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3 **The relationship between membrane damage, release of protein and loss of**

4 **viability in *Escherichia coli* exposed to high hydrostatic pressure**

5

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Abstract

The aim of this work was to examine a possible association between resistance of two *Escherichia coli* strains to high hydrostatic pressure and the susceptibility of their cell membranes to pressure-induced damage. Cells were exposed to pressures between 100 and 700 MPa at room temperature (~20°C) in phosphate-buffered-saline. In the more pressure-sensitive strain *E. coli* 8164, loss of viability occurred at pressures between 100 MPa and 300 MPa and coincided with irreversible loss of membrane integrity as indicated by uptake of propidium iodide (PI) and leakage of protein of molecular mass between 9 and 78 kDa from the cells. Protein release increased to a maximum at 400 MPa then decreased, possibly due to intracellular aggregation at the higher pressures. In the pressure-resistant strain *E. coli* J1, PI was taken up during pressure treatment but not after decompression indicating that cells were able to reseal their membranes. Loss of viability in strain J1 coincided with the transient loss of membrane integrity between approximately 200 MPa and 600 MPa. In *E. coli* J1 leakage of protein occurred before loss of viability and the released protein was of low molecular mass, between 8 and 11 kDa and may have been of periplasmic origin. In these two strains differences in pressure resistance appeared to be related to differences in the ability of their membranes to withstand disruption by pressure. However it appears that transient loss of membrane integrity during pressure can lead to cell death irrespective of whether cells can reseal their membranes afterwards.

Key words: *E. coli*, hydrostatic pressure, membrane damage, protein leakage

Abbreviated title: Release of protein from pressure-treated *Escherichia coli*

48 **1. Introduction**

49

50 A range of non-thermal methods for preserving food have been investigated to
51 satisfy growing consumer demands for minimally-processed high-quality foods that
52 contain little or no chemical preservatives but are safe to eat (Mañas and Pagán 2005).
53 High hydrostatic pressure (HHP) is generally regarded as one of the more promising of
54 these emerging technologies and many new products have appeared on the market
55 including fruit juices, smoothies, guacamole, seafood, snacks and prepared meals
56 (Rastogi et al., 2007). HHP can inactivate vegetative microorganisms but is largely
57 ineffective against spores, at least when applied at ambient temperatures (San Martín et
58 al., 2002). In this sense it is essentially a pasteurization process and it is therefore
59 essential that pressure treatments used in food preservation can inactivate the most
60 resistant vegetative foodborne pathogens. To this end, considerable effort has been
61 spent to determine the intrinsic pressure resistance of different microorganisms and to
62 understand the physiological, environmental and processing factors that modify that
63 resistance (Smelt, 1998; Hoover, et al., 1989; Rastogi et al., 2007). Resistance to high
64 pressure varies between species of microorganism but does not always correlate with
65 resistance to other preservation treatments such as heat (Metrick et al., 1989; Alpas,
66 2000). Strains within a given species can also differ widely in pressure resistance. This
67 is particularly true of *E. coli,* some strains of which are inactivated by pressures as low
68 as 200 MPa whereas others can survive exposure to 600 MPa in neutral media (Benito
69 et al., 1999; Robey et al., 2001). This is of considerable practical importance because
70 some strains of *E. coli* O157 are among the most pressure resistant vegetative cells
71 examined to date (Patterson et al., 1995; Benito et al., 1999).

72 Many cellular components are affected by pressure including cell membranes
73 and membrane proteins, enzymes, ribosomes and the nucleoid (Hoover et al., 1989;

74 Mackey and Mañas, 2008). Details of the mechanisms of inactivation have been
75 investigated in several bacterial species but the particular events leading to loss of
76 viability are not known for certain. In *E. coli* three processes seem to be especially
77 important. These are protein denaturation, oxidative stress and disruption of the
78 cytoplasmic membrane (Mackey and Mañas, 2008). Several lines of evidence point to
79 the importance of protein denaturation in microbial cell death. At the thermodynamic
80 level pressure-temperature diagrams of cellular inactivation rates of *E. coli* and other
81 microorganisms show a strong resemblance to the elliptic pressure-temperature phase
82 diagrams for protein denaturation (Sonoike, 1992). Supporting evidence comes from
83 biochemical studies showing that heat shock proteins are synthesized in cells during
84 exposure to sublethal pressures and in cells recovering from pressure treatment (Welch
85 et al., 1993; Aertsen et al., 2004). The heat shock proteins synthesized include
86 chaperones (DnaK, GrpE, GroES, and GroEL) and proteases that are involved in
87 degradation of denatured proteins (ClpB, ClpP and Lon). There is also strong
88 circumstantial evidence that protection against protein denaturation may enhance
89 cellular pressure resistance since exposure to mild heat shock increases resistance to
90 pressure whilst pressure-resistant mutants of *E. coli* selected by successive cycles of
91 pressure treatment and outgrowth had increased levels of heat-shock proteins (Aertsen
92 et al., 2004).

