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

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1 **Abstract**

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

15

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

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31 1. INTRODUCTION

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

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

47 Working with *Bacillus subtilis* vegetative cells in ACES (pH 8.5), Shen, Urrutia Benet, Brul, and Knorr
48 (2005) showed that a 20 s treatment at 250 and 350 MPa at -25 °C on frozen cell suspensions
49 resulted in more than 4 log reduction of CFU·ml⁻¹. The authors suggested that solid–solid phase
50 transitions were mainly responsible for the observed drop in cell viability.

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

58 Su, Yu, Ramaswamy, Hu, Xu, and Zhu (2014) investigated HHP death kinetics of *Escherichia coli* strain
59 ATCC 25922 in frozen and unfrozen nutrient broth. Pressure treatment at 400 MPa with zero holding
60 time or a pressure treatment at 350 MPa for 1 min inactivated *E. coli* strain ATCC 25922 in frozen
61 (-20 °C) nutrient broth by more than 6 log CFU·ml⁻¹ cycles, whereas the inactivation in unfrozen
62 nutrient broth was less than 1.0 log of CFU·ml⁻¹. The same research group, (Zhu, Wang, Ramaswamy,

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

69 Shiba and Furushita (2017) created a thermal buffer zone around the plastic bags of bacterial
70 suspensions in 1% skim milk by using two polytetrafluoroethylene plates. The bacterial suspensions
71 held between the polytetrafluoroethylene plates were brought to -50 °C before a 20 min pressure
72 treatment at 200 MPa and 4 °C, which resulted in a reduction of 8 log cycles of *E. coli* strain ATCC
73 10798. The authors suggested that a homogeneous temperature within the sample achieved by an
74 initial temperature that was low enough to keep the sample temperature below the melting point
75 during compression, and the completion of melting within the holding time were necessary to take
76 the advantage of combined subzero temperature and high pressure during pressure-assisted
77 thawing.

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

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

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

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

99 FDA guideline for the fruit juice industry (FDA, 2004) states that a 5-log reduction must be targeted
100 for the pertinent pathogen which is the most resistant microorganism of public health concern that
101 may occur in the fruit juice. FDA also states that the pertinent pathogen may vary with the type of
102 juice and the type of treatment used, though typically it could be *Salmonella* or *Escherichia coli*
103 O157:H7 (FDA, 2001). FDA does not have a definitive suggestion as a test organism for HHP
104 processing however, *E. coli* O157:H7 is the main microorganism of concern that should be studied
105 for process development, challenge work and process validation (Institute of Food Technologists,
106 2000). However, it was shown that *E. coli* O157:H7 (ATCC 700728) in orange juice was more sensitive
107 to pressure compared to *E. coli* K12 (Torres, González-M, Klotz, & Rodrigo, 2016), suggesting that *E.*
108 *coli* K12 could be a more suitable strain for HHP studies.

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

117 2. MATERIALS AND METHODS

118 2.1. Bacterial strain and growth conditions

119 A pure culture of *E. coli* strain K12 was obtained from the culture collection of the Department of
120 Food and Nutritional Sciences, University of Reading. The stock culture was kept at -80°C in 15%
121 (v/v) glycerol and prior to experiments a sample was transferred onto LB Agar (Lab M limited, UK)
122 agar plates which were incubated at 37 °C overnight. The inoculum was prepared by transferring
123 three isolated colonies from the latter plates into 200 ml sterile LB Broth (Lab M limited, UK) in 500
124 ml sterile flasks and incubated at 37 °C for 18 h to give approximately 10⁹ CFU·ml⁻¹ at stationary
125 phase. Subsequently, cells were harvested by centrifugation, (7000 × g for 5 min) and washed twice

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

129 **2.2. Preparation of inoculated PBS samples**

130 By using a heat sealer, small plastic bags were prepared out of sterile stomacher bags. Subsequently,
131 12 ± 1 ml of *E. coli* strain K12 stock suspension were transferred with a sterile syringe in the bags
132 which were sealed and placed into a larger sterile stomacher bags by using a vacuum sealer to
133 prevent contamination of the pressurization medium in case of rupture during pressurization.
134 Samples were then stored under 3 different conditions overnight as follows: i) at 4°C in a laboratory
135 refrigerator (Labocold, Chineham, UK), ii) at -24°C in a laboratory freezer (Labcold, Chineham, UK)
136 and iii) at -80°C in a laboratory freezer (New Brunswick U570HEF, UK).

