Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli.*

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Abstract.

Many strains of verotoxigenic *Escherichia coli* (VTEC) are highly resistant to pressure. To facilitate future studies to improve the elimination of VTEC by pressure processing of food, this study developed and validated a cocktail of non-pathogenic strains of *E. coli* with equal or higher resistance to pressure when compared to pressure resistant strains of VTEC. Strains of *E. coli* obtained from a beef processing plant were screened for their resistance to heat and pressure. Treatments were carried out in LB broth. Cell counts of 3 out of 16 strains were reduced by 5-6 log (cfu/mL) after 30 min at 60°C, and cell counts of 10 out of 16 strains were reduced by 5-6 log (cfu/mL) after 30 min at 40°C and 400 MPa. All highly heat resistant strains were also pressure resistant but not all pressure resistant strains were also heat resistant. Pressure resistant and –sensitive strains of *E. coli* were treated in presence of 0 or 2% NaCl and at 3, 20, or 40°C. The effect of these parameters on the lethality of pressure treatments was comparable for all strains. The addition of 2% NaCl did not increase pressure resistance. The bactericidal effect of treatments at 3 and 20°C and 600 MPa was comparable but inactivation of *E. coli* was faster at 40°C and 600 MPa. The resistance to treatment with 600 MPa at 20°C of a cocktail of 5 non-pathogenic strains of *E. coli* was compared to a 5 strain cocktail of pressure resistant VTEC. Treatments were performed in ground beef containing 15% fat. Survival and sublethal injury of the two cocktails was comparable; cell counts of beef inoculated with either cocktail were reduced by about 4 log(cfu/mL) after 30 min of treatment. In conclusion, this study validated a cocktail of non-pathogenic strains of *E. coli* for use as surrogate organisms in studies on the elimination of *E. coli* by pressure.

Keywords: *Escherichia coli*, EHEC, STEC, VTEC, O157, high pressure.

1. Introduction

Verotoxin-producing *Escherichia coli* (VTEC) remain an unsolved problem for food safety. The most virulent strains of VTEC combine verotoxin (Shiga-like toxin) production with virulence factors that mediate adhesion and colonization of the intestine. VTEC cause the hemolytic uremic syndrome with substantial morbidity and mortality (Croxen et al., 2013). Over 100 serotypes of VTEC have been linked to human illness (Grant et al., 2011; Johnson et al., 2006; Mathusa et al., 2010). Ruminants constitute the main reservoir of VTEC as the toxin provides protection against predatory protozoa that are part of ruminant intestinal microbiota (Lainhart et al., 2009). Accordingly, consumption of beef is a major contributor to foodborne VTEC infections (Greig and Ravel, 2009). Ground beef is contaminated with *E. coli* originating from the animal hide as well as the beef-packing environment (Aslam et al., 2004; Gill, 2009).

Pathogen intervention methods in beef abattoirs commonly include dry aging, hide washes, steam vacuuming, steam pasteurization, hot water washes, and lactic acid sprays (Algino et al., 2007; Corantin et al., 2005; Gill, 2009; Ingham et al., 2010; Rajic et al., 2007). However, the heat resistance in *E. coli* is highly variable (Dlusskaya et al., 2011; Jin et al., 2008) and *E. coli* AW1.7, an isolate obtained from beef after application of steam and lactic acid washes in a commercial processing facility, exhibited an exceptional resistance to heat (Dlusskaya et al., 2011).

Meat preservation is generally based on high and low temperature, addition of salt, and / or acidification (Cotter and Hill, 2003; Duche et al., 2002). New technologies for food preservation include high hydrostatic pressure (HP) processing, which has been adopted by the meat industry in the last few years. Pressure in the range of 200 to 600 MPa inactivates some foodborne pathogens and spoilage microorganisms to enhance food safety and to extend the storage life of

65 the product (Considine et al., 2008; Hsu et al., 2015; Knorr et al., 1993; Trujillo et al., 2002).
66 However, some strains of *E. coli,* including a substantial proportion of strains of VTEC, resist
67 the application of 600 MPa in meat with minimal reduction of cell counts (Liu et al., 2012,
68 2015). Moreover, *E. coli* readily develops resistance to pressure after consecutive cycles of lethal
69 pressure, followed by resuscitation and outgrowth of surviving cells (Hauben et al., 1997;
70 Vanlint et al., 2011).

