Effect of the food matrix on pressure resistance of Shiga-toxin producing *Escherichia coli*

Hui Li[1], Rigoberto Garcia-Hernandez[1], Darcy Driedger[2], Lynn M. McMullen[1], and Michael Gänzle[1,3,*]

[1] University of Alberta, Department of Agricultural, Food and Nutritional Science, Edmonton, Canada

[2]Alberta Agriculture and Forestry, Food Processing Development Centre, Leduc, Canada

[3]Hubei University of Technology, School of Food and Pharmaceutical Engineering, Wuhan, China

* Corresponding author footnote:

Michael Gänzle

University of Alberta, Dept. of Agricultural, Food and Nutritional Science,

4-10 Ag/For Centre Edmonton, AB,

Canada, T6G 2P5.

Tel, + 1 780 492 0774; fax: + 1 780 492 4265

E-mail: mgaenzle@ualberta.ca.

19 **Abstract**

20 The pressure resistance of Shiga-toxin producing *Escherichia coli* (STEC) depends on food matrix.
21 This study compared the resistance of two five-strain *E. coli* cocktails, as well as the pressure
22 resistant strain *E. coli* AW 1.7, to hydrostatic pressure application in bruschetta, tzatziki, yoghurt and
23 ground beef at 600 MPa, 20 °C for 3 min and during post-pressure survival at 4 °C. Pressure reduced
24 STEC in plant and dairy products by more than 5 logs (cfu/ml) but not in ground beef. The pH
25 affected the resistance of STEC to pressure as well as the post-pressure survival. *E. coli* with food
26 constituents including calcium, magnesium, glutamate, caffeic acid and acetic acid were treated at
27 600 MPa, 20 °C. All compounds exhibited a protective effect on *E. coli*. The antimicrobial
28 compounds ethanol and phenylethanol enhanced the inactivation by pressure. Calcium and
29 magnesium also performed protective effects on *E. coli* during storage. Glutamate, glutamine or
30 glutathione did not significantly influence the post-pressure survival over 12 days. Preliminary
31 investigation on cell membrane was further performed through the use of fluorescence probe
32 1-N-phenylnaphthylamine. Pressure effectively permeabilised cell membrane, whereas calcium
33 showed no effects on membrane permeabilisation.

34 **Keywords**

35 High pressure; *Escherichia coli*; Shiga-toxin producing *E. coli* (STEC); Food matrix; Membrane

36

1. Introduction

The application of hydrostatic pressure for food preservation experiences worldwide commercial growth (Balasubramaniam et al., 2015; Georget et al., 2015). Pressure ranging from 400 – 600 MPa eliminates pressure-sensitive pathogens and spoilage organisms (Patterson et al.,. 1995, Balasubramaniam et al., 2015; Georget et al., 2015); however, some foodborne pathogens including *Staphylococcus aureus* and Shiga-toxin producing *Escherichia coli* (STEC) are highly resistant to pressure (Hauben et al., 1997; Tassou et al., 2008; Liu et al., 2015, Gänzle and Liu, 2015). STEC cause severe foodborne disease; they are primarily associated with ruminants but plant foods including fruit juice and produce are also recognized as vectors for their transmission (Frenzen et al., 2005; Karch et al., 2005). Pressure treatments aiming to eliminate pathogens in fresh meat or plant products thus target STEC. The pressure resistance of *E. coli* is variable (Hauben et al., 1997; Liu et al., 2015). The lethality of 600 MPa towards 100 strains of STEC differed by more than 5 log(cfu/mL) and approximately 30% of strains of STEC were highly pressure resistant (Liu et al., 2015). The food matrix, process temperature, and pH also influence the pressure resistance of *E. coli* (Gänzle and Liu, 2015). The pressure resistance of several strains of *E. coli* was assessed in different food products; however, the comparison of literature data is confounded by the use of different process parameters in different studies (Garcia-Graells et al., 1998; Lavinas et al., 2008; Liu et al., 2012 and 2015; Reineke et al., 2015).

As pressure processing alone does not sufficiently inactivate STEC, the use of additional antimicrobial hurdles is necessary. The targeted design of improved pressure processes necessitates an improved understanding of the role of matrix constituents on pressure resistance. Multiple

58 pressure-sensitive targets have been described in *E. coli*. Pressure permeabilises the outer membrane
59 of Gram-negative bacteria (Gänzle and Vogel, 2001; Ritz et al., 2000). Pressure also induces a phase
60 transition in the cytoplasmic membrane (Casadei et al., 2002), resulting in the dissipation of the
61 proton motive force (Wouters et al., 1998; Winter, 2002; Kilimann et al., 2005), and the elimination
62 of acid resistance (Garcia-Graells et al., 1998). Ribosomes, protein folding, and the disposal of
63 misfolded proteins also are pressure-sensitive targets in *E. coli* (Niven et al, 1999; Aertsen et al.,
64 2004; Govers et al., 2014). Moreover, pressure induces oxidative stress in *E. coli* which enhances
65 pressure-mediated inactivation (Aertsen et al., 2005). In keeping with pressure-induced oxidative
66 stress as "suicide mechanism" in *E.* coli, thiol reactive antimicrobials exhibited a strong synergistic
67 bactericidal activity with pressure (Feyaerts et al., 2015).

68 The use of hurdle technology in food included combinations of pressure with high (40 – 60°C)
69 temperature (Liu et al., 2012, Reineke et al., 2015). However, even moderately elevated temperatures
70 in the range of 40 – 60°C may alter food quality when combined with high pressure (Omama et al.,
71 2011). The pressure treatment at low pH also eliminates *E. coli* after pressure treatment (Alpas et al.,
72 2000; Garcia-Graells et al., 1998) but not all food products can be acidified. The synergistic activity
73 of antimicrobial compounds, including thiol-reactive antimicrobials and bacteriocins, was
74 demonstrated in model systems but rarely in food. This study therefore aimed to compare the
75 pressure resistance of *E. coli* in foods and to assess the matrix effect on pressure resistance.
76 Experiments were performed with a cocktail of 5 pathogenic *E. coli* and a cocktail of non-pathogenic
77 strains (Garcia-Hernandez et al., 2015). Moreover, model studies were carried out in buffer systems
78 with the heat- and pressure resistant *E. coli* AW1.7 (Dlusskaya et al., 2011; Liu et al., 2012).

2. Materials and Methods

2.1 Bacterial strains and culture conditions.

This study employed two cocktails each containing five strains of *E. coli* (Garcia-Hernandez et al., 2015). One strain cocktail was composed of four strains of STEC (05-6544, 03-2832, 03-6430 and C0283) and the enteropathogenic *E. coli* O145:NM PARC 449. These strains were selected to represent the most pressure resistant strains of more than 100 strains of STEC (Liu et al., 2015). *E. coli* PARC 449 harbors the locus of enterocyte effacement but not the gene coding for the shiga-like toxin (Liu et al., 2015; Mercer et al., 2015). The second strain cocktail was composed of the non-pathogenic *E. coli* AW1.7, AW1.3, GM16.6, DM18.3 and MG1655. *E. coli* strains were streaked from the frozen (−80 °C) stock cultures onto Luria-Bertani (LB) agar (Difco, Sparks, MD, USA) and incubated for 24 h at 37 °C. Strains were subcultured in LB broth and incubated at 37 °C and 200 rpm for 16-18 h. Equal volumes of each of the five single cultures were mixed to form the respective strain cocktails.