137 **2.3. Preparation of orange juice samples**

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

154 **2.4. HHP treatment**

155 A HHP system (Stansted Fluid Power Products Ltd., Harlow, UK) having a 16 mm internal diameter and
156 55 ml working volume was used to treat the samples. Alcohol (80% v/v) mixed with castor oil (20% v/v)

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

168 **2.5. Enumeration of microorganisms**

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

176 **2.6. Scanning Electron Microscopy (SEM) imaging**

177 Suspensions (200 µl) from each sample were pipetted onto poly-L-lysine coated glass coverslips for
178 15 min. The adhered bacteria were fixed with a solution of 2% paraformaldehyde and 2.5%
179 glutaraldehyde (pH 7.0) for 15 min and subsequently washed with sterile distilled water. The samples
180 were then dehydrated through a series of ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%)
181 prepared with deionised water. Then the samples were dried in a critical point dryer (Balzers 030
182 Critical Point Dryer) and coated with a thin layer of gold (Edwards S150B Sputter Coater). The
183 scanning electron micrographs were obtained using a FEI Quanta 600 FEG SEM equipped with the
184 xTm user interface (version 2.4).

185 **2.7. Determination of DNA and RNA concentration in PBS**

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

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

194 **2.8. Statistical analysis**

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

205 For the experiments with orange juice inoculated with *E. coli*, a factorial design was chosen where 3
206 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
207 replicates. Data were plotted by OriginPro v. 2019 (OriginLab Corporation, Northampton, MA, USA).
208 Microsoft® Excel® 2016 (Microsoft Corporation, USA) was used for calculation of standard deviations
209 (StDev).

210 **2.9. Validations**

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

216 3. RESULTS AND DISCUSSIONS

217 3.1. Inactivation of *E. coli* K12 in PBS and mechanism of inactivation

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

223 3.1.1. Effect of freezing on *E. coli* K12 in PBS

224 Freezing on its own can inflict damage on microbial cells. Therefore, it was necessary to understand
225 the effect of freezing and thawing on *E. coli* K12 under atmospheric conditions. Our results showed
226 that freezing at $-24\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ overnight followed by thawing at room temperature resulted in
227 1.73 ± 0.12 and 1.49 ± 0.07 log reduction, respectively. Freezing is known to cause inactivation of
228 microorganisms and the extent of the damage to bacterial cells depends on mainly the food matrix
229 and the freezing speed (temperature). Damage to microbial cell due to atmospheric freezing could be
230 due to ice crystal formation and growth within the cytoplasm that could result in a decrease in pH
231 (due to freeze concentration), which eventually could cause protein (enzyme) denaturation and
232 death of microbial cells. It was shown that slow freezing caused more death of *E. coli* cells compared
233 to rapid freezing (Souzu, 1980).

234 3.1.2. Temperature profile during pressurization

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

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

253 3.1.3. Effect of pressure and pressurization time on microbial inactivation

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

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

$$263 \text{Log reduction (CFU}\cdot\text{ml}^{-1}\text{)} = 0.022 + 1.62 \times 10^{-5} \times P - 0.068 \times t + 6.356 \times 10^{-4} \times P \times t \quad (\text{Eq.1})$$

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

265 Overnight freezing at -24 °C and -80°C prior to pressurization enhanced bacterial inactivation
266 significantly as seen in Figure 2b and Figure 2c. For frozen samples the time and pressure interaction
267 terms were not significant ($p>.05$) and the response surfaces were produced by linear models.

268 Response surface for the samples frozen at -24 °C (model $r^2=0.93$, $p<.0001$) produced the following
269 equation where pressure ($p=.0089$) and time ($p<.0001$) were the significant terms.

$$270 \text{Log reduction (CFU}\cdot\text{ml}^{-1}\text{)} = 1.346 + 4.37 \times 10^{-3} \times P + 0.309 \times t \quad (\text{Eq.2})$$

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

$$273 \text{Log reduction (cfu}\cdot\text{ml}^{-1}\text{)} = 0.861 + 8.55 \times 10^{-3} \times P + 0.334 \times t \quad (\text{Eq.3})$$