71 The resistance of *E. coli* to pressure is strongly affected by the food matrix (Huang., et al 2013;
72 Linton et al., 1999; Liu et al., 2012; Morales et al., 2008; Rodriguez et al., 2005), the process
73 temperature (Sonoike et al., 1992) and the osmotic pressure (Van Opstal et al., 2003). Therefore,
74 the validation of novel high pressure processes targeting *E. coli* necessitates in plant challenge
75 studies to verify process efficacy. However, such challenge studies are not possible with
76 pathogenic strains; moreover, biosafety and bioterrorism legislation prevents sharing of strains of
77 VTEC across international borders (Anonymous, 2014). Non-pathogenic strains of *E. coli* are
78 required for use as surrogate organisms that behave similarly to the target pathogen when
79 exposed to processing conditions (Ingham et al., 2010). However, surrogate strains of *E. coli* to
80 match the resistance of VTEC against intervention methods such as heat and pressure remain to
81 be identified (Anonymous, 2006). It was therefore the aim of this study to evaluate heat and
82 pressure resistance of VTEC and non-VTEC in laboratory media and ground beef. The impact of
83 NaCl on the lethality of heat and pressure was determined in LB broth; information on cell
84 viability and sublethal injury was also obtained on pressure treated cells in ground beef.

2. Materials and methods

2.1 Bacterial strains and culture conditions.

Bacterial strains and their origin are listed in Table 1. *E. coli* were cultivated at 37 °C in Luria–Bertani (LB) broth (Difco; BD, Sparks, MD, USA) containing 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl unless otherwise noted. Stock cultures were stored at −80 °C, subcultured by streaking on LB agar (Difco; BD), followed by a second subculture in LB broth and incubation for 16 – 18h with agitation (200 rpm) in 25 mL of LB broth in 50 mL conical tubes. For preparation of strain cocktails, equal volumes of individual cultures was mixed to form a five-strain cocktail composed of four strains of VTEC (05-6544, 03-2832, 03-6430, and C0283) and the enteropathogenic *E. coli* PARC 449, and a five-strain cocktail composed of the non-pathogenic *E. coli* AW1.7, AW1.3, GM16.6, DM18.3 and MG1655.

2.2 Determination of heat resistance.

To determine heat resistance, overnight cultures (100 µL) were placed in a 200 µL PCR tube and heated in a PCR thermal cycler at 60 °C. The treatment temperature of 60°C was chosen because thermal death time data is available for a large number of strains (Hauben et al., 1997; Dlusskaya et al., 2011; Liu et al., 2015); the treatment time was adjusted depending on the heat resistance of the individual strains. *E. coli* AW1.7, AW1.3, GM16.6 and DM18.3 were treated for 10 to 70 min; *E. coli* MB2.1, GM3.4, GM9.8, GM11.5, GM18.3, GM11.9 and GGG10 were heated for 1 to 8 min. Heat treated and untreated cultures were placed on ice until cell counts were determined by surface plating. Serial dilutions of treated and untreated cultures in 0.1% buffered peptone water were plated on LB agar plates using a spiral platter (Don Whitely Scientific, Shipely, UK). Plates were incubated at 37 °C for 24 h. Experiments were performed in triplicate.

107 2.3 Determination of HP resistance.

108 Pressure treatments were carried out as described previously (Liu et al., 2011). In brief, overnight
109 cultures (250 μL) were packed into 3-cm R3603 tubing (Tygon, Akron, PA, USA) and heat
110 sealed after exclusion of air bubbles. The samples were inserted in a 2-mL cryovial (Wheaton,
111 Millville, NJ) filled with 10% bleach and subjected to 400 and 600 MPa at 40 °C for 5, 15, 30,
112 45, 60, 75, or 90 min in a U111 Multivessel Apparatus (Unipress Equipment, Warsaw, Poland).
113 The temperature of the unit was maintained by a thermostat jacket coupled to an external water
114 bath. Polyethylene glycol was used as pressure transferring fluid. The vessel was compressed to
115 the target pressure of 400 or 600 MPa in about 1 min and decompressed in about 30 sec. Cell
116 counts of treated and untreated cultures were determined by surface plating on LB agar. Plates
117 were incubated at 37 °C for 24 h. Experiments were performed in triplicate.

118 2.4 Effect of NaCl on heat and pressure resistance.

119 To evaluate the effect of NaCl on heat and pressure resistance, strains of *E. coli* were grown in
120 LB broth without NaCl or with addition of 2 or 4 % (w/v) NaCl. Aliquots of overnight cultures
121 grown in LB with 0%, 2%, or 4 % NaCl were heated at 60 °C for 0 to 40 min or treated at 600
122 MPa and 20 °C for 0 to 15 min. Surviving cells were enumerated as described above.
123 Experiments were performed in triplicate.