2.2 Preparation of samples for pressure treatment.

Bruschetta (pH 4.1) and tzatziki (pH 4.0) were obtained from Food Processing and Development Centre located in Leduc of Alberta, Canada. The formulation of the products is shown in Table 1. Plain low-fat yoghurt (pH 4.0, Astro, Canada) and ground beef (20% fat) were purchased from a local supermarket. Products were used as obtained, or after adjusting the pH to 5.5 or 7.5. Cell counts of each batch of each food product were quantified by surface plating on LB agar; all cell counts were less than 2.6 log(cfu/g). Strain cocktails or the pressure resistant strain *E. coli* AW1.7

99 (1.5 ml) were inoculated into the food products (10 ml or g) to an initial population of around
100 10^7-10^8 cfu/ml. The inoculated food products were homogenized for 2 min. Subsamples of 250 µL or
101 µg were packed into 3-cm R3603 tygon tubes (Akron, PA, USA) and heat-sealed after exclusion of
102 air. Prior to pressure treatment, tubes were placed into a 2-ml Cryovial (Wheaton, Millville, NJ)
103 filled with 10% bleach.

104 2.3 Pressure treatments of food samples

105 Pressure treatments were carried out as described previously (Liu et al., 2012). Samples were treated
106 in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) at 600 MPa and 20°C for 3
107 min. After the pressure treatment, the cell counts were determined by serial 10-fold dilution and
108 surface plating on LB agar. Lactic acid bacteria in untreated or pressure treated yoghurt were
109 enumerated by surface plating on modified de Man Rogosa Sharpe medium. Samples were stored at
110 4 °C over 16 days and cell counts were determined during storage. Cell counts of uninoculated and
111 untreated as well as uninoculated and pressure-treated samples were used as controls. During
112 enumeration of the colonies, the colony morphology was noted to determine whether it matched the
113 colony morphology of the *E. coli* inoculum. All experiments were performed in triplicate.

114 2.4 Effect of food constituents on pressure resistance of *E. coli*.

115 The effect of the following food constituents on the pressure resistance of *E. coli* was evaluated:
116 calcium, magnesium, glutamate, acetic acid and caffeic acid. Experiments were carried out in 100
117 mmol/L MES (Fisher, Ottawa, Canada) buffer at pH 5.5. The food constituents were used at the
118 following concentration: 10 mmol/L calcium chloride (Sigma, new Jersey, USA), 10 mmol/L

magnesium sulfate heptahydrate (Sigma, new Jersey, USA), 10 mmol/L L-glutamic acid monosodium salt hydrate (Sigma, new Jersey, USA), 1 g/L caffeic acid (Sigma, St. Louis, USA) and 0.1% acetic acid in MES buffer. MES buffer or MES buffer supplemented with the respective compounds was mixed with an overnight culture of *E. coli* AW 1.7 in a volumetric ratio of 9:1 (vol:vol). Samples were prepared for pressure treatment as described above and treated at 600 MPa and 20 °C for 0 to 16 min. Cell counts of untreated and pressure-treated samples were determined by surface plating on LB agar. Experiments were performed in triplicate.

2.5 Determination of effects of ethanol and phenylethanol on pressure resistance.

The effect of ethanol and phenylethanol on pressure resistance was evaluated in acetate:MES:MOPS buffer (Sigma-Aldrich, St. Louis, MS, USA). The use of three buffering components with different pKa allows changing the buffer pH without changing the buffering component. The pH of the buffer was adjusted to 5.5. Ethanol and 2-phenylethanol (Sigma) were added to the buffer to a final concentration of 2% and 20 mM, respectively. Addition of *E. coli* AW1.7, and preparation and treatment of cultures was performed as described above. Cell counts of untreated and pressure-treated samples were determined on LB and Violet Red Bile agar (Difco) plates to enumerate the surviving with or without injury. Experiments were performed in triplicate.

2.6 Effect of food constituents on survival of *E. coli* during post-pressure refrigerated storage.

Cultures of *E. coli* AW1.7 were washed twice with imidazole buffer (pH 5.5) and supplemented with 10 mmol/L of calcium, magnesium, L-glutamine (Fluka, Seelze, Germany), L-glutamic acid, or L-glutathione (Sigma-Aldrich). Treatment was performed at 600 MPa pressure at 20 °C for 3 min,

139 followed by refrigerated storage at 4 ℃ over 12 days. Cell counts were obtained as described in 2.5.
140 Experiments were performed in triplicate.

141 2.7 Effects of calcium on permeability of cell membrane.

142 Outer membrane permeability was determined with the probe 1-*N*-phenylnaphtylamine (NPN)
143 (Helander and Matila-Sandholm, 2000). In brief, a solution of 10 mmol/L NPN in ethanol was
144 diluted to 20 μmol/L in imidazole (IM) buffer. *E. coli* AW1.7 cultures suspended in IM buffer (pH
145 5.5) supplemented with 10 mmol/L calcium, or not, were treated with 100, 300, or 500 MPa for 3
146 min at 20℃. Aliquots of 100 μL of pressure treated samples were mixed with 100 μL of the NPN
147 solution and the fluorescence intensity was measured using a fluorescence spectrofluorometer
148 (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada) at an excitation and emission
149 wavelength of 340 and 420 nm, respectively. Each assay was performed in triplicate. Results were
150 calculated by correcting the relative fluorescence of cultures with the reagent blank (28 ±1 RFU) and
151 dividing the fluorescence of treated cells by the fluorescence of untreated cells, and reported as NPN
152 uptake factor.

153 2.8 Statistical analysis.

154 Significant differences between cell counts were determined by two way analysis of variance in SAS.
155 A Student Newman Keuls test was used to determine differences among means within each
156 time.Significance was assessed at an error probability of 5% ($p<0.05$).

157 **3. Results**

158 3.1 The effects of food matrix on pressure resistance.

159 We were initially interested in the survival of two pressure resistant strain cocktails of *E. coli* in
160 bruschetta, a tomato-based sauce, and tzatziki, a sauce containing yoghurt, cucumbers, and garlic.
161 The products were inoculated with two strain cocktails and treated at conditions matching current
162 industrial practice. Cells counts of both *E. coli* cocktails in bruschetta and tzatziki after pressure
163 treatment were reduced by more than 5 log(cfu/ml) and remained below the detection limit during
164 storage (Figure 1). Similar cell counts were observed in products inoculated with the cocktail
165 composed of pathogenic strains and the cocktail composed of surrogate strains. Cell counts after
166 pressure treatment were not different from the uninoculated control. Moreover, the colony
167 morphology of cells cultured after pressure treatment demonstrated that these counts originated from
168 background microbiota rather than surviving *E. coli* (Figure 1).