274 Freezing PBS suspension of *E. coli* K12 prior to HHP treatment increased microbial inactivation
275 remarkably. For example, after a 9 min pressure treatment at 400 MPa and 4 °C, a 6.83 log reduction
276 of CFU·ml⁻¹ was obtained in frozen PBS suspension (-80 °C) while, only 1.83 the log reduction of
277 CFU·ml⁻¹ was observed in unfrozen PBS suspension (4 °C). More interestingly, significant levels of
278 inactivation were observed in frozen PBS samples even after a very brief exposure to pressure. After
279 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
280 samples frozen at -24 and -80 °C, respectively. On the other hand, inactivation in unfrozen samples
281 (4 °C) was insignificant (0.01 log CFU·ml⁻¹). Similar observations were reported previously by Su, et al.
282 (2014) who showed more than 6 log reduction in CFU·ml⁻¹ for *E. coli* ATCC 25922 in frozen nutrient
283 broth after 1 min at 350 MPa and room temperature. In previous work (Bulut, 2014b) we reported a
284 3.8 log reduction in CFU·ml⁻¹ of *E. coli* ATCC 25922 in frozen milk after 1 min exposure to 300 MPa at
285 -3 °C.

286 Increased microbial inactivation as a result of freezing the samples prior to pressure treatment could
287 be explained mainly by the mechanical stress associated with the solid–solid phase transitions of ice I
288 to other ice polymorphs (Luscher, et al., 2004). As the density of ice-I (0.92 g·cm⁻³) is lower than the
289 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
290 other forms of ice results in a volume decrease (Chaplin, 2018). In our experiments we observed this
291 volume decrease at about 315 MPa where a sudden pressure drop of about 55 MPa took place
292 accompanied by a 4-5 °C temperature drop (Figure 1) as a result of the endothermic nature of
293 transitions from ice-I to other forms of ice.

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

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

310 **3.1.4. Validation of models obtained by response surface methodology**

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

313 **3.1.5. Effect of freezing and pressure on morphology of *E. coli* K12 cells**

314 The freeze damage that caused inactivation of *E. coli* K12 as detected by microbiological counts was
315 visible in SEM images. As seen in Figure 3b and 3c, thawing after freezing at $-24\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$
316 resulted in bacterial morphology changes which could be explained by the effects of freezing and
317 thawing.

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

328 **3.1.6. DNA and RNA leakage from *E. coli* K12 cells after pressure treatments**

329 As seen in SEM micrographs, significant disruption to bacterial cell membrane was observed in
330 samples that were frozen before pressure treatment. In order to verify possible leakage of
331 intracellular material DNA and RNA of treated and non-treated cells was determined by NanoDrop
332 spectroscopy. Small amounts of DNA ($28.43 \pm 0.57\text{ ng }\mu\text{l}^{-1}$) and RNA ($24.37 \pm 0.06\text{ ng }\mu\text{l}^{-1}$) were found
333 in control samples (Figure 4). DNA and RNA concentrations in frozen and thawed samples were
334 181.37 ± 3.07 and $151.40 \pm 1.93\text{ ng }\mu\text{l}^{-1}$ respectively. After pressurization of frozen samples, DNA and

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

339 **3.2. Inactivation of *E. coli* K12 in orange juice**

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

344 **3.2.1. Microbial load of orange juice**

345 No microbial growth was observed on LB agar for the samples of orange juice before inoculation with
346 *E. coli* (min detection level <10 CFU·ml $^{-1}$) indicating absence of *E. coli* and other organisms. After
347 inoculation with *E. coli* K12 and overnight storage at 4 °C, the number of *E. coli* in unpressurized
348 control samples ranged between 2.2×10^8 CFU·ml $^{-1}$ and 5.3×10^8 CFU·ml $^{-1}$ for the samples prepared
349 on different days. The pH of orange juice did not have a significant effect on counts of *E. coli* during
350 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
351 ± 0.04 log CFU·ml $^{-1}$ (n=9).