124 2.5 Effect of temperature during pressure treatment at 600 MPa

125 To evaluate the effect of temperature at 600 MPa, overnight cultures were treated at 600 MPa
126 and 3 or 20 °C for 5, 10, 20, and 30 min, and 40 °C for 2, 4, 6, and 8 min. The temperature inside
127 the pressure vessel was monitored continuously during each pressure treatment by internal
128 thermocouples. The temperature change during compression and decompression was less than 3

°C. Samples were placed into the vessel for 3 min before pressure treatment to equilibrate the sample temperature to the process temperature. Depressurization times were not included in the pressure-holding time because of their relatively smaller magnitude in relation with the pressure holding times. Cell counts were determined by plating serial dilutions on LB agar. Plates were incubated at 37 °C for 24 h. Experiments were performed in triplicate.

2.6 Pressure inactivation of VTEC and Non-VTEC in ground beef.

Lean ground beef (15% fat) was purchased from a local supermarket, divided into approximately 10-g portions which were stored in plastic bags at -18 °C until use. Cell counts of non-inoculated samples for each batch were determined by surface plating on LB agar and Violet Red Bile Agar (VRBA; Difco, BD). Cell counts on LB agar and VRBA were less than 2.6 log (cfu/g) and less than 2 log (cfu/g), respectively. Meat (6 g) was inoculated with a fresh 5-strain cocktail (1 mL) to a final cell count of 7.68 ± 0.33 log (cfu/g) for the non-VTEC cocktail and 7.63 ± 0.64 log (cfu/g) for the VTEC cocktail, and manually homogenized for 2 min. The sample was placed into 3-cm tube and both ends were sealed. Treatment conditions were 600 MPa for 2, 5, 15 and 30 min at 20 °C. After treatment the tubes were opened aseptically and the contents were diluted with sterile 0.1% peptone water. Cell counts of uninoculated, untreated and pressure treated samples were determined by plating serial dilutions on LB agar and VRBA to enumerate the survivors with and without injured cells. Plates were incubated at 37 °C for 24 h. Experiments were performed in triplicate.

2.7 Statistical analysis.

Significant differences between means of triplicate experiments were determined by using Student's T-test and an error probability of 5% ($P<0.05$).

151 **3. Results**

152 3.1. Heat and pressure resistance of *E. coli*

153 To determine the heat or pressure resistance of slaughter plant isolates of *E. coli*, eleven strains
154 of *E. coli* were heat treated at 60 °C or pressure treated at 600 MPa in LB broth with 1% NaCl.
155 The heat- and pressure resistant strain *E. coli* AW1.7 was used as reference (Dlusskaya et al.,
156 2011; Liu et al., 2012). Survivor curves are shown in Figure 1. Three strains, *E. coli* AW1.3,
157 DM18.3 and GM16.6, showed heat resistance comparable to *E. coli* AW1.7. Cell counts of these
158 strains were reduced by less than 5 log (cfu/mL) after 20 min at 60 °C. The pressure resistance of
159 ten strains of *E. coli* was comparable to *E. coli* AW1.7, corresponding to a reduction of cell
160 counts of less than 6 log (cfu/mL) after 15 min at 400 MPa and 40 °C. *E. coli* GGG10 was
161 sensitive to pressure (Figure 2). Four heat resistant strains, *E. coli* AW1.7, AW1.3, DM18.3 and
162 GM16.6, and three heat sensitive strains, *E. coli* GM18.3, GM11.5 and GGG10, were selected
163 for further experiments. *E. coli* MG1655 was added as a reference strain.

164 3.2. Effect of NaCl on heat and pressure resistance

165 Supplementation of media with NaCl increased the heat resistance of *E. coli* AW1.7 (Pleitner et
166 al., 2012). To determine whether NaCl has a comparable effect on the resistance of other strains
167 of *E. coli,* the heat and pressure resistance was determined after addition of 0 to 4% NaCl to LB
168 broth. The addition of NaCl increased the heat resistance of *E. coli* AW1.3, DM18.3, GM16.6,
169 GM18.3, GM11.5 and MG1655, comparable to the effect of NaCl on the heat resistance of *E.*
170 *coli* AW1.7 and GGG10 (Figure 3). Omission of NaCl in the growth and treatment medium
171 reduced the heat resistance of all *E. coli* strains. For example, cell counts of *E. coli* AW1.7
172 decreased about 5.5 log (cfu/ml) in the absence of NaCl and about 2.2 log (cfu/ml) in the
173 presence of 2 or 4 % NaCl after treatment at 60 °C for 40 min. Interestingly, the addition of 2

174 and 4 % NaCl did not affect the resistance of *E. coli* AW1.3, DM18.3, GM16.6, GM18.3,
175 GM11.5 and MG1655, to treatment at 400 MPa at 40 °C, or to treatment at 600 MPa and 20°C
176 (data not shown).