93 Oxidative stress appears to play an important role in cell death under some
94 circumstances. The lethality effect of pressure was increased by mutations in *oxyR* and
95 *soxS,* coding for oxidative stress regulatory elements, and in *katE* and *sodAB* coding for
96 HPII hydroperoxidase and superoxide dismutase respectively (Aertsen et al., 2005).
97 Conversely recovery of pressure-treated cells under anaerobic conditions enhanced
98 survival. It has been suggested that pressure treatment results in the release of iron

99 from Fe-S clusters leading to the generation of hydroxyl free radical via the Fenton
100 reaction (Malone et al., 2006).
101 Finally, there is strong evidence that membrane damage can lead to cell death.
102 Exponentially growing cells of *E. coli* are much more pressure sensitive than stationary
103 phase ones and in these cells loss of viability coincides with irreversible disruption of
104 cytoplasmic membrane integrity as measured by uptake of the non-permeant fluorescent
105 dye propidium iodide (PI) and loss of osmotic responsiveness (Pagán and Mackey,
106 2000, Mañas and Mackey 2004). Pressure resistance is influenced by membrane fluidity
107 and fatty acid composition such that cells with more fluid membranes are more pressure
108 resistant (Casadei et al., 2002). In stationary-phase cells the picture is more
109 complicated. Some weak strains undergo irreversible disruption of the cytoplasmic
110 membrane similar to that in exponential phase cells but more robust strains are able to
111 re-seal their membranes after decompression (Pagán and Mackey, 2000). The role of
112 membrane damage in stationary phase cells of the more pressure resistant strains of *E.*
113 *coli* is thus far from clear.
114 Further work is needed to unravel the contribution of the three types of
115 mechanism outlined above to cell death which may depend on the properties of
116 individual strains, their physiological state at the time of exposure to pressure and the
117 conditions during pressure treatment and recovery. The aim of this work was to
118 investigate the role of membrane damage in cell death of stationary-phase cells,
119 specifically to examine the relationship between loss of membrane integrity and loss of
120 viability in a two strains of *E. coli* with wide differences in pressure resistance. Two
121 different indicators of membrane damage were used: uptake of PI and loss of protein
122 from the cell. A preliminary characterization of released protein was also carried out
123 using 1-D gel electrophoresis.
124

125 **2. Materials and methods**

126

127 *2.1. Bacterial strain and growth conditions.*

128

129 *Escherichia coli* NCTC 8164, *E. coli* J1 and *E. coli* NCTC 8003 were stored at -
130 70°C in bead vials (Protect Technical Service Consultants Limited, Lancashire, United
131 Kingdom). *Escherichia coli* NCTC 8164 was used in previous studies of the
132 mechanisms of thermal inactivation (Mackey et al., 1991), the role of membrane fluidity
133 in pressure resistance (Casadei et al., 2002) and kinetics of inactivation by pressure
134 (Klotz et al., 2007). *Escherichia coli* J1 is a commensal strain with high pressure
135 resistance used previously to study morphological changes caused by exposure to high
136 pressures (Mañas and Mackey, 2004). *Escherichia coli* NCTC 8003 was previously used
137 in studies of membrane damage in pressure-treated cells (Pagán and Mackey, 2000).
138 To activate the strains one frozen bead was transferred to 9 ml Tryptone Soya Broth
139 (TSB; Oxoid CM129, Basingstoke, United Kingdom) and incubated in shaken culture
140 (140 rpm; Aquatron, Infors UK, Reigate, Surrey, United Kingdom) at 37°C for
141 approximately 6 h. The culture was then diluted 1:1000 into 100 ml fresh TSB and
142 incubated in shaken flasks (250 mL) at 37°C for approximately 18 h. The resulting
143 stationary-phase culture contained approximately 3×10^9 cells/mL

144

145 *2.2. Pressure treatment.*

146

147 Samples of stationary-phase cells were centrifuged at 2800 x g for 15 min at 5°C
148 (Biofuge 28 RS15; Heraeus Sepatech, Osterode, Germany), resuspended in an equal
149 amount of phosphate-buffered-saline (PBS; Oxoid BR0014, Basingstoke, United
150 Kingdom) and dispensed in volumes of 2 mL in plastic sachets, heat sealed, and placed

151 on ice before treatment. Samples were treated in a 300 ml pressure-vessel (Foodlab
152 Plunger Press model S-FL-850-9W; Stansted Fluid Power, Stansted, Essex, United
153 Kingdom). The pressure- transmitting fluid was ethanol: castor oil (80:20). The come-up
154 rate was approximately 330 MPa / min and the deviation at targeted pressure was ± 10
155 MPa. After treatment, the pressure was released quickly in two steps. In the first step
156 the pressure decreases to 30 MPa in about 15 seconds. The total decompression takes
157 about 35 seconds. The transient increase in temperature of the pressurization fluid due
158 to adiabatic heat during the treatment is measured with a thermocouple located near the
159 vessel closures attached to the inside of the vessel lid. The average temperature rise
160 was 4.3 (± 0.4) ^{o}C/100 MPa. Experiments were carried out at room temperature.
161
162 *2.2. Viable counts*
163
164 Sample bags were opened with sterile scissors and cell suspensions were diluted
165 ten-fold in Maximum Recovery Diluent (MRD; Oxoid CM733, Basingstoke, United
166 Kingdom). Appropriate dilutions were plated on TSA plus 0.1% sodium pyruvate as
167 recovery medium and colonies were counted after incubation at 37°C for 24 and 48 h.
168 Two to four counts at relevant dilutions were performed for each sample. The mean was
169 calculated and expressed as CFU/mL (colony-forming unit per mL sample). The lower
170 limit of accurate measurements was 25 CFU/mL.
171
172 *2.3. Preparation of the supernatant from suspensions of pressure-treated cells.*
173
174 Cultures were centrifuged for at 5°C for 15 min at 2800 x g, resuspended in PBS,
175 dispensed in sterile stomacher bags (Seward Limited, Worthing, West Sussex, United
176 Kingdom), heat-sealed without head space, and placed on ice. Pouches were pressure-