352 **3.2.2. Effect of freezing on *E. coli* K12**

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

359 **3.2.3. Effect of pressurization time and pH on inactivation of *E. coli* K12**

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

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

365 On the other hand, up to 3.84 ± 0.33 log reduction of CFU·ml⁻¹ was obtained in unfrozen orange juice
366 having a pH of 3.2 after a 15 min pressurization. Enhanced inactivation at lower pH could be
367 explained by increased antimicrobial activity of organic acids present in orange juice. Kelebek, et al.
368 (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
369 malic acid, 4.28 mg·L⁻¹ hydroxybenzoic acids (gallic and protocatechuic acid) and 60.38 mg·L⁻¹
370 hydroxycinnamic acids (caffeic, chlorogenic, p-Coumaric, ferulic and sinapic acid). It should be noted
371 that, we used citric acid which is naturally present in orange juice to lower the pH of orange juice
372 used in our experiments. Weak organic acids are most effective on microorganisms in their
373 undissociated form (Erkmen & Bozoglu, 2016) as they can pass through the membrane and acidify
374 the cytoplasm of the microbial cells. Organic acids are more effective at low pH values, where
375 solutions have increased concentrations of undissociated acids (Lambert & Stratford, 1999). Smelt
376 (1998) suggested that undissociated organic acid molecules could be more active under pressure.
377 Alpas, Kalchayanand, Bozoglu, and Ray (2000) demonstrated that inactivation of *E. coli* O157:H7 933
378 in 1% peptone water following a pressurization at 345 MPa for 5 min increased from 1.86 to 3.82 log
379 reduction of CFU·ml⁻¹ when the pH of the medium was reduced from 6.5 to 4.5 by addition of citric
380 acid.

381 As seen in Figure 5b, inactivation of *E. coli* K12 in orange juice increased significantly when the
382 samples were frozen before pressure treatment and this increase was more pronounced for the
383 samples having pH 4.5 and 5.8. For example, freezing orange juice with pH 3.2 increased the bacterial
384 inactivation from 3.84 (unfrozen) to 4.88 log CFU·ml⁻¹ after a 15 min pressure treatment. In
385 comparison, when the samples were frozen before the same pressure treatment, bacterial
386 inactivation increased from 0.35 (unfrozen) to 4.22 log CFU·ml⁻¹ for samples with pH 4.5, and from
387 0.42 (unfrozen) to 4.41 log CFU·ml⁻¹ for the samples with pH 5.8. About 4 log unit increase in
388 bacterial inactivation as a result of freezing orange juice with pH of 4.5 and 5.8 before pressurization,
389 indicates that the main mechanism of inactivation in frozen orange juice is partly different from that
390 in unfrozen orange juice where log reduction remained below 0.42 log CFU·ml⁻¹. Therefore, the main
391 mechanism of bacterial inactivation in frozen orange juice is most likely to be due to disintegration of
392 cell membrane as in the case of frozen BPS.

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

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

398 **3.2.4. Effect of temperature of pressurization and pH on inactivation of *E. coli* K12**

399 In order to maximize the bactericidal potential of organic acids in orange juice during the
400 pressurization process, further pressurization experiments were conducted where orange juice
401 samples containing *E. coli* K12 were frozen at $-80\text{ }^{\circ}\text{C}$ and then pressurized at $20\text{ }^{\circ}\text{C}$ (temperature of
402 PTM). Figure 6 shows inactivation of *E. coli* K12 in frozen orange juice samples after pressurization at
403 $4\text{ }^{\circ}\text{C}$ and $20\text{ }^{\circ}\text{C}$. As seen in the figure, pressurization of frozen ($-80\text{ }^{\circ}\text{C}$) orange juice samples at $20\text{ }^{\circ}\text{C}$
404 resulted in similar inactivation of *E. coli*, compared to pressure treatment at $4\text{ }^{\circ}\text{C}$.

405 The inactivation of *E. coli* in frozen orange juice having pH of 3.2 was slightly higher at $20\text{ }^{\circ}\text{C}$ (5.0 log
406 reduction of $\text{CFU}\cdot\text{ml}^{-1}$), compared to $4\text{ }^{\circ}\text{C}$ (4.6 log reduction $\text{CFU}\cdot\text{ml}^{-1}$). This could be explained by the
407 fact that compared to pressurization at $4\text{ }^{\circ}\text{C}$, the sample melts faster under pressure at $20\text{ }^{\circ}\text{C}$,
408 meaning that it will remain for longer in the liquid phase under the latter temperature. During a
409 typical pressurization of frozen samples at $20\text{ }^{\circ}\text{C}$, the temperature measured through PTM increases
410 to a max of $32.4\text{ }^{\circ}\text{C}$ within 50 s of pressure build up period. The temperature gradually drops to
411 $21.7\text{ }^{\circ}\text{C}$ by the end of 10 min pressurization time and then instantly drops to $12.5\text{ }^{\circ}\text{C}$ upon releasing
412 the pressure.

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

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

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

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

441 **4. Conclusions**

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

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

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

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

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