169 The sensitivity to pressure of the two strain cocktails in bruschetta and tzatziki was greater when
170 compared to the survival of the same cocktails in beef (Garcia-Hernandez et al., 2015). To determine
171 whether the low pH accounts for this difference, the pH of bruschetta and tzatziki was adjusted to 5.5,
172 equivalent to the pH of ground beef. Bruschetta was inoculated with the two strain cocktails; tzatziki
173 was inoculated only with the non-VTEC cocktail. Products were subjected to pressure treatment,
174 followed by refrigerated storage (Figure 2). Treatments in ground beef served as comparison (Figure
175 2C). Increasing the pH increased pressure resistance of *E. coli* slightly (bruschetta, Fig. 2A) or
176 substantially (tzatziki, Figure 2B). The lethality of pressure treatment in tzatziki was similar to that
177 of pressure treatment in ground beef; however, cell counts of *E. coli* in tzatziki were reduced to
178 levels below the detection limit after storage while cell counts of *E. coli* in ground beef were reduced
179 by less than 90%. These results demonstrate that the food matrix differentially affects survival during

180 pressure treatment and survival during post-pressure refrigerated storage even if the pH is adjusted to
181 the same value.

182 To further confirm the role of pH on survival of *E. coli*, treatments were performed with bruschetta
183 and tzatziki at a pH of 7.5, and with plain yoghurt after adjustment to pH 4.0 (unadjusted), 5.5, and
184 7.5. The two strain cocktails composed of pathogenic and non-pathogenic strains exhibited similar
185 survival during and after pressure treatment in previous experiments, and among the surrogate strains,
186 *E. coli* AW 1.7 is a typical pressure resistant strain. Therefore, subsequent experiments were carried
187 out only with *E. coli* AW 1.7. Adjusting the pH of bruschetta and tzatziki to 7.5 did not substantially
188 alter the lethality of pressure treatment (Fig. 2 and data not shown). Pressure treatments in yoghurt
189 demonstrated the effect pH on the lethality of pressure and post-pressure refrigerated storage (Figure
190 3). At pH 4.0, pressure treatment reduced cell counts of *E. coli* by more than 5 log(cfu/mL). At pH
191 5.5, the resistance of *E. coli* to pressure was substantially increased but cell counts were reduced to
192 less than 2 log(cfu/mL) after 4 d of refrigerated storage. Treatments in yoghurt at pH 7.5 did not
193 change the lethality of pressure when compared to treatments at pH 5.5, however, cell counts
194 remained unchanged during storage (Fig 3). Of note, cell counts of lactic acid bacteria were below
195 the detection limit after pressure treatment at any pH (data not shown), indicating that *Streptococcus*
196 *thermophilus* and *Lactobacillus delbrueckii* are substantially more pressure sensitive than *E. coli*.

197 3.2 Effect of food constituents on pressure resistance of *E. coli*.

198 Above data demonstrate that food constituents other than the pH affect survival of *E. coli* after
199 pressure treatment and refrigerated storage. To identify food constituents that account for these

200 effects, we designed model experiments with or without addition of individual compounds. Calcium,
201 magnesium, and glutamate were chosen because they were suggested to exert protective effects
202 (Niven et al., 1999; Kilimann et al., 2005) and occur in meat or dairy products but at a lower
203 concentration in plant foods. Acetic and caffeic acids were selected as antimicrobial organic acids
204 with a potential synergistic effect (Sanchez-Maldonado et al., 2011) that are present in plant foods.
205 Experiments in buffer systems were carried out with *E. coli* AW1.7 as pressure-resistant model
206 organism. Addition of magnesium, or glutamate protected *E. coli* AW 1.7 against pressure-induced
207 inactivation (Figure 4A). Surprisingly, caffeic acid and acetic acid also protected *E. coli* after 16 min
208 of treatment when compared to the control without additives (Figure 4A). The biophysical properties
209 of the membrane play a decisive role in the pressure resistance of *E. coli* (Casadei et al., 2002;
210 Charoenwong et al., 2011), therefore, further experimentation manipulated membrane properties of *E.*
211 *coli* by addition of ethanol or phenylethanol. Ethanol and phenylethanol strongly enhanced the lethal
212 effect of pressure on *E. coli* AW1.7 although the concentrations used, 2% and 20 mmol / L, are not
213 lethal or inhibitory to *E. coli* (Figure 4B).

214 3.3 Effect of food constituents on survival of *E. coli* during post-pressure refrigerated storage.

215 Because individual food products differentially affected the resistance of *E. coli* during pressure
216 treatment and post-pressure refrigerated storage, we additionally explored the role of selected food
217 constituents on post-pressure survival. The selection of compounds focused on potentially protective
218 compounds that occur in meat, i.e. calcium, magnesium, glutamine, glutamate, and glutathione.
219 None of these compounds affected survival of *E. coli* after 3 min at 600 MPa (Fig. 5). However, cell
220 counts of *E. coli* in buffer at pH 5.5 were reduced by more than 5 log(cfu/mL) over 12 days of

221 post-pressure refrigerated storage (Fig. 5A and 5B). Survival was improved by addition of calcium
222 or magnesium (Fig. 5A); other compounds had no effect on survival of *E. coli* after pressure
223 treatment (Fig. 5B).

224 3.4 Effects of calcium on the integrity of the outer membrane.

225 Divalent cations interact with multiple cellular components, including ribosomes, the cytoplasmic
226 membrane, and the outer membrane. The outer membrane is a pressure sensitive target in *E. coli* that
227 is perturbed by less than 300 MPa (Gänzle and Vogel, 2001). To determine whether the protective
228 effect of calcium related to stabilization of the outer membrane, we used NPN to probe the integrity
229 of the outer membrane of *E. coli* AW1.7 that was pressure treated in presence or absence of 10
230 mmol/L calcium (Table 2). Pressure fully permeabilised outer membrane of *E. coli* after treatment
231 with 300 MPa or higher (Table 2). The addition of calcium did not influence the permeability of the
232 outer membrane of pressure treated cells.

233 **4. Discussion**

234 The resistance of *E. coli* to pressure is strain-, pH-, and matrix-dependent (Garcia-Graells et al., 1998;
235 Alpas et al., 2000; Liu et al., 2015; Garcia-Hernandez et al., 2015; Reineke, et al., 2015; Gänzle and
236 Liu, 2015). This study demonstrated that treatment with 600 MPa for 3 min in bruschetta or tzatziki
237 reduce cell counts of two strain cocktails by more than 5 log(cfu/mL). The pathogenic and surrogate
238 strain cocktails exhibited a comparable resistance to pressure in bruschetta and dairy products; in
239 keeping with prior results that were obtained in ground beef (Garcia-Hernandez et al., 2015). The
240 strain cocktail composed of surrogate non-pathogenic strain is thus useful for validation of pressure

241 processes in a wider range of products. However, we also demonstrated that the lethality of the same
242 pressure treatment on the same strains differs by up to 4 log(cfu/mL) when applied to different foods
243 or at different pH values.