177 treated in the range of 125 to 700 MPa. After decompression, pouches were removed
178 from the unit and wiped clean of any residual pressurising fluid. The bags were opened
179 with a sterile scissors and the content was centrifuged (2800 x g, 15 min, 5°C). The
180 supernatant was collected, filtered (Minisart High Flow syringe filters, 0.2 µm; Sartorius
181 Mechatronics UK Limited, Epsom, Surrey, United Kingdom) and stored at -70°C for
182 protein electrophoretic analysis.
183
184 *2.4. Osmotic shock treatment*
185
186 The osmotic shock treatment was performed according to Vázquez-Laslop et al.
187 (2001). Samples of stationary-phase *E. coli* NCTC 8164 cultures were centrifuged (2800
188 x g, 15 min, 5°C) and resuspended to an OD_{680} of 10 in ice-cold TSE buffer (10 mM Tris-
189 HCl, pH 7.5, 20% sucrose, 2.5 mM Na-EDTA). After 10 min incubation on ice, cells were
190 centrifuged for 10 min at 5000 x g at 4°C. The supernatant was decanted and the
191 pelleted cells were resuspended in an equal amount of ice-cold water. After 10 min
192 incubation on ice cell suspensions were centrifuged again and the supernatant with the
193 released proteins was collected, filtered (Sartorius Minisart High Flow syringe filters, 0.2
194 µm,), and saved for electrophoretic analysis at -70°C.
195
196 *2.5. Determination of the protein content of supernatants*
197
198 The protein determination was performed using the Bradford Reagent according
199 to the Micro 2 mL assay protocol described in the technical bulletin (B 6916, Sigma-
200 Aldrich Company Ltd., Gillingham, Dorset, United Kingdom.). One ml of Bradford reagent
201 was added to 1 mL sample containing 1-10 µg protein and the samples were incubated
202 at room temperature for 5 min. The absorbance was measured at 595 nm in a

203 spectrophotometer (model CE 2020, Cecil Instruments Ltd., Cambridge, United
204 Kingdom). Bovine serum albumin (BSA; Sigma-Aldrich P-0834) was used as the protein
205 standard at concentrations between 1-10 μg/mL.
206
207 *2.6. Electrophoretic analysis of proteins*
208
209 Characterisation of the proteins present in the supernatant was performed with
210 pre-cast gels (Novex, 1.0 mm x 10 well, Invitrogen Ltd., Paisley, United Kingdom.)
211 according to the manufacturer's electrophoresis guide. Tricine gels (Novex) were used
212 for low molecular weight peptides and proteins. The protein gels were stained using a
213 silver staining kit (Cat. no.161-0449; Bio-Rad Laboratories, Hemel Hempstead, United
214 Kingdom). The amount of extract from strain J1 loaded onto the gels was twice that from
215 NCTC 8164 to allow protein bands to be visualized at similar densities. The silver
216 stained gels were digitally photographed with the computer-based automated gel
217 imaging system Gene Snap from Syngene V. 3.00.15 (Syngene, Cambridge, UK). The
218 gray-scale files were quantified with Gene Tools from Syngene with the subtraction of
219 the background. According to the gel resolution and characteristics the software was
220 operated automatically or manually. Results were expressed in Microsoft Excel charts.
221
222 *2.7. Assessment of cell membrane damage*
223
224 The fluorescent dye propidium iodide (PI; Sigma-Aldrich, 287075) was used to
225 evaluate cell membrane damage in stationary-phase cultures of *E. coli* NCTC 8164 and
226 J1. A stock solution of 1 mg PI in 1 mL water (ISO grade 2) was prepared. Samples of
227 cell suspensions in PBS with an OD_{680} of 0.2 (spectrophotometer model CE 2020, Cecil
228 Instruments) were mixed with PI solution to a final concentration of 2.9 μM before or

229 after pressure treatment for 10 min at 100, 125, 150, 200, 300, 400, 500, 600, or 700
230 MPa. For evaluation of PI uptake after pressure treatment, cells were incubated with PI
231 for 10 min, then centrifuged (10 000 x g) at 4°C and washed twice in PBS. When PI was
232 present during pressure treatment the cells were centrifuged and washed immediately
233 after decompression. Fluorescence was measured at an excitation wavelength of 495
234 nm and an emission wavelength of 615 nm in a fluorimeter (Model LS-5B, PerkinElmer,
235 Massachusetts, USA). The data were normalized by subtracting fluorescence values
236 obtained from untreated cells and against OD_{680}. The normalized data were plotted as
237 percentages of PI uptake during and after pressure treatment at different pressures.
238
239
240 **3. Results**
241
242 *3.1. Pressure resistance of stationary-phase cells of E. coli NCTC 8164 and E. coli J1*
243
244 The two strains showed large intrinsic differences in pressure resistance. The
245 onset of extensive cell inactivation occurred at a pressure that was about 200 MPa
246 higher in *E. coli* J1 than in *E. coli* NCTC 8164 (Fig 1). To reduce viable numbers of *E.*
247 *coli* NCTC 8164 by 90% required a pressure treatment of only 300 MPa for 10 min,
248 compared with 500 MPa needed to achieve the same effect in strain J1.
249
250 *3.2. Loss of membrane integrity*
251
252 The uptake of the PI by pressure-treated cells is shown in Fig 2. The dye was
253 added to the cell suspensions either before pressure treatment or after decompression.
254 Uptake of dye added before pressure treatment was taken to indicate loss of cytoplasmic

