli* and pressurized in frozen and unfrozen state.

2. MATERIALS AND METHODS

2.1. Bacterial strain and growth conditions

A pure culture of *E. coli* strain K12 was obtained from the culture collection of the Department of Food and Nutritional Sciences, University of Reading. The stock culture was kept at −80°C in 15% (v/v) glycerol and prior to experiments a sample was transferred onto LB Agar (Lab M limited, UK) agar plates which were incubated at 37 °C overnight. The inoculum was prepared by transferring three isolated colonies from the latter plates into 200 ml sterile LB Broth (Lab M limited, UK) in 500 ml sterile flasks and incubated at 37 °C for 18 h to give approximately 10^9 CFU·ml^{-1} at stationary phase. Subsequently, cells were harvested by centrifugation, (7000 × g for 5 min) and washed twice

in sterile phosphate buffered saline solution (PBS, Oxoid, Basingstoke, UK) before they were resuspended in sterile PBS (pH 7.1) at a concentration of approximately 10^9 CFU·ml^{-1} (stock suspension).

2.2. Preparation of inoculated PBS samples

By using a heat sealer, small plastic bags were prepared out of sterile stomacher bags. Subsequently, 12 ± 1 ml of *E. coli* strain K12 stock suspension were transferred with a sterile syringe in the bags which were sealed and placed into a larger sterile stomacher bags by using a vacuum sealer to prevent contamination of the pressurization medium in case of rupture during pressurization. Samples were then stored under 3 different conditions overnight as follows: i) at 4°C in a laboratory refrigerator (Labocold, Chineham, UK), ii) at −24°C in a laboratory freezer (Labcold, Chineham, UK) and iii) at −80°C in a laboratory freezer (New Brunswick U570HEF, UK).

2.3. Preparation of orange juice samples

In order to compare the inactivation of *E. coli* K12 in PBS with a real food system, experiments were conducted in orange juice inoculated with *E. coli* K12. For this purpose, oranges bought from a local supermarket were sprayed with %70 (v/v) alcohol and then wiped with paper towel after 1 min. Then, oranges were cut in half with a sterile knife by hand using gloves sanitized with %70 (v/v). Half oranges were squeezed manually and filtered through a sterilised sieve (315 µm aperture) into a sterile bowl. Samples of orange juice (3 × 5 ml) were taken for microbiological analysis before it was inoculated with *E. coli* K12 at a level of about 10^9 CFU·ml^{-1}. For inoculation of orange juice, stock suspension of *E. coli* K12 was centrifuged at 7000 × g and the supernatant was discarded to eliminate the buffering potential of PBS. About 30 ml orange juice was added into a centrifuge tube and then vortexed for 1 min to disperse *E. coli* cells in the orange juice. The tubes were then poured into a bowl holding orange juice and mixed for 1 min with a sterile spatula. After inoculation, the pH of orange juice was adjusted to 3.2, 4.5 and 5.8 by either adding sterile citric acid (0.2 M) or sodium hydroxide (1.0 M) solution. The orange juice was then packed into sterile stomacher bags (12±1 ml) by using a heat sealer. The samples were then packed for the second time by using a vacuum sealer. Half of the samples were kept in a walk-in fridge operating at 4±1°C and the rest were kept at -80 °C in a freezer.

2.4. HHP treatment

A HHP system (Stansted Fluid Power Products Ltd., Harlow, UK) having a 16 mm internal diameter and 55 ml working volume was used to treat the samples. Alcohol (80% v/v) mixed with castor oil (20% v/v)

was used as the pressure transmitting medium (PTM) and the temperature of the medium inside the pressure chamber was controlled with a water jacket surrounding the pressure vessel connected to a cooling circulator (Grant LTD6G, UK). All pressurization experiments were carried out at 4 ± 1 °C, while temperature was measured by a K-type thermocouple mounted to the head of the machine and being in contact with the pressurization liquid to monitor the internal temperature in the pressure chamber. Samples were quickly placed in the pressure chamber which was previously brought to test temperature (4 ± 1 °C). The time to remove the samples from the fridge or freezer and the start of pressurization was less than 30 s. After a pre-set pressurization time, the pressure was automatically released in less than 5 s to atmospheric pressure. The temperature of PTM was recorded by a data logger during the experiments. After pressure treatment, all the samples were taken to a refrigerator operating at 4 ± 1 °C until the time of analysis on the same day.