244 The effect of pH on the lethality of pressure treatment is well documented. Pressure inactivates
245 bacterial F_0F_1-ATPases thus impairs ability to maintain a transmembrane pH gradient (ΔpH)
246 (Wouters et al., 1998, Kilimann et al., 2005). The pressure mediated loss of pH gradients and acid
247 resistance eliminates *E. coli* in acidic food products after pressure treatment (Garcia-Graells et al.,
248 1998; Jordan et al., 2001, Pagán et al., 2001). We demonstrated that this elimination of *E. coli* after
249 pressure treatment occurs even at modest levels of acidity, i.e. pH 5.5, but not at pH values near
250 neutral. However, the pH only partially accounted for the different resistance of *E. coli* in different
251 foods, demonstrating that other food constituents account for this effect. Our analysis of possible
252 constituents accounting for this effect was guided by the differences in food composition as well as
253 literature data on pressure resistance in *E. coli*.

254 Glutathione contributes to redox homeostasis in *E. coli* (Carmel-Harel & Storz, 2000), and may thus
255 counteract the pressure mediated "oxidative suicide" of *E. coli* (Aertsen et al., 2005, Malone et al.,
256 2006). Meat but not dairy products or tomatoes are rich in low-molecular weight thiols. However,
257 glutathione did not change the pressure resistance or the post-pressure survival of *E. coli*. Caffeic
258 acid, used as representative of antimicrobial plant phenolic compounds, and acetic acid, a food
259 preservative, exhibited a modest protective effect on pressure resistance of *E. coli*. Organic acids
260 alter the pressure-induced pH shift but cosmotropic and specific ion effects additionally play a role,
261 making the effect of ions difficult to interpret (Gayán et al, 2013, Molina-Gutierrez et al., 2002). The

262 protective effect of caffeic acid is nevertheless remarkable because caffeic acid was used at 1 g/L, a
263 concentration which exceeds the MIC against *E. coli* AW1.7 (Sánchez-Maldonado et al., 2011).
264 Acidification of the cytoplasm by undissociated caffeic acid (Choi and Gu, 2001; Cueva et al., 2010;
265 Sánchez-Maldonado et al., 2011) may support the pressure-mediated acidification of the cytoplasm.
266 However, caffeic acid also influences the fluidity of the cytoplasmic membrane (Keweloh et al.,
267 1991) and this interaction may account for its protective effect during pressure treatment. The
268 divergent effect of the antimicrobial compounds nisin and reutericyclin on pressure-assisted
269 inactivation of *Bacillus* and *Clostridium* endospores has been related to their divergent effects on
270 spore membrane fluidity (Hofstetter et al., 2013).

271 Glutamate decarboxylation is the most effective system for pH homeostasis of acid challenged
272 *E. coli*. Glutamate decarboxylation consumes intracellular protons, exports negative charges and thus
273 contributes to generation of the pmf (Foster 2004; Feehily and Karatzas, 2012; Teixeira et al., 2014).
274 Glutamate mediated acid resistance was more pressure resistant than glucose-mediated acid
275 resistance and thus improved survival during post-pressure acid challenge (Kilimann et al., 2005). In
276 food, glutamate dependent acid resistance is complemented by glutamine deamination, which also
277 consumes an intracellular proton (Lu et al., 2013). Surprisingly, glutamate addition did not affect
278 post-pressure survival of *E. coli*. Refrigerated storage of *E. coli* may have reduced the rate of
279 glutamate decarboxylation; prior studies incubated *E. coli* at a temperature permitting growth and
280 metabolism (Kilimann et al., 2005).

281 The accumulation of cyclopropane fatty acids in the membrane of *E. coli* increases its pressure
282 resistance (Casadei et al., 2002; Charoenwong et al., 2011). Ethanol and phenylethanol fluidize the

283 membrane and thus antagonize pressure effects on bacterial membranes (Welch and Bartlett, 1998;
284 Huffer et al, 2011); however, membrane-bound proteins are more sensitive to pressure-mediated
285 denaturation when embedded in a liquid crystalline membrane (Ulmer et al., 2002).

286 Divalent cations such as calcium and magnesium protect *E. coli* against pressure inactivation
287 (Hauben et al., 1998; Gayán et al., 2013). For example, Ca^{2+} in concentrations ranging from 0.5 to 80
288 mmol/L increased the pressure resistance of *E. coli* at 300 MPa, and this effect increased
289 proportional to the calcium concentration (Hauben et al., 1998). Our data conform with Hauben et al.,
290 (1998) who concluded that the protective effect of Ca^{2+} is not related to the stabilization of the outer
291 membrane. We extend prior data by demonstrating that the effect of Ca^{2+} and Mg^{2+} on the
292 post-pressure survival is more pronounced than the effect on survival during pressure treatment. The
293 protective effect of Ca^{2+} and Mg^{2+} may thus partially explain the relative resistance of *E. coli* in meat
294 (rich in magnesium), dairy products (rich in calcium and magnesium) and bruschetta (low levels of
295 divalent cations).

296 In conclusion, the food matrix strongly influenced the pressure-mediated inactivation of STEC and
297 EPEC. The product pH influenced both the survival of *E. coli* during pressure treatment and the
298 survival after pressure treatment. However, differences in the product pH did not explain the
299 product-specific effect on pressure resistance of *E. coli*. Remarkably, divalent cations exhibited a
300 protective effect on *E. coli* during post-pressure refrigerated storage. In combination with pH effect,
301 the presence of divalent cations in dairy and meat products accounts for the higher resistance of *E.*
302 *coli*. Membrane-active antimicrobial compounds that increase the membrane fluidity exhibit
303 synergistic activity with pressure-mediated elimination of *E. coli* in food.

Acknowledgements

Financial support for this project was provided by the Alberta Crop Industry Development Funds (QFH-11-031), Alberta Innovates Biosolutions (2013R048R), and the Alberta Livestock and Meat Agency (2013R048R, 2012F137R). Hui Li is supported by China Scholarship Council, Rigoberto Garcia Hernandez was supported by the Mexican National Council for Science and Technology.

References

Aertsen, A., Spiegeleer, P.D., Vanoirbeek, K., Lavilla, M., Michiels, C. W., 2005. Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli.* Appl. Microbiol. Biotechnol. 71, 2226-2231.

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. Int. J. Food Microbiol. 60, 33-42.

Balasubramaniam, V.M., Martinez-Monteagudo, S.I., and Gupta, R., 2015. Principles and application of high pressure - based technologies in the food industry. Ann. Rev. Food Sci. Technol. 6, 19.1-19.28.

Carmel-Harel, O., Storz, G., 2000. Roles of the glutathione- and thioredoxin- dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress.