255 membrane permeability under pressure whilst uptake of dye added after decompression
256 was taken to indicate permanent loss of membrane integrity. Uptake of PI began
257 between 100 and 125 MPa in *E. coli* NCTC 8164 and between 200 and 300 MPa in *E.*
258 *coli* J1. In *E. coli* NCTC 8164 there was little difference in the amount of PI taken up
259 during or after pressure treatment indicating a permanent loss of membrane integrity. In
260 *E. coli* J1 PI was also taken up during pressure treatment but very little after indicating
261 that the permeability barrier to PI was restored after decompression.
262
263 *3.3. Uptake of propidium iodide by single cells*
264
265 Propidium iodide staining of single cells of *E. coli* J1 is shown in Fig 3. Cells
266 were pressure treated at 400 MPa for 10 min and PI was added either before pressure
267 treatment (Fig 3A) or after decompression (Fig 3B). Under these conditions some
268 individual cells in the population take up PI during pressure treatment, but few cells do
269 so after decompression.
270
271 *3.4. Release of protein from pressure-treated cells*
272
273 Loss of protein into the extracellular fluid began at 100-125 MPa in *E. coli* 8164
274 and 125-150 MPa in *E. coli* J1 but the total amount of protein released was greater in *E.*
275 *coli* NCTC 8164 than in *E. coli* J1 (Fig 4). In *E. coli* NCTC 8164 the amount of protein
276 released increased to a maximum at 300-400 MPa then decreased whereas in *E. coli* J1
277 the amount of protein released increased to a maximum at 200 MPa then remained
278 constant.
279

280 *3.5. Relationship between loss of membrane integrity, loss of protein and cell death in E.*
281 *coli strains.*
282
283 The relationship between loss of membrane integrity, loss of cellular protein and
284 loss of viability in *E. coli* strains J1 and NCTC 8164 is shown in Figs 5A and 5B
285 respectively. In *E. coli* J1 loss of viability coincided with uptake of PI during pressure
286 treatment but not with uptake of PI after pressure treatment, which occurred at higher
287 pressures; or with release of protein, which occurred at lower pressures. In *E. coli*
288 NCTC 8164 loss of viability, uptake of PI and release of protein all occurred over more or
289 less the same pressure range although uptake of PI after pressure took place at
290 somewhat higher pressures than the other measured events. The only event that was
291 correlated with loss of viability in both strains was therefore the uptake of PI during
292 pressure treatment.
293 The relationship between uptake of PI during pressure treatment and loss of
294 viability in *E. coli* J1, *E. coli* 8164 and an additional strain, *E. coli* NCTC 8003, is shown
295 in Fig 6. The correlation between PI uptake under pressure and loss of viability was
296 reasonable for the combined data (coefficient of determination = 0.94), consistent with
297 there being an association between loss of membrane integrity during pressure
298 treatment and cell death in all three of the tested strains of *E. coli*.
299
300 *3.6. Characterisation of proteins released from E. coli strains during pressure treatment*
301
302 The electrophoretic profiles of the proteins released from *E. coli* J1 and *E. coli*
303 NCTC 8164 and are shown in Figs. 7A and 7B. Twelve protein bands were identified
304 from *E. coli* NCTC 8164 and sixteen from *E. coli* J1 (Table 1). The approximate

305 molecular masses ranged from 6 to 64 kDa in *E. coli* J1 and from 9 to 78 kDa in *E. coli*
306 8164. Ten of the proteins released were of similar molecular mass in both strains.
307 Protein release started between about 100-150 MPa in both strains with three
308 proteins from *E. coli* 8164 being released and one from *E. coli* J1. Visual inspection of
309 the gel showed that the protein from *E. coli* J1 was a 9 kDa protein that comprised most
310 of the released material from this strain. Further groups of proteins were released from
311 each strain at successively higher pressures though the pattern was different in each
312 strain (Table 1).
313 The amounts of each protein released at different pressures were estimated by
314 measuring the optical density of the bands. This is only semi-quantitative but does give
315 an indication of the relative amounts of particular proteins released at different
316 pressures. With many proteins, the amount released increased with pressure, as for
317 example those in strain 8164 with an apparent molecular mass of 15-16, 19-20, 20-
318 21,21-22 and 22-23 kDa. In other cases the amounts released increased initially but
319 then decreased at higher pressures. This was the case for the higher molecular mass
320 proteins in strain 8164, for example the bands at 52-53, 56-65, and 66-78 kDa. In
321 general more different proteins were released from *E. coli* NCTC 8164 than from *E. coli*
322 J1 and much of the protein released from *E. coli* J1 was of low molecular mass (Fig 7A
323 and 7B).
324
325 *3.7. A comparison of proteins released by pressure and osmotic shock treatment*
326
327 Figure 8 shows a comparison of the proteins released by pressure and osmotic
328 shock from *E. coli* NCTC 8164. Proteins of molecular mass 64-65, 49, 41, 28, 24 and 9
329 kDa were present in the supernatant of both pressure-treated and osmotically-shocked