2.5. Enumeration of microorganisms

By using sterile PBS (pH 7.1), dilutions were prepared from control and pressure-treated samples. Dilutions were plated on LB agar by the spot plate method in duplicate and the average values from two counts were used for calculations. The plates were then incubated at 37 °C for 24 h for control samples and 48 h for pressurized samples before counting the colonies. Microbial reduction was expressed in terms of logarithmic reduction corresponding to the difference between the logarithms of the initial number of microorganism before pressure treatment and that of the number of microorganisms surviving after pressure treatment.

2.6. Scanning Electron Microscopy (SEM) imaging

Suspensions (200 µl) from each sample were pipetted onto poly-L-lysine coated glass coverslips for 15 min. The adhered bacteria were fixed with a solution of 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.0) for 15 min and subsequently washed with sterile distilled water. The samples were then dehydrated through a series of ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%) prepared with deionised water. Then the samples were dried in a critical point dryer (Balzers 030 Critical Point Dryer) and coated with a thin layer of gold (Edwards S150B Sputter Coater). The scanning electron micrographs were obtained using a FEI Quanta 600 FEG SEM equipped with the xTm user interface (version 2.4).

2.7. Determination of DNA and RNA concentration in PBS

A NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, ND-1000) controlled by ND-1000 v.3.8.1 software was used for determination of DNA and RNA concentration in PBS. For this purpose,

following pressure treatment, bacterial suspensions were centrifuged at 13 000 rpm (Thermo Fisher Scientific, Pico 17) for 10 min at room temperature. The supernatant was separated and 2 µl was used for the determination of DNA and RNA spectrophotometrically at 260 nm as described by (Desjardins & Conklin, 2010). Unprocessed samples that were kept at 4 °C and –80 °C overnight were used as controls. Frozen samples were thawed before measurements. Three readings were taken from each of the samples and the averages of the readings were used for plotting the graph.

2.8. Statistical analysis

The surface response experimental was designed and analysed by employing Design-Expert v.10 Software (Stat-Ease Inc. Minneapolis, USA). For experiments with PBS inoculated with *E. coli* K12, a rotatable central composite design (α=1.4142) with 3 replicates of central point was used, where variable parameters were pressure (159 - 441 MPa) and time (1-11 min). The experimental design with randomized run order and the resulting response variables are given in Table 2. Standard errors were determined by repletion (n=3) of the central point of the design (300 MPa, 5 min). All data were subjected to Analysis of Variance (ANOVA). Sequential model sum of squares and lack of fit tests were conducted for determination of best model (linear, quadratic or two factor interaction) for each response variable. Level of significance was set for $p<.05$ and the significance of each response variable was assessed by F-test.

For the experiments with orange juice inoculated with *E. coli*, a factorial design was chosen where 3 levels of pH (3.2, 4.5, 5.8) and 3 levels of time (5, 10, 15 min) were tested at 250 MPa with 3 replicates. Data were plotted by OriginPro v. 2019 (OriginLab Corporation, Northampton, MA, USA). Microsoft® Excel® 2016 (Microsoft Corporation, USA) was used for calculation of standard deviations (StDev).

2.9. Validations

Based on the models produced by RSM, the optimization function of Design-Expert v.11 Software predicted that a 9 min pressure treatment of frozen PBS samples (–24 °C or –80 °C) at 180 or 250 MPa could result in more than 5 log reduction in number of *E. coli* K12. Therefore, validations were carried out at 180 and 250 MPa as the aim of the study was to use the lowest possible pressures that could result in about 5 log reduction in number of *E. coli* K12.

3. RESULTS AND DISCUSSIONS

3.1. Inactivation of *E. coli* K12 in PBS and mechanism of inactivation

A limited number of studies as well as our work presented here show that microbial inactivation could be significantly increased when pressure is applied on a frozen material. However, lack of evidence on mechanism behind such an increased inactivation hinders better exploitation of this technology. Therefore, in the first part of this study we focused on understanding the mechanisms behind enhanced microbial inactivation in HHP treated frozen PBS.