Casadei, M.A., Manas, P., Nilven, G., Needs, E., Mackey, B.M., 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Appl. Environ. Microbiol. 68, 5965-5972.

323 Charoenwong, D., Andrews, S., Mackey, B., 2011. Role of RpoS in the development of cell envelope
324 resilience and pressure resistance in stationary-phase *Escherichia coli.* Appl. Environ. Microbiol. 77,
325 5220-5229.

326 Choi, S.H., Gu, M.B., 2001. Phenolic toxicity e detection and classification through the use of a
327 recombinant bioluminescent *Escherichia coli*. Environ. Toxicol. Chem. 20, 248-255.

328 Cueva, C., Moreno-Arribas, M.V., Martín-Álvarez, P.J., Bills, G., Vicente, M.F., Basilio, A., Rivas,
329 C.L., Requena, T., Rodríguez, J.M., Bartolomé, B., 2010. Antimicrobial activity of phenolic acids
330 against commensal, probiotic and pathogenic bacteria. Res. Microbiol. 161, 372-382.

331 Dlusskaya, E., McMullen, L.M., Gänzle, M.G. 2011. Characterization of an extremely heat resistant
332 *Escherichia coli* obtained from a beef processing facility. J. Appl. Microbiol. 110, 840–849.

333 Feehily, C., Karatzas, K.A.G., 2012. Role of glutamate metabolism in bacterial responses towards
334 acid and other stresses. J. Appl. Microbiol. 114, 11-24.

335 Feyaerts, J., Rogiers, G., Corthouts, J., Michiels, C.W. 2015. Thiol-reactive natural antimicrobials
336 and high pressure treatment synergistically enhance bacterial inactivation. Innov. Food Sci. Emerg.
337 Technol. 27, 26-34.

338 Foster, J.W., 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat. Rev.
339 Microbiol. 2, 898-907.

340 Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working
341 Group., 2005. Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States.

342 J. Food Prot. 68, 2623-2630.

343 Gänzle, M.G., Vogel, R.F., 2001. On-line fluorescence determination of pressure mediated outer
344 membrane damage in *Escherichia coli.* System. Appl. Microbiol, 24: 477-485.

345 Gänzle, M.G., Liu, Y., 2015. Mechanisms of pressure-mediated cell death and injury in Escherichia
346 coli: from fundamentals to food applications. Frontiers Food Microbiol. 6:599

347 Garcia-Graells, C., Hauben, K.J.A., Michiels, C.W., 1998. High-pressure inactivation and sublethal
348 injury of pressure-resistant *Escherichia coli* mutants in fruit juices. Appl. Environ. Microbiol. 64,
349 1566-1568.

350 Garcia-Hernandez, R., McMullen, L., Gänzle, M.G., 2015. Development and validation of a
351 surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli.*
352 Int. J. Food Microbiol. 205, 16-22.

353 Gayán, E., Condó, S., Álvarez, I., Nabakabaya, M., Bernard, M., 2013. Effect of pressure-induced
354 changes in the ionization equilibria of buffers on inactivation of *Escherichia coli* and *Staphylococcus*
355 *aureus* by high hydrostatic pressure. Appl. Environ. Microbiol. 79, 4041-4047.

356 Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., Rauh, C., Knorr, D.,
357 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices: A
358 review. Innov. Food Sci. Emerg. Technol. 27, 1-14.

359 Govers, S.K., Dutré, P., Aertsen, A. 2014. In vivo disassembly and reassembly of protein aggregates
360 in *Escherichia coli*. J. Bacteriol. 196, 2325-2332

Hauben, K.J., Bartlett, D.H., Soontjens, C.C., Cornelis, K., Wuytack, E.Y., Michiels, C. W. (1997). *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl. Environ. Microbiol. 63, 945-950.

Hauben, K.J.A., Bernaerts, K., Michiels, C.W., 1998. Protective effect of calcium on inactivation of *Escherichia coli* by high hydrostatic pressure. J. Appl. Microbiol. 85, 678-684.

Helander, I.M., Mattila-Sandholm, T., 2000. Fluorometric assessment of Gram-negative bacterial permeabilization. J. Appl. Microbiol. 88, 213-219.

Hofstetter, S., Winter, R., McMullen, L.M., Gänzle, M.G. 2013. In situ determination of membrane fluidity of *Clostridium* spp. during pressure-assisted thermal processing in combination with nisin or reutericyclin. Appl. Environ. Microbiol. 79, 2103-2106.

Huffer, S., Clark, M.E., Ning, J.C., Blanch, H.W., Clark, D.S., 2011. Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria, and archaea. Appl. Environ. Microbiol. 77, 6400-6408.

Jordan, S.L., Pascual, C., Bracey, E., Mackey, B.M. (2001). Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. J. Appl. Microbiol. 91, 463-469.

Karch, H., Tarr, P.I., Bielaszewaka, M., 2005. Enterohaemorrhagic *Escherichia coli* in human medicine. Int. J. Med. Microbiol. 295, 405-418.

Keweloh, H., Diefenbach, R., Rehm, H., 1991. Increase of phenol tolerance of *Escherichia coli* by

alternations of the fatty acid composition of the membrane lipids. Arch. Microbiol. 157, 49-53.

Kilimann, K.V., Hartmann, C., Vogel, R.F., Gänzle, M.G., 2005. Differential inactivation of glucose- and glutamate-dependent acid resistance of *Escherichia coli* TMW 2.497 by high pressure treatments. Syst. Appl. Microbiol. 28, 663-671.

Lavinas, F.C., Miguel, M.A.L., Lopes, M.L.M., Valente-Mesquita, V.L. 2008. Effect of high hydrostatic pressure on cashew apple (*Anacardium occidentale* L.) juice preservation. J. Food Sci. 73, 273–277.

Liu, Y., Betti, M., Gänzle, M.G., 2012. High pressure inactivation of *Escherichia coli*, *Campylobacter jejuni*, and spoilage microbiota on poultry meat. J. Food Prot. 75, 497-503.

Liu, Y, Gill, A, McMullen, L., Gänzle, M.G., 2015. Variation in heat and pressure resistance of verotoxigenic and non-toxigenic *Escherichia coli*. J. Food Prot. 78, 111-120.

Lu, P., Ma, D., Chen, Y., Guo, Y., Chen, G., Deng, H., Shi, Y., 2013. L-Glutamine provides acid resistance for *Escherichia coli* through enzymatic release of ammonia. Cell Res. 23, 635-644.

Malone, A.S., Chuang, Y-K., Yousef, A.E. 2006. Genes of *Escherichia coli* O157:H7 that are involved in high-pressure resistance. Appl. Environ. Microbiol. 72, 2661-2671.

Mercer, R.G., Zheng, J., Garcia-Hernandez, R., Ruan, L., Gänzle, M.G., McMullen, L.M., 2015. Genetic determinants of heat resistance in *Escherichia coli*. Front Microbiol. 6, 932.