330 cells but additional bands at 37, 21 and 15 kDa were present only in the supernatant
331 from pressure-treated cells.
332
333 **4. Discussion**
334
335 The large difference in pressure-resistance between stationary phase cells of *E.*
336 *coli* NCTC 8164 and *E. coli* J1 appears to be due to a difference in the resilience of their
337 cytoplasmic membranes towards high pressure stress. The membrane of *E. coli* NCTC
338 8164 became disrupted at a lower pressures than that of *E. coli* J1 and was unable to
339 reseal after release of pressure whereas the cell membrane of *E. coli* J1 appeared to
340 undergo less severe disruption and could reseal afterwards. Previous work by Pagán
341 and Mackey (2000) showed that stationary phase cells of *E. coli* O157 strain C9490 and
342 *E. coli* NCTC 8003 underwent transient membrane permeabilization during pressure
343 treatment. Cells of strain C9490 retained their ability to plasmolyse and remained alive
344 whilst in cells of strain NCTC 8003, 50% of the cells lost their osmotic responsiveness
345 and 99% of the cells died. Mañas and Mackey (2004) showed that at high pressures
346 above 400 MPa a proportion of stationary phase cells of *E. coli* J1 died without loss of
347 osmotic responsiveness. Moussa et al. (2007) found that pressure treatment: at subzero
348 temperatures induced mainly reversible permeabilization in *E. coli* while both reversible
349 and irreversible permeabilization occurred at room temperature. A qualitative relationship
350 was noted between membrane permeabilization and cell death. In the present work a
351 direct relationship was observed between transient loss of membrane integrity and cell
352 death in three different strains of *E. coli.*
353 From the above findings we can discern a spectrum in the resilience of
354 stationary-phase *E. coli* membranes towards pressure. At one extreme we have strains
355 such as *E. coli* NCTC 8164 which have fragile cell membranes that undergo permanent

356 disruption under pressure. These strains bear some resemblance to exponential phase
357 cells which are also unable to reseal after decompression (Benito et al., 1999; Pagán
358 and Mackey, 2000). In another group of strains cells undergo transient permeabilization
359 but nevertheless die; whilst at the other extreme we have unusually resistant strains
360 such as *E. coli* O157 C9490 which are able to recover from transient permeabilization
361 (Pagán and Mackey, 2000). The basis of this spectrum of behaviour among the different
362 strains is unknown. Although physical integrity of the cell membrane can apparently be
363 regained under some circumstances, there may be other irreversible changes that can
364 lead to cell death. Possibilities are: subtle changes in permeability control preventing
365 restoration of homeostasis; disruption of electron transport components leading to
366 oxidative stress; denaturation of critical membrane or cytoplasmic proteins; loss of
367 critical intracellular components or an irreversible change in the intracellular environment
368 that prevents recovery. With regard to the last point it is interesting that near-complete
369 recovery of *E. coli* after electroporation is possible if cells are quickly transferred from
370 electroporation buffer to recovery medium, but if cells remain in the electroporation
371 medium viability is rapidly lost (Dower et al., 1988). The composition of the suspending
372 medium may thus be critical in survival of transiently permeabilized cells. Though not
373 investigated here, transient changes in the outer membrane of *E. coli* have also been
374 reported (Hauben et al., 1996; Chilton et al., 2001; Ganzle and Vogel, 2001). Outer
375 membrane damage is not believed to be lethal but does allow entry of antimicrobial
376 substances such as lysozyme or nisin that can enhance lethality of pressure treatments
377 (Garcia-Graells, 1999).

378 In Gram-positive bacteria the relationship between membrane damage and death
379 of pressure-treated cells is unclear. Pressure-treated populations of *Listeria*
380 *monocytogenes*, *Lactobacillus rhamnosus,* and *Staphylococcus aureus* in which more
381 than 99% of cells were dead, still contained appreciable proportions of cells with intact

382 membranes as indicated by lack of staining with propidium iodide (Arroyo et al., 1999;
383 Ritz et al., 2001; Ananta and Knorr 2009). Ulmer et al. (2000) concluded from their
384 studies of the kinetics of pressure inactivation and PI uptake in *L. plantarum* that
385 irreversible membrane damage occurred after cell death. By contrast Smelt et al. (1994)
386 reported a very good correlation between PI uptake and cell death in *L. plantarum*. The
387 possible effects of growth phase and transient membrane permeabilization on survival
388 after pressure treatment appear not to have been investigated in Gram-positive bacteria
389 and it may be significant that Smelt et al. (1994) used exponential phase cells whereas
390 the other authors used cells in stationary phase. Studies using pulsed electric fields at
391 pH 7.0 found that Gram-positive bacteria were able to recover after transient
392 permeabilization whereas Gram-negative ones were not (Garcia et al., 2006). These
393 studies also suggest that an ability to reseal cell membranes is necessary but not
394 sufficient for cell survival.

395 Both *E. coli* strains released cellular proteins into the suspending medium as a
396 result of pressure treatment but the amount of protein released from *E. coli* J1 was
397 considerably less than from *E. coli* NCTC 8164 and the proteins were fewer and smaller
398 in size. This supports the conclusion that damage to the cytoplasmic membrane in *E.*
399 *coli* J1 was less extensive than in *E. coli* NCTC 8164. Loss of protein was coincident
400 with loss of cytoplasmic membrane integrity in *E. coli* NCTC 8164 but not in *E. coli* J1. A
401 large proportion of the total protein leaking from strain J1 consisted of a protein of 9 kDa
402 that appeared in the supernatant before any uptake of PI or loss of viability. This small
403 protein may therefore have come from the periplasm or outer membrane and its loss
404 does not appear to be lethal to the cell.