3.1.1. Effect of freezing on *E. coli* K12 in PBS

Freezing on its own can inflict damage on microbial cells. Therefore, it was necessary to understand the effect of freezing and thawing on *E. coli* K12 under atmospheric conditions. Our results showed that freezing at −24 °C and −80 °C overnight followed by thawing at room temperature resulted in 1.73 ± 0.12 and 1.49 ± 0.07 log reduction, respectively. Freezing is known to cause inactivation of microorganisms and the extent of the damage to bacterial cells depends on mainly the food matrix and the freezing speed (temperature). Damage to microbial cell due to atmospheric freezing could be due to ice crystal formation and growth within the cytoplasm that could result in a decrease in pH (due to freeze concentration), which eventually could cause protein (enzyme) denaturation and death of microbial cells. It was shown that slow freezing caused more death of *E. coli* cells compared to rapid freezing (Souzu, 1980).

3.1.2. Temperature profile during pressurization

Temperature of PTM was measured during the pressurization. As seen in Figure 1, in a typical experiment the temperature of the pressure chamber measured through PTM was 4.5 °C before the insertion of a frozen sample. The temperature of PTM dropped to about 1.4 °C, before it started to increase by the onset of pressurization. At around 315 MPa, there was about 55 MPa pressure drop accompanied by a 2.5 °C temperature drop. This pressure drop was systematic but the magnitude of it varied depending on the temperature of the samples (–24 °C or –80 °C) and the set maximum pressure. The drop was instantaneous and took place during the pressure build up period for the experiments that employed pressures above 300 MPa. For the experiments that was carried out at lower pressures, the magnitude of the pressure drop was less and took place during the pressure holding period.

In a typical experiment, the temperature of the PTM gradually approached to about 3 °C after 10 min of pressurization. Upon releasing the pressure, the temperature instantaneously dropped to −10.6 °C due to expansion. The samples were removed from the pressure chamber within 10 seconds of pressure release. The samples removed from the pressure chamber were either melted or partially frozen, depending on the initial temperature of the samples (−24 or −80 °C), intensity of pressure and pressurization time. As a general observation, for pressure treatments that were less than 5 min, the samples were not completely melted. For higher pressure intensities and/or longer treatment times the samples were in liquid state when they were removed from the pressure chamber.

3.1.3. Effect of pressure and pressurization time on microbial inactivation

In order to understand the impact of pressure and pressurization time on the inactivation of *E. coli* in PBS, we constructed a response surface for log reductions of *E. coli a*s a function of pressure and pressurization time for the samples that were kept in a refrigerator overnight (4 °C) , frozen at −24 °C and −80 °C for overnight (Figure 2).

Bacterial inactivation increased to a maximum of 1.83 log CFU·ml^{-1} at the most severe pressure conditions (400 MPa, 9 min) for the samples that were kept in a refrigerator at 4 °C (Figure 2a). Log reduction CFU·ml^{-1} was strongly correlated with pressure (p=.0011), time (p<.0001) and interaction of pressure and time (p=.0198). The response surface (model r^2=0.94, p=.002) produced by two factor interaction model had the following equation of surface:

$$Log\ reduction\ (CFU{\cdot}ml^{-1}) = 0.022 + 1.62 \times 10^{-5} \times P - 0.068 \times t + 6.356 \times 10^{-4} \times P \times t \quad \textbf{(Eq.1)}$$

Where, P is pressure in MPa and t is time in min.