177 3.3 Effect of temperature during HP treatment at 600 MPa

178 To determine the effect of temperature during pressure inactivation, the resistance of *E. coli* to
179 treatment at 600 MPa was determined at 3, 20 and 40 °C in LB broth. Pressure death time data
180 are shown for *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 at each temperature in Figure 4. All
181 strains of *E. coli* were least resistant to pressure at 40 °C and most resistant to pressure at 3 °C
182 (Figure 4). After 5 min of treatment at 40 °C and 600 MPa, cell counts of all strains were
183 reduced to less than 2 log (cfu/ml). Pronounced tailing was observed when samples were treated
184 at 3 °C and 600 MPa. Cell counts of all four strains of *E. coli* remained higher than 3 log
185 (cfu/mL) after treatment at 3 °C and 600 MPa for up to 30 min (Figure 4).

186 3.4 Pressure inactivation of VTEC and Non-VTEC on ground beef.

187 To validate pressure resistance data in a food model system, and to compare the pressure
188 resistance of meat isolates with VTEC, treatments at 600 MPa and 20 °C were performed with
189 two five-strain cocktails in ground beef. The VTEC strain cocktail contained five pressure
190 resistant strains of VTEC that were identified after screening of 102 VTEC (Liu et al., 2015).
191 Surviving cells were enumerated on LB agar to quantify total viable cells; the low initial cell
192 counts of the meat used (less than 400 cfu/g) allowed the accurate quantification of the inoculum
193 without interference of indigenous microbiota. Surviving cells were also enumerated on VRBA,
194 which inhibits growth of sublethally injured cells with a permeabilized outer membrane (Hauben
195 et al., 1996). Survival of both strain cocktails was generally equivalent (Figure 5); a significant
196 difference between total cell counts of the two cocktails was observed after 2 min of treatment

197 but cell counts at other treatment times or cell counts on VRBA were not significantly different.
198 Both strain cocktails exhibited a substantial resistance to pressure. The reduction of cell counts
199 was about 2 and 5 log (cfu/g) after 5 and 30 min, respectively. Cell counts on VRBA were
200 reduced below 2 log (cfu/g) after 15 min, indicating that surviving cells were sublethally injured.

201 **4. Discussion**

202 The tolerance of *E. coli* and related organisms to pathogen interventions such as heat, pressure,
203 and low pH differs substantially among strains (Benito et al., 1999; Erkmen and Doğan, 2004;
204 Liu et al., 2012; Tahiri et al., 2006). A substantial proportion of VTEC are highly resistant to
205 pressure and their elimination from low acid food products at ambient temperature therefore
206 necessitates additional process development (Liu et al., 2015). This study evaluated the pressure
207 resistance of non-pathogenic strains of *E. coli* to validate a cocktail of surrogate strains with
208 equal resistance to pressure when compared to pressure-resistant STEC. The strain selection
209 focused on beef isolates. Pressure resistance was evaluated at 400 and 600 MPa and different
210 process temperatures and NaCl levels to encompass a variety of different process parameters,
211 and compared to heat resistance.

212 *E. coli* AW1.7 was described as an exceptionally heat resistant strain; its cell counts are reduced
213 by only 2 and 4 log (cfu/g) when inoculated into ground beef patties cooked to a core
214 temperature of 63 and 71°C, respectively (Dlusskaya et al., 2011, Liu et al., 2015). The current
215 study demonstrated that the heat resistance of this strain is not exceptional, but was matched by 3
216 of the 11 tested strains of *E. coli*. The pressure resistance of *E. coli* AW1.7 was matched by 10
217 additional strains of *E. coli*. The direct comparison of the pressure resistance of mutant strains
218 generated by multiple cycles of sublethal pressure treatment and sub-culturing of surviving cells
219 (Hauben et al., 1997; Vanlint et al., 2011) to the pressure resistance of *E. coli* AW1.7