405 Release of protein from *E. coli* under pressure was previously reported by Mañas
406 and Mackey (2004) but the proteins were not characterized and there appears to be no
407 information on this in the literature. The preliminary analysis of released proteins by 1-D

408 gel electrophoresis revealed sixteen protein bands from *E. coli* NCTC 8164 and thirteen
409 from *E. coli* J1. Several proteins released from *E. coli* NCTC 8164 had the same
410 molecular mass as those released by osmotic shock and may therefore have come from
411 the periplasm. These include the protein of approximately 9 kDa; however, given the
412 disruption of the cytoplasmic membrane in *E. coli* NCTC 8164 it is likely that some of the
413 released proteins were of cytoplasmic origin. The real number of different proteins
414 released under pressure is likely to be greater than that detectable on 1-D gels and
415 further studies using 2-D gel electrophoresis are desirable to identify the proteins and
416 their origin.

417 All of the proteins released from pressure-treated cells had a molecular mass
418 below 80 kDa. This cut-off point is consistent with the suggestion of Vázquez-Laslop et
419 al. (2001) that the peptidoglycan of the cell wall acts as a molecular sieve for proteins
420 leaking from bacterial cells. The results obtained by Vázquez-Laslop et al., (2001) in a
421 study of osmotically-shocked cells indicated that the peptidoglycan mesh was
422 comparable in porosity to a 100 kDa cut-off cellulose membrane. The amount of protein
423 released from *E. coli* NCTC 8164 increased with pressure intensity up to 300-400 MPa
424 then decreased. We believe this may be due to the formation of intracellular aggregates
425 at the higher pressures that are unable to pass through the peptidoglycan. In *E. coli* J1
426 the amount of protein released increased with pressure then remained more or less
427 constant. This would be consistent with the released proteins originating from a region
428 outside the peptidoglycan. In this case the release of any aggregated protein would not
429 be impeded by the sieving effect of the peptidoglycan so no reduction in released protein
430 would be expected at high pressures.

431 Although the membrane disruption by high pressure is acknowledged as a critical
432 event in microbial inactivation by pressure the role of membrane damage in death of
433 stationary phase cells has been unclear. This work shows that stationary phase

434 membranes of different *E. coli* strains differ quite widely in their ability to resist disruption
435 by pressure treatment and in their ability to recover integrity after decompression. This
436 has a major influence on the ability of cells to survive high hydrostatic pressure. It is now
437 clear that the pressure at which membrane disruption begins is more important for cell
438 survival than the ability to reseal membranes after decompression. Even temporary loss
439 of membrane integrity can lead to cell death. Since the degree of membrane
440 permeabilization, protein loss and resealing varies between different strains of *E. coli*,
441 differences in the efficacy of combined processes which rely in the entrance of an
442 antimicrobial substances during pressurization might be expected. It would be
443 interesting for example to examine whether such combined treatments could overcome
444 the pressure resistance of strains that have more resilient cell membranes.
445
446 **Acknowledgements**
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450 Bogota, Colombia.

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561 **Legends to Figures.**

562 **Fig 1.** Loss of viability of *E. coli* J1 (■) and *E. coli* NCTC 8164 (●) after treatment for 10
563 min at different pressures. Plotted values are means from three replicate trials ±
564 standard deviation.

565 **Fig 2.** Uptake of propidium iodide during (closed symbols) and after (open symbols)
566 treatment of *E. coli* J1 (■,□) and *E. coli* NCTC 8164 (●,O) for 10 min at different
567 pressures. Plotted values are means from three replicate trials ± standard deviation.

568 **Fig 3.** Microscopy of *E. coli* J1 cells stained with propidium iodide present during (A)
569 and after (B) pressure treatment at 400 MPa for 10 min. Bar marker 1 μm.

570 **Fig 4.** Release of protein from cells of *E. coli* J1 (■) and *E. coli* NCTC 8164 (●) after
571 treatment for 10 min at different pressures. Plotted values are means from three
572 replicate trials ± standard deviation.

573 **Fig 5.** Relationship between loss of viability (O), uptake of propidium iodide during
574 pressure treatment (■), uptake of propidium iodide after pressure treatment (□) and
575 release of protein (▲) in *E. coli* NCTC 8164 (A) and *E. coli* J1 (B).

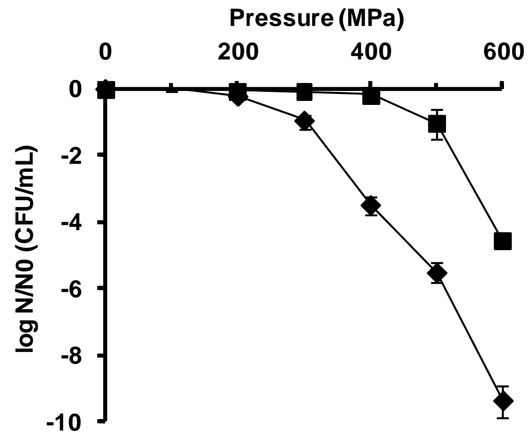
576 **Fig 6.** Relationship between propidium iodide uptake during pressure treatment and
577 loss of viability in *E. coli* strains J1, NCTC 8164 and NCTC 8003.

578 **Fig 7.** Gel electrophoresis of proteins released following 10 min treatment at different
579 pressures from *E. coli* J1 (A) and *E. coli* NCTC 8164 (B). Indicated pressures are in
580 MPa. Molecular mass markers are shown in the right hand lane.

581 **Fig 8.** Comparison of proteins released from *E. coli* NCTC 8164 after pressure treatment
582 at 400 MPa for 10 min (solid bars) or osmotic shock induced by transfer from TSE buffer
583 containing 20% sucrose to distilled water (open bars).