Overnight freezing at −24 °C and −80°C prior to pressurization enhanced bacterial inactivation significantly as seen in Figure 2b and Figure 2c. For frozen samples the time and pressure interaction terms were not significant (p>.05) and the response surfaces were produced by linear models. Response surface for the samples frozen at −24 °C (model r^2=0.93, p<.0001) produced the following equation where pressure (p=.0089) and time (p<.0001) were the significant terms.

$$Log\ reduction\ (CFU{\cdot}ml^{-1}) = 1.346 + 4.37 \times 10^{-3} \times P + 0.309 \times t \quad \textbf{(Eq.2)}$$

The linear response surface for the samples frozen at −80°C (model r^2=0.93, p<.0001) produced the following equation where pressure (p=.0005) and time (p<.0001) were the significant terms.

$$Log\ reduction\ (cfu{\cdot}ml^{-1}) = 0.861 + 8.55 \times 10^{-3} \times P + 0.334 \times t \quad \textbf{(Eq.3)}$$

Freezing PBS suspension of *E. coli* K12 prior to HHP treatment increased microbial inactivation remarkably. For example, after a 9 min pressure treatment at 400 MPa and 4 °C, a 6.83 log reduction of CFU·ml^{-1} was obtained in frozen PBS suspension (−80 °C) while, only 1.83 the log reduction of CFU·ml^{-1} was observed in unfrozen PBS suspension (4 °C). More interestingly, significant levels of inactivation were observed in frozen PBS samples even after a very brief exposure to pressure. After a 1 s pressurization at 300 MPa, the log reductions in CFU·ml^{-1} of *E. coli* were 2.60 and 3.52 for the samples frozen at −24 and −80 °C, respectively. On the other hand, inactivation in unfrozen samples (4 °C) was insignificant (0.01 log CFU·ml^{-1}). Similar observations were reported previously by Su, et al. (2014) who showed more than 6 log reduction in CFU·ml^{-1} for *E. coli* ATCC 25922 in frozen nutrient broth after 1 min at 350 MPa and room temperature. In previous work (Bulut, 2014b) we reported a 3.8 log reduction in CFU·ml^{-1} of *E. coli* ATCC 25922 in frozen milk after 1 min exposure to 300 MPa at −3 °C.

Increased microbial inactivation as a result of freezing the samples prior to pressure treatment could be explained mainly by the mechanical stress associated with the solid–solid phase transitions of ice I to other ice polymorphs (Luscher, et al., 2004). As the density of ice-I (0.92 g·cm^{-3}) is lower than the density of Ice-II (1.16 g·cm^{-3}), ice-III (1.16 g·cm^{-3}) and ice-V (1.24 g·cm^{-3}), a phase change from ice-I to other forms of ice results in a volume decrease (Chaplin, 2018). In our experiments we observed this volume decrease at about 315 MPa where a sudden pressure drop of about 55 MPa took place accompanied by a 4-5 °C temperature drop (Figure 1) as a result of the endothermic nature of transitions from ice-I to other forms of ice.

The temperature drop (to about −12 to −4 °C) upon release of the pressure could cause pressure shift freezing, which could contribute to increased bacterial inactivation as suggested by some authors (Alizadeh, Chapleau, de Lamballerie, & Le-Bail, 2007; Picart, Dumay, Guiraud, & Cheftel, 2004; Picart, Dumay, Guiraud, & Cheftel, 2005; Volkert, Ananta, Luscher, & Knorr, 2008). In our experiments, only the frozen samples that were exposed to moderate pressures (100-300 MPa) for short periods of time (<5 min) were partially frozen by the end of the pressure cycle, which infers that either the samples were not melted thoroughly during the pressure cycle and/or pressure shift freezing took place. There was no evidence of pressure shift freezing in pressure treated unfrozen samples (4 °C) when they were removed from the pressure chamber right after the pressure release. This was probably due to a very short time exposure (less than 10 s) to low temperatures (−8 to -4 °C) after releasing of the pressure.

Our results show that when samples were frozen at −80 °C before the pressurization, a higher microbial inactivation was observed compared to samples that were frozen at −24 °C (Figure 2). This could be explained by increased rigidity of bacterial cell wall at −80 °C and higher number of solid to solid phase transitions taking place as per the water phase diagram under pressure (Chaplin, 2018; LeBail, Chevalier, Mussa, & Ghoul, 2002; Luscher, et al., 2004; Zhu, et al., 2017).

3.1.4. Validation of models obtained by response surface methodology

As shown in Table 1, the validation experiments showed that the predicted values by the models presented by Eq. 1-3 were in reasonable agreement with the actual results.