Molina-Gutierrez, A., Stippl, V., Delgado, A., Gänzle, M.G., Vogel, R.F., 2002. In situ determination of the intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment.

399 Appl. Environ. Microbiol., 68, 4399-4406.

400 Niven, G.W., Miles, C.A., Mackey, B.M., 1999. The effects of hydrostatic pressure on ribosome
401 conformation in *Escherichia coli*: an *in vivo* study using differential scanning calorimetry.
402 Microbiology 145, 419-425.

403 Omana, D.A., Plastow, G., Betti, M. 2011. Effect of different ingredients on color and oxidative
404 characteristics of high pressure processed chicken breast meat with special emphasis on use of
405 β-glucan as a partial salt replacer. Innov. Food Sci. Emerg. Technol. 12, 244–254.

406 Pagán, R., Jordan, S., Benito, A., Mackey, B., 2001. Enhanced acid sensitivity of pressure-damaged
407 *Escherichia coli* O157 cells. Appl. Environ. Microbiol. 67, 1983-1985.

408 Patterson, M.F., Quinn, M., Simpson, R., Gilmour, A. 1995. Sensitivity of vegetative pathogens to
409 high hydrostatic pressure treatment in phosphate-buffered saline and foods. J. Food Prot. 58,
410 524-529.

411 Reineke, K., Sevenich, R., Hertwig, C., JanBen, T., Fröhling, A., Knorr, D., Wieler, L. H., Schlüter,
412 O., 2015. Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing
413 *Escherichia coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. Food
414 Microbiol. 46: 184-194.

415 Ritz, M., Feulet, M., Orange, N., Federighi, M., 2000. Effects of high hydrostatic pressure on
416 membrane proteins of *Salmonella typhimurium*. Int. J. Food Microbiol. 55, 115-119.

417 Sánchez-Maldonado, A.F., Schieber, A., Gänzle, M.G., 2011. Structure-function relationships of the

418 antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. J. Appl.
419 Microbiol. 111, 1176-1184.

420 Tassou, C.C., Panagou, E.Z., Samaras, F.J., Galiatsatou, P., Mallidis, C.G. (2008).
421 Temperature-assisted high hydrostatic pressure inactivation of *Staphylococcus aureus* in a ham
422 model system: evaluation in selective and nonselective medium. J. Appl. Microbiol. 104, 1764-1773.

423 Teixeira, J.S., Seeras, A., Sanchez-Maldonado, A.F., Zhang, C., Su, M.S., Gänzle, M.G., 2014.
424 Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. Food Microbiol.
425 42, 172-180.

426 Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R., Vogel, R.F. 2002. Effects of
427 pressure induced membrane phase transitions on HorA inactivation in *Lactobacillus plantarum*. Appl.
428 Environ. Microbiol., 68, 1088-1095.

429 Welch, T.J., Bartlett, D.H. 1998. Identification of a regulatory protein required for
430 pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9.
431 Mol. Microbiol. 27, 977-985.

432 Winter, R., 2002. Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid
433 mesophases, model biomembranes and proteins in solution at high pressure. Biochim Biophys. Acta.
434 1595:160-184.

435 Wouters, P. C., Glaasker, E., Smelt, J.P.P.M., 1998. Effects of high pressure on inactivation kinetics
436 and events related to proton efflux in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64,
437 509-514

Figure legends

Figure 1. Cell counts of bruschetta **(Panel A)** and tzatziki **(Panel B)** during storage at 4°C. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was used as control (△); note that the open triangles are partially obscured by the symbol representing inoculated and pressure treated products. Prior to storage, products were treated at 600 MPa and 20°C for 3 min (closed symbols) or at 0.1 MPa and 20°C (untreated control, open symbols). Data are shown as mean ± standard deviation of three independent experiments. Lines dropping below the x-axis indicate cell counts below the detection limit for microbial counts. The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

Figure 2. Cell counts of bruschetta **(Panel A)**, tzatziki **(Panel B)** and ground beef **(Panel C)** during storage at 4°C. The pH of bruschetta and tzatziki was adjusted to 5.5 prior to inoculation and treatment to match the pH of ground beef. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was used as control (△). Prior to storage, products were treated at 600 MPa and 20°C (closed symbols) or at 0.1 MPa and 20°C (untreated control, open symbols). Note that the treatment time for bruschetta and tzatziki (panels A and B) was 3 min while the treatment time in for ground beef (panel C) was 5 min. Data are shown as mean ±

standard deviation of three independent experiments. Lines dropping below the x-axis indicate cell counts below the detection limit. The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

Figure 3. Cell counts of yoghourt during storage at 4°C. The initial pH of yoghurt was 4.0 **(Panel A)**; the pH was also adjusted to 5.5 **(Panel B)** or 7.5 **(Panel C)** prior to inoculation and treatment. Products were inoculated with *E. coli* AW 1.7. Uninoculated product was used as control (△). Prior to storage, products were treated at 600 MPa and 20°C for 3 min (▼); untreated products were used as reference (○). Data are shown as mean ± standard deviation of three independent experiments. Cell counts of lactic acid bacteria in un-treated samples were around 8.4 log(cfu/ml); cell counts in all pressure treated samples were below the detection limit (data not shown). The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

Figure 4. Cell counts of *E. coli* AW1.7 after pressure treatment in buffer (pH 5.5) with or without additions of food constituents. For Panel A, the following compounds were added to the MES buffer: 10 mmol/L calcium (●), 10 mmol/L magnesium (▲), 10 mmol/L glutamate (▲), 1 g/L acetic acid (■) or 1 g / L caffeic acid (■); For Panel B, ethanol (2%, ▲) or phenylethanol (20mmol/L, ■) were added to the acetate:MES:MOPS buffer. Treatment in buffer without addition was used as control (○). Samples were treated with at 600 MPa and 20°C. The treatment effect is expressed as cell count

reduction [log(N_0/N)] where N_0 represents initial cell count and N represents cell counts after high pressure. Data are shown as mean ± standard deviation of three independent experiments.

Figure 5. Cell counts of *E. coli* AW1.7 after treatment at 600 MPa for 3 min at 20°C in imidazole buffer at a of pH 5.5. Treatments were performed in buffer without additives (white bars) or with addition of 10 mM calcium (grey bars), magnesium (black bars) **(Panel A)**, or with addition of glutamine (light grey), glutamate (dark grey), or glutathione (black) (**Panel B**). Viable cell counts were enumerated on LB agar before treatment, after 3 min pressure treatment, and after pressure treatment and 3, 6, or 12 days of refrigerated storage. Without pressure treatment, cell counts of *E. coli* were not significantly reduced during refrigerated storage (data not shown). Survival of *E. coli* in controls shown in Panel A and B was not significantly different ($P>0.05$). Data are shown as mean ± standard deviation of three independent experiments. Values in the same panel that were obtained at the same storage time and do not share a common superscript differ significantly ($P<0.05$).