584

585 **Fig 1.**



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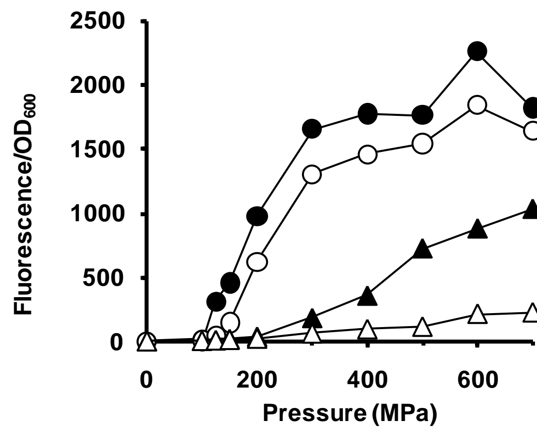
589 **Fig. 2**



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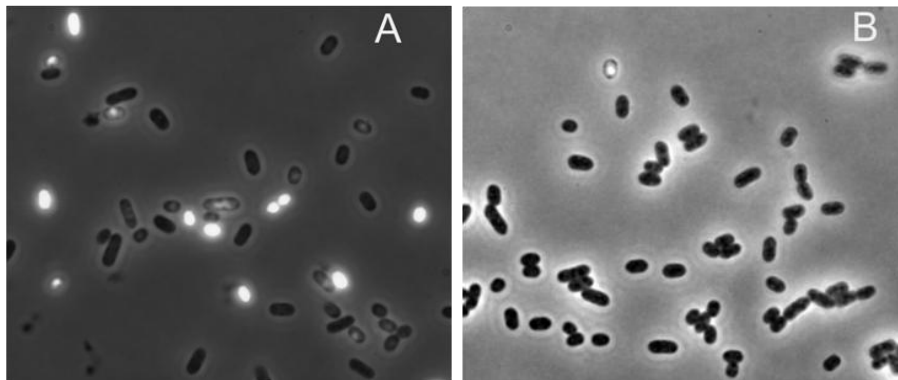
593 **Fig 3.**



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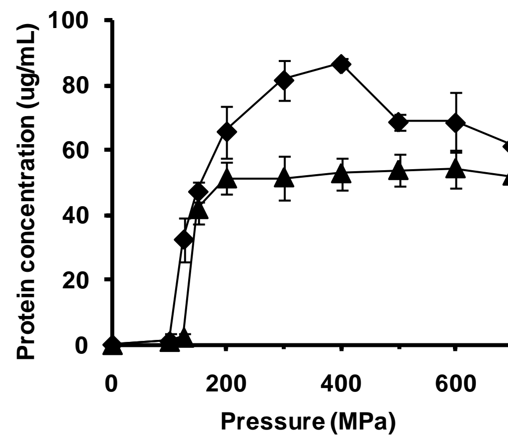
596 **Fig 4.**



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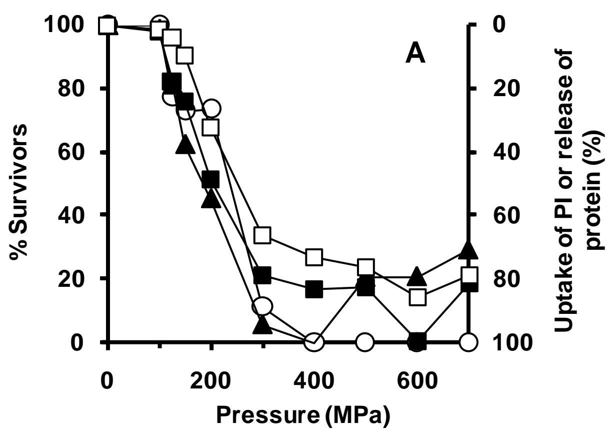
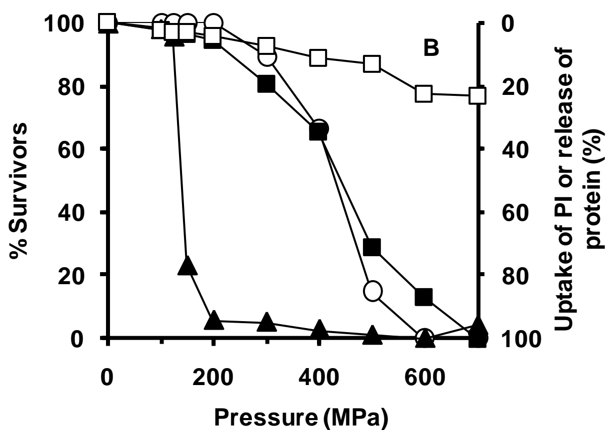
599 **Fig 5.**





600 **Fig 6.**

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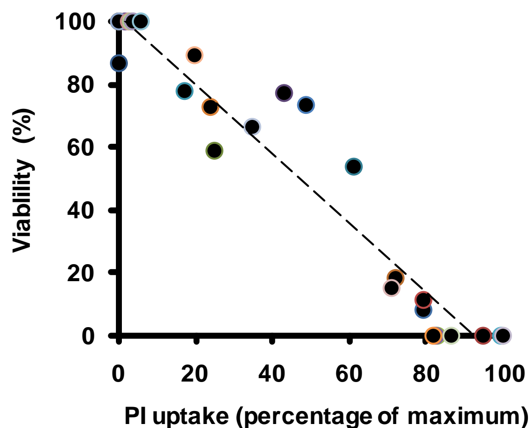