3.1.5. Effect of freezing and pressure on morphology of *E. coli* K12 cells

The freeze damage that caused inactivation of *E. coli* K12 as detected by microbiological counts was visible in SEM images. As seen in Figure 3b and 3c, thawing after freezing at −24 °C and -80 °C resulted in bacterial morphology changes which could be explained by the effects of freezing and thawing.

Major disintegration of *E. coli* cell wall structure was observed in samples that were frozen before the pressurization (Figure 3e and 3f), whereas there were no signs of damage to bacterial cell wall in unfrozen samples pressurized under the same conditions (Figure 3d). Zhou, Zhang, Wang, Dong, Hu, and Zhang (2019) also did not observe cell disintegration or a significant change in morphology of *Escherichia coli* O157:H7 in physiological saline after a 450 MPa pressure treatment at room temperature. Therefore, under normal conditions, cell membrane damage is not a major contributor in the bacterial inactivation process as suggested by Rivalain, Roquain, and Demazeau (2010). On the contrary, cell membrane damage appears to be the major cause of bacterial inactivation in pressurized frozen samples. To the best of our knowledge this is the first time such clear SEM micrographs show the extent of damage to bacterial cell walls in frozen samples after HHP.

3.1.6. DNA and RNA leakage from *E. coli* K12 cells after pressure treatments

As seen in SEM micrographs, significant disruption to bacterial cell membrane was observed in samples that were frozen before pressure treatment. In order to verify possible leakage of intracellular material DNA and RNA of treated and non-treated cells was determined by NanoDrop spectroscopy. Small amounts of DNA (28.43 ± 0.57 ng μl^{-1}) and RNA (24.37 ± 0.06 ng μl^{-1}) were found in control samples (Figure 4). DNA and RNA concentrations in frozen and thawed samples were 181.37 ± 3.07 and 151.40 ± 1.93 ng μl^{-1} respectively. After pressurization of frozen samples, DNA and

RNA concentrations were 537.73 ± 7.60 and 450.60 ± 6.56 ng μl^{-1} respectively. Significantly increased levels of DNA and RNA in pressure-treated frozen PBS samples suggest loss of cell wall integrity, which was also evident in SEM images providing strong evidence that membrane damage is the major cause of bacterial inactivation during HHP treatment of frozen PBS suspension.

3.2. Inactivation of *E. coli* K12 in orange juice

In order to see if this technique could be applied in a real food system, orange juice was employed as a test medium. Orange and other fruit juices are some of the products where HHP is commonly used. Currently, beverages are the fastest growing segment of food products among the HHP processed foods, worldwide (2020).

3.2.1. Microbial load of orange juice

No microbial growth was observed on LB agar for the samples of orange juice before inoculation with *E. coli* (min detection level <10 CFU·ml^{-1}) indicating absence of *E. coli* and other organisms. After inoculation with *E. coli* K12 and overnight storage at 4 °C, the number of *E. coli* in unpressurized control samples ranged between 2.2×10^8 CFU·ml^{-1} and 5.3×10^8 CFU·ml^{-1} for the samples prepared on different days. The pH of orange juice did not have a significant effect on counts of *E. coli* during the overnight storage at 4 °C and the average counts of orange juice with pH 3.2, 4.5 and 5.8 was 8.5 ± 0.04 log CFU·ml^{-1} (n=9).

3.2.2. Effect of freezing on *E. coli* K12

Freezing at −80 °C overnight followed by thawing at room temperature did not result in significant change in the numbers of *E. coli* K12 in orange juice at all the pH values and the average of counts were 8.51 ± 0.03 CFU·ml^{-1}. This could be explained by the protective effect of soluble solids and fibres in orange juice. Although the proximate composition analysis of orange juice used for our experiments was not performed, it has been reported that orange juice contains about 120.19 g·L^{-1} total sugar (Kelebek, Selli, Canbas, & Cabaroglu, 2009).

3.2.3. Effect of pressurization time and pH on inactivation of *E. coli* K12

In this work we also assessed the inactivation of *E. coli* K12 in orange juice as a function of pH and pressurisation time for unfrozen (4 °C; Figure 5a) and frozen (−80 °C; Figure 5b) samples at 250 MPa, as preliminary experiments showed that a-5 log reduction is possible at this pressure.