Table 1. Product composition of bruschetta and tzatziki

Bruschetta (pH 4.1)	**%**	**Tzatziki (pH 4.0)**	**%**
Tomato	94.821	Cucumber	24.093
Balsamic Vinegar (6% acidic acid)	1.546	Sour Cream (14%)	34.36
Olive Oil	1.288	Plain Yogurt	34.36
Garlic (diced in oil)	1.031	Olive Oil	4.014
Basil Paste	0.644	Lemon Juice	1.608
Salt	0.386	Garlic (pre-chopped)	0.964
Black Pepper (80 mesh)	0.077	Salt	0.45
Xanthan Gum	0.155	Pepper	0.063
Crushed Chilis	0.052	Xanthan Gum	0.088

Table 2. Relative fluorescence of *E. coli* AW1.7 stained with 1-N-phenylnaphthylamine (NPN) before or after pressure treatment. Cells were treated at 100 - 500 MPa for 3 min at 20°C. Values are shown as means ± standard deviation of three independent experiments.

Sample	Relative Fluorescence	NPN uptake factor[a)]
Untreated cells	76±4	1
100 MPa	135±10	2.2
100 MPa + Ca	117±17	1.8
300 MPa	264±17	4.9
300 MPa + Ca	272±19	5
500 MPa	337±9	6.4
500 MPa + Ca	362±22	6.9

[a)]The NPN uptake factor was calculated by correcting the relative fluorescence of cultures with the reagent blank (28 ±1 RFU) and dividing the fluorescence of treated cells by the fluorescence of untreated cells.

Figure 1. Cell counts of bruschetta **(Panel A)** and tzatziki **(Panel B)** during storage at 4°C. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was used as control (△); note that the open triangles are partially obscured by the symbol representing inoculated and pressure treated products. Prior to storage, products were treated at 600 MPa and 20°C for 3 min (closed symbols) or at 0.1 MPa and 20°C (untreated control, open symbols). Data are shown as mean ± standard deviation of three independent experiments. Lines dropping below the x-axis indicate cell counts below the detection limit for microbial counts. The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

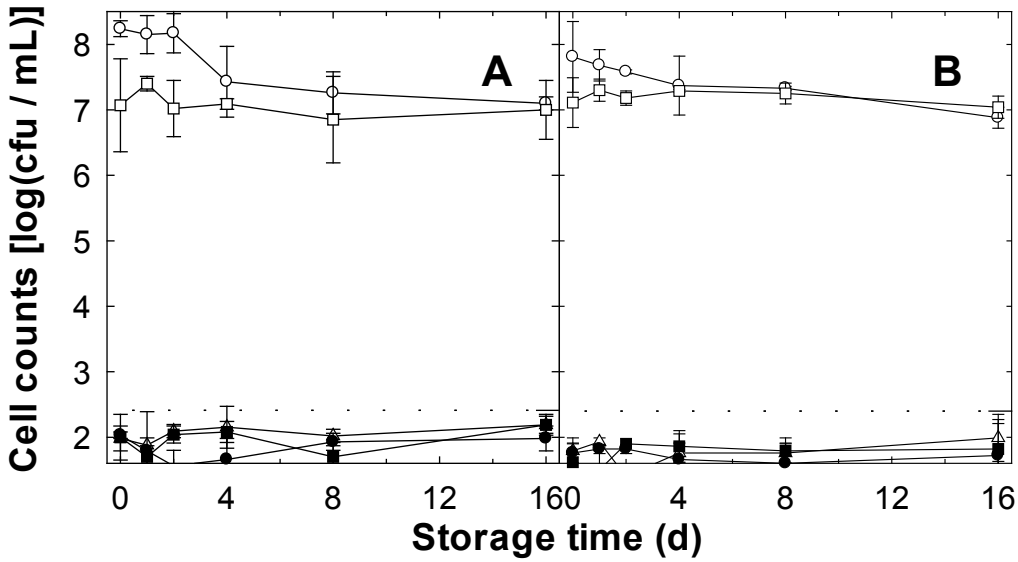

Figure 2. Cell counts of bruschetta **(Panel A)**, tzatziki **(Panel B)** and ground beef **(Panel C)** during storage at 4°C. The pH of bruschetta and tzatziki was adjusted to 5.5 prior to inoculation and treatment to match the pH of ground beef. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was used as control (△). Prior to storage, products were treated at 600 MPa and 20°C (closed symbols) or at 0.1 MPa and 20°C (untreated control, open symbols). Note that the treatment time for bruschetta and tzatziki (panels A and B) was 3 min while the treatment time in for ground beef (panel C) was 5 min. Data are shown as mean ± standard deviation of three independent experiments. Lines dropping below the x-axis indicate cell counts below the detection limit. The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

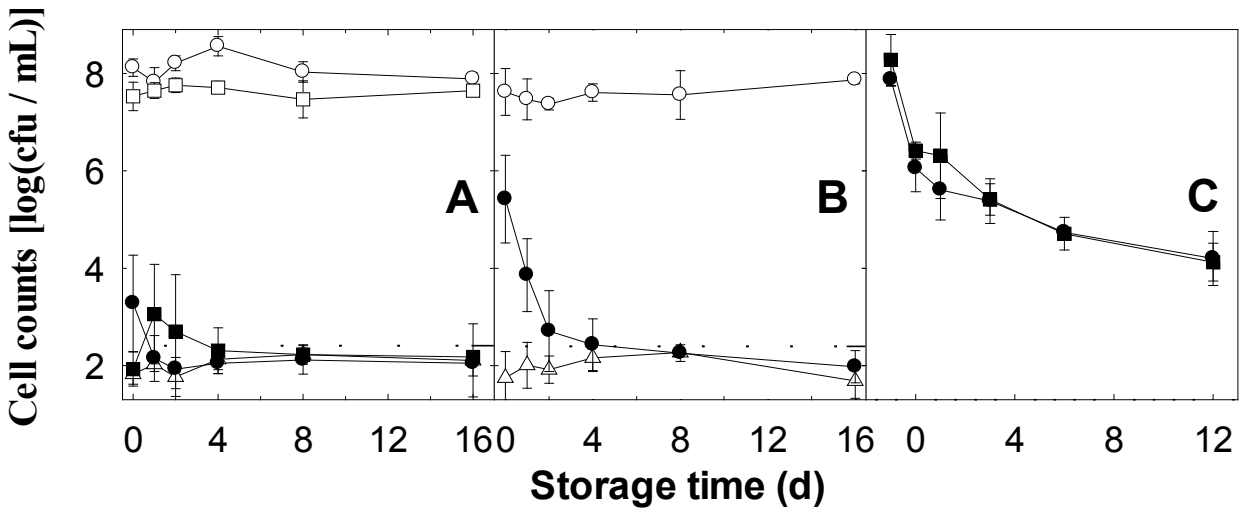

Figure 3. Cell counts of yoghurt during storage at 4°C. The initial pH of yoghurt was 4.0 **(Panel A)**; the pH was also adjusted to 5.5 **(Panel B)** or 7.5 **(Panel C)** prior to inoculation and treatment. Products were inoculated with *E. coli* AW 1.7. Uninoculated product was used as control (△). Prior to storage, products were treated at 600 MPa and 20°C for 3 min (▼); untreated products were used as reference (○). Data are shown as mean ± standard deviation of three independent experiments. Cell counts of lactic acid bacteria in un-treated samples were around 8.4 log(cfu/ml); cell counts in all pressure treated samples were below the detection limit (data not shown). The dashed reference lines indicate detection limit for *Escherichia coli*; counts below that line were indistinguishable from product microbiota. The colony morphology of all counts below the dashed reference lines also demonstrated that counts below the dashed line represent background microbiota.