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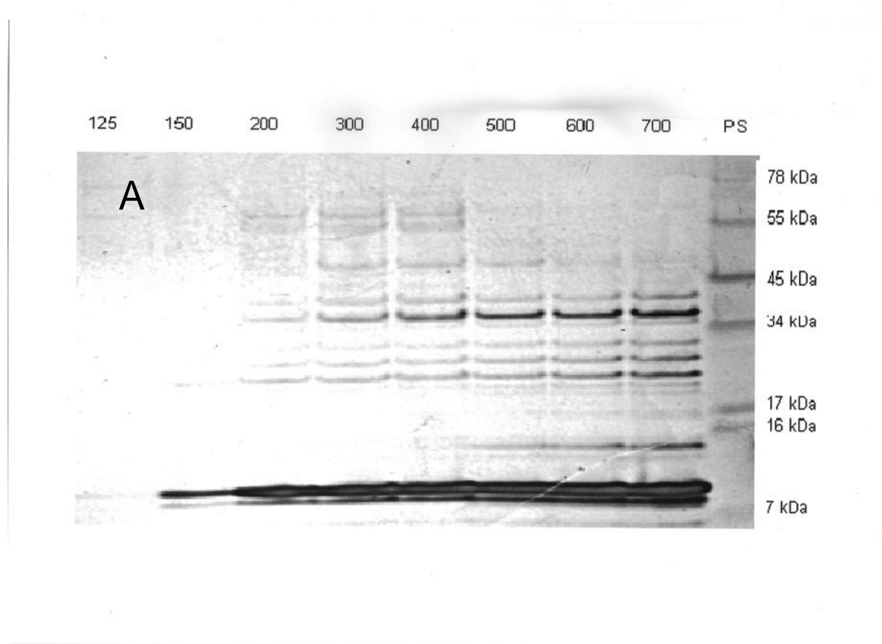
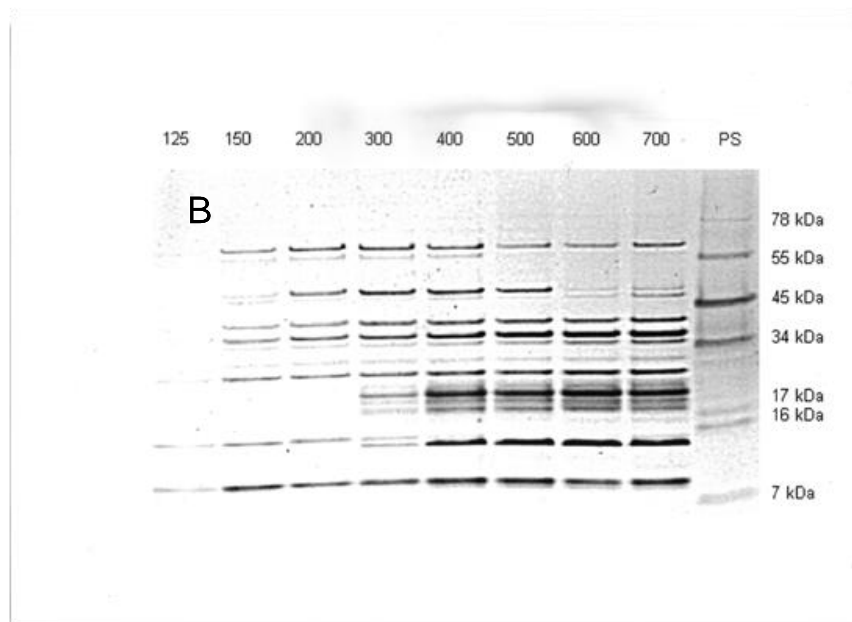
603 **Fig 7A**



604 **Fig 7B.**



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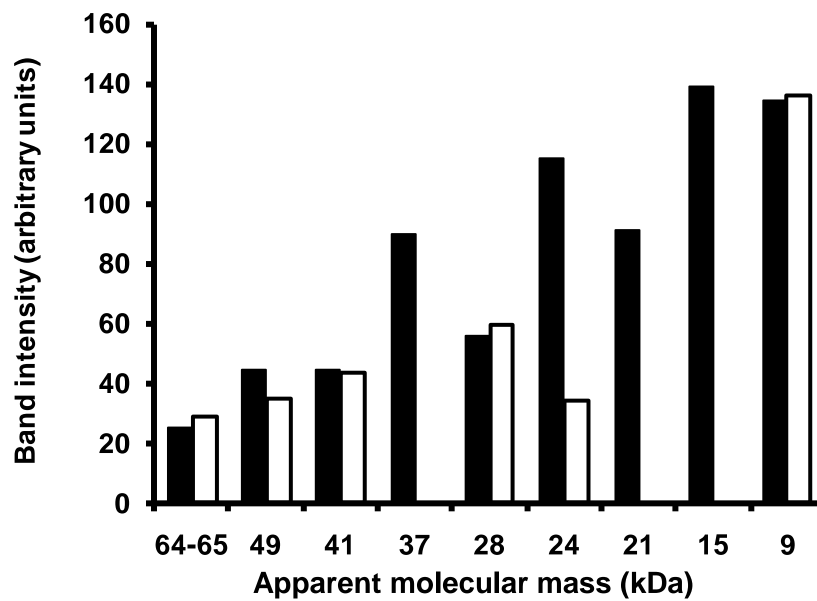
610 **Fig 8.**



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