Pressure treatments of unfrozen orange juice samples with pH values of 4.5 and 5.8 resulted in insignificant reductions of less than 0.42 log CFU·ml^{-1} in number of *E. coli* at all pressurizations times.

On the other hand, up to 3.84 ± 0.33 log reduction of CFU·ml^{-1} was obtained in unfrozen orange juice having a pH of 3.2 after a 15 min pressurization. Enhanced inactivation at lower pH could be explained by increased antimicrobial activity of organic acids present in orange juice. Kelebek, et al. (2009) reported that orange juice contained 12.66 g·L^{-1} citric acid, 0.49 g·L^{-1} ascorbic acid, 1.06 g·L^{-1} malic acid, 4.28 mg·L^{-1} hydroxybenzoic acids (gallic and protocatechuic acid) and 60.38 mg·L^{-1} hydroxycinnamic acids (caffeic, chlorogenic, p-Coumaric, ferulic and sinapic acid). It should be noted that, we used citric acid which is naturally present in orange juice to lower the pH of orange juice used in our experiments. Weak organic acids are most effective on microorganisms in their undissociated form (Erkmen & Bozoglu, 2016) as they can pass through the membrane and acidify the cytoplasm of the microbial cells. Organic acids are more effective at low pH values, where solutions have increased concentrations of undissociated acids (Lambert & Stratford, 1999). Smelt (1998) suggested that undissociated organic acid molecules could be more active under pressure. Alpas, Kalchayanand, Bozoglu, and Ray (2000) demonstrated that inactivation of *E. coli* O157:H7 933 in 1% peptone water following a pressurization at 345 MPa for 5 min increased from 1.86 to 3.82 log reduction of CFU·ml^{-1} when the pH of the medium was reduced from 6.5 to 4.5 by addition of citric acid.

As seen in Figure 5b, inactivation of *E. coli* K12 in orange juice increased significantly when the samples were frozen before pressure treatment and this increase was more pronounced for the samples having pH 4.5 and 5.8. For example, freezing orange juice with pH 3.2 increased the bacterial inactivation from 3.84 (unfrozen) to 4.88 log CFU·ml^{-1} after a 15 min pressure treatment. In comparison, when the samples were frozen before the same pressure treatment, bacterial inactivation increased from 0.35 (unfrozen) to 4.22 log CFU·ml^{-1} for samples with pH 4.5, and from 0.42 (unfrozen) to 4.41 log CFU·ml^{-1} for the samples with pH 5.8. About 4 log unit increase in bacterial inactivation as a result of freezing orange juice with pH of 4.5 and 5.8 before pressurization, indicates that the main mechanism of inactivation in frozen orange juice is partly different from that in unfrozen orange juice where log reduction remained below 0.42 log CFU·ml^{-1}. Therefore, the main mechanism of bacterial inactivation in frozen orange juice is most likely to be due to disintegration of cell membrane as in the case of frozen BPS.

The results indicate that the pH is a less critical factor for inactivation of *E. coli* in orange juice by HHP when samples are frozen. This observation could be important, as freezing of liquid foods (fruit and vegetable juices) before pressure treatment may remove the necessity of a strict control and/or

adjustment of the pH of the juices, provided that other measures (hurdles) are in place to prevent the growth of spore forming pathogenic bacteria.

3.2.4. Effect of temperature of pressurization and pH on inactivation of *E. coli* K12

In order to maximize the bactericidal potential of organic acids in orange juice during the pressurization process, further pressurization experiments were conducted where orange juice samples containing *E. coli* K12 were frozen at −80 °C and then pressurized at 20 °C (temperature of PTM). Figure 6 shows inactivation of *E. coli* K12 in frozen orange juice samples after pressurization at 4 °C and 20 °C. As seen in the figure, pressurization of frozen (−80 °C) orange juice samples at 20 °C resulted in similar inactivation of *E. coli*, compared to pressure treatment at 4 °C.