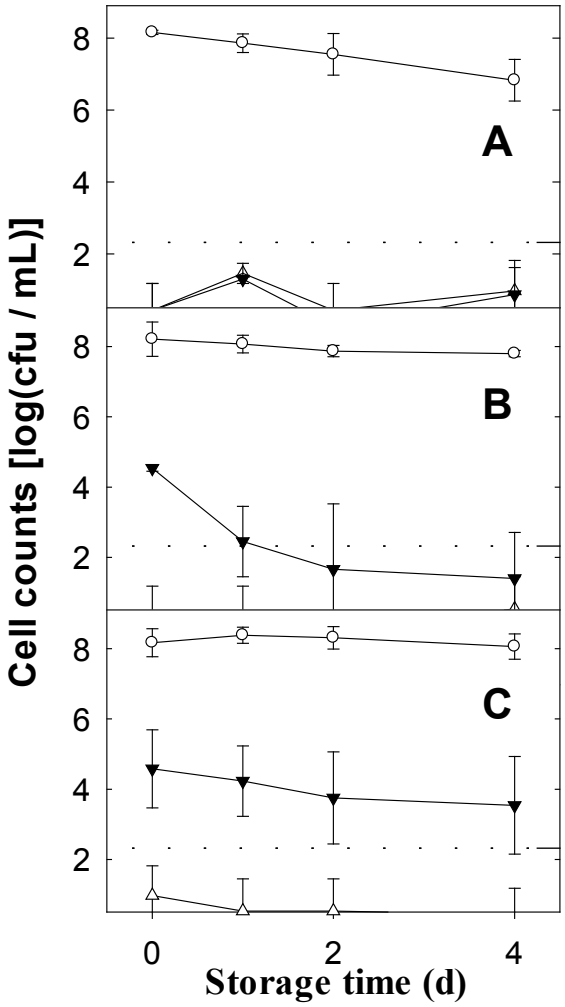

Figure 4. Cell counts of *E. coli* AW1.7 after pressure treatment in buffer (pH 5.5) with or without additions of food constituents. For Panel A, the following compounds were added to the MES buffer: 10 mmol/L calcium (●), 10 mmol/L magnesium (▲), 10 mmol/L glutamate (▲), 1 g/L acetic acid (■) or 1 g / L caffeic acid (■); For Panel B, ethanol (2%, ▲) or phenylethanol (20mmol/L, ■) were added to the acetate:MES:MOPS buffer. Treatment in buffer without addition was used as control (○). Samples were treated with at 600 MPa and 20°C. The treatment effect is expressed as cell count reduction [log(N_0/N)] where N_0 represents initial cell count and N represents cell counts after high pressure. Data are shown as mean ± standard deviation of three independent experiments.

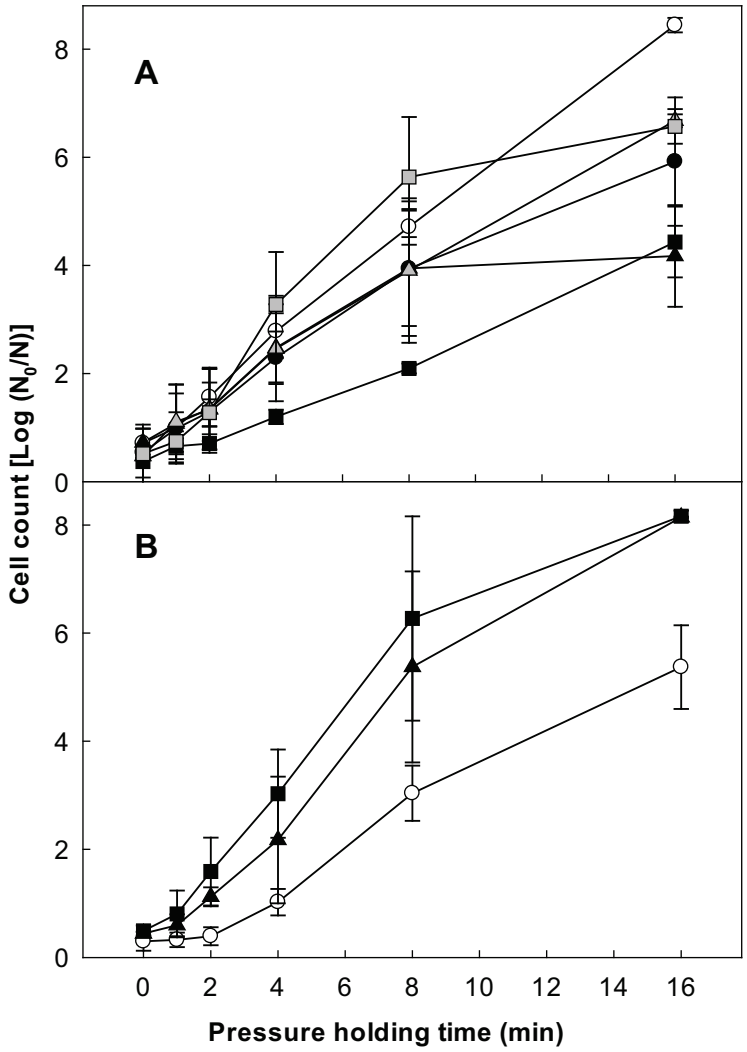

Figure 5. Cell counts of *E. coli* AW1.7 after treatment at 600 MPa for 3 min at 20°C in imidazole buffer at a of pH 5.5. Treatments were performed in buffer without additives (white bars) or with addition of 10 mM calcium (grey bars), magnesium (black bars) **(Panel A)**, or with addition of glutamine (light grey), glutamate (dark grey), or glutathione (black) (**Panel B**). Viable cell counts were enumerated on LB agar before treatment, after 3 min pressure treatment, and after pressure treatment and 3, 6, or 12 days of refrigerated storage. Without pressure treatment, cell counts of *E. coli* were not significantly reduced during refrigerated storage (data not shown). Survival of *E. coli* in controls shown in Panel A and B was not significantly different ($P>0.05$). Data are shown as mean ± standard deviation of three independent experiments. Values in the same panel that were obtained at the same storage time and do not share a common superscript differ significantly ($P<0.05$).