220 demonstrated that the pressure resistance of the wild type *E. coli* AW1.7 in poultry meat or beef
221 matches or exceeds the resistance of pressure-resistant mutant strains (Liu et al., 2012; Liu et al.,
222 2015). The heat- and pressure resistance of *E. coli* strains isolated from meat or a meat
223 processing plant suggests that beef may be contaminated with *E. coli* strains that are resistant to
224 heat and pressure. The screening of 100 strains of STEC revealed that about 30% of STEC are
225 pressure resistant while heat resistant strains of STEC were less frequent (Liu et al., 2015). This
226 study also observed a higher prevalence of pressure resistant strains among non-pathogenic *E.*
227 *coli*. Pressure resistant mutant strains of *E. coli* have a marginal cross-resistance to heat (Hauben
228 et al., 1996; Vanlint et al., 2011) and *E. coli* AW1.7 is both heat- and pressure resistant. The σ^H
229 mediated heat shock response and the σ^S mediated general stress response contribute to both
230 pressure and heat resistance (Aertsen et al., 2004; Robey et al., 2001). Exposure to pressure
231 selects for increased σ^S activity and also increases thermotolerance in *E. coli* O157:H7 (Vanlint
232 et al., 2013). However, sequential exposure to sublethal pressure, followed by cultivation of
233 surviving cells readily generates pressure resistant mutants of *E. coli* while the same strategy
234 failed to produce heat resistant derivatives (Vanlint et al., 2012). Taken together, pressure
235 resistant strains of *E. coli* occur relatively frequently and mechanisms of resistance are likely
236 multi-factorial while resistance to heat (60 °C) is a less frequent trait.

237 The heat resistance of *E. coli* AW1.7 is linked to ribosome stability and accumulation of
238 compatible solutes (Pleitner et al., 2012; Ruan et al., 2011). Accumulation of disaccharides in
239 response to a high external osmolarity also protects vegetative bacteria against pressure-mediated
240 cell death (Lange and Hengge-Aronis, 1994; Molina-Höppner et al., 2004; Van Opstal et al.,
241 2003). *E. coli* AW1.7 accumulates higher levels of amino acids and trehalose in response to
242 NaCl when compared to heat sensitive strains (Liu et al., 2012; Pleitner et al., 2012). In this

study addition of NaCl increased heat resistance in all strains of *E. coli* including K12, indicating that NaCl generally confers a protective effect against lethal heat treatment. Interestingly, increasing NaCl in the growth medium did not increase pressure resistance in the same strains of *E. coli,* again indicating that mechanisms of heat- and pressure resistance only partially overlap.

Commercial applications of pressure for food preservation are generally performed at ambient temperature. An increase of the process temperature to 30 to 50 °C accelerates pressure inactivation of microorganisms (Erkmen and Doğan, 2004). However, the effect of low temperature is not as consistent. Sonoike et al. (1992) suggested that pressure treatment of *E. coli* at lower temperatures also accelerates inactivation of *E. coli*; however, other reports indicate that *E. coli* and *S. aureus* were more resistant to pressure application at 4°C than to the same pressure at 25°C (Trujillo et al., 2002). Pressurization at subzero temperatures without freezing significantly enhanced the lethal effect of pressure in *L. plantarum* and *S. cerevisiae* (Perrier-Cornet et al., 2005). *E. coli* MG1655 is more pressure resistant at 5 °C when compared to treatments at 20 °C or higher (Van Opstal et al., 2005). During the first few minutes of pressure treatment, we observed no major differences in the resistance of *E. coli* when treated at 3 or 20°C at 600 MPa but extended pressure treatment at 20°C was consistently more lethal when compared to treatments at 3°C. Prior studies demonstrate that tailing in pressure-death time curves of *Listeria monocytogenes* and *E. coli* is influenced by the process temperature (Simpson and Gilmour, 1997; Van Opstal et al., 2005). All four strains of *E. coli* that were investigated in this study responded similarly to a change of the temperature of pressure treatments.

Data on the pressure resistance of non-pathogenic strains of *E. coli* was used to select strains included in a cocktail of five non-pathogenic strains. The resistance of *E. coli* O157:H7 and other VTEC to heat or other environmental stresses is not generally different from that of other *E. coli*

(Ingham et al., 2010; Large et al., 2005); however, because of the large strain-to-strain variability of the stress resistance of *E. coli*, strain cocktails of non-pathogenic *E. coli* for use in challenge studies have to be validated with cocktails of pathogenic strains (Ingham et