The inactivation of *E. coli* in frozen orange juice having pH of 3.2 was slightly higher at 20 °C (5.0 log reduction of $CFU \cdot ml^{-1}$), compared to 4 °C (4.6 log reduction $CFU \cdot ml^{-1}$). This could be explained by the fact that compared to pressurization at 4 °C, the sample melts faster under pressure at 20 ^{o}C, meaning that it will remain for longer in the liquid phase under the latter temperature. During a typical pressurization of frozen samples at 20 °C, the temperature measured through PTM increases to a max of 32.4 °C within 50 s of pressure build up period. The temperature gradually drops to 21.7 °C by the end of 10 min pressurization time and then instantly drops to 12.5 °C upon releasing the pressure.

In order for organic acids to diffuse through the cell membrane a liquid phase is required. Following the solid-solid phase transitions and melting, the sample remains for longer periods of time in liquid phase at a higher temperature (20 ^{o}C). As a result, organic acids in low pH orange juice could more effectively interact with *E. coli* cells which is further enhanced by the pressure. This observation could have important implications in industrial applications, as it may not be necessary to cool the pressure chamber and PTM to a low temperature of 4 °C, (orange juice must frozen before the pressurization) in order to obtain required microbial inactivation, which in turn could reduce the running costs of HHP processing.

Achievement of a 5-log reduction for a pressure resistant *E. coli* K12 at a very low pressure of 250 MPa, compared to the commonly used commercially 600 MPa, is significant. To put things in perspective from an economic point of view, a specific reduction in the required pressure would not result in a proportional reduction in manufacturing cost of the equipment (informal discussions with equipment manufacturers). This is due to the fact that the components and materials cost, as well as the complexity of construction technologies increase significantly, as the operation pressure

increases. Another advantage of HHP equipment operating at lower pressures is that, it easily allows construction of equipment with larger volumes. This is important as one of the main disadvantages of HHP equipment is their limited capacity (compared to conventional processes).

In terms of the freezing cost prior to HHP, it could be stated that most foods processed by HHP are required to be chilled to refrigeration temperatures, which requires energy expenditure and time. Thus, while cooling the products after the pressure treatment, the quality and shelf life of the food could be adversely affected. In case of pressurization of orange juice in frozen state, the cost of freezing could be justified as the orange juice comes out from HHP at near zero temperatures and it could to be shipped without any delay for cooling. Furthermore, the freezing cost per unit is very low as the products remain for only a few hours at this temperature. It is worth mentioning that in some cases increasing the pressure beyond a certain point could cause irreversible damage to some biological molecules such as proteins and this could impact the functionality of end product. Other adverse effects of HHP, such as undesirable changes in colour and functionality of foods and food ingredients could also be minimised by this approach.

4. Conclusions

Clear evidence by SEM micrographs and measurement of nucleic acids concentrations in PBS revealed that the significantly increased microbial inactivation in pressure treated frozen PBS is mainly due to physical damage of *E. coli* cells. Further studies with orange juice, showed that this remarkable bacterial inactivation in pressure treated frozen orange juice was not significantly pH dependent, whereas the limited inactivation in unfrozen orange juice was strongly pH dependant. This result could be exploited for HHP of low acid foods and biological materials.

High reduction in number of *E. coli* (5.0 log reduction of $CFU \cdot ml^{-1}$) in frozen orange juice samples with pH 3.2 after a 10 min at 250 MPa and 20 °C implies that for industrial applications lowering the process temperature near zero may not be necessary as long as the fruit juice is frozen before pressurizing under optimal set of parameters. Pressurization can be carried out at 4 °C or 20 °C, while the freezing of the food could take place separately at an earlier stage. This set up makes the pressurization of some liquid foods in frozen sate commercially feasible as a 5-fold decrease in the initial population of pertinent pathogens could be achieved as per the recommendations of FDA.

This study establishes some ground for further studies that need to focus on testing different pathogenic microorganisms and enzymes in various liquid foods. Studies on maximizing the bacterial reduction while keeping the optimum quality and shelf life of foods needed. Packaging size of the

pressurised liquid food could be a critical factor, which needs to be further investigated for optimization of HHP of frozen liquid foods at an industrial scale. The balance between the economy of lower cost of equipment manufacturing and running costs as a result of operating at significantly reduced pressures and the cost increase due to freezing prior to pressurization also needs to be looked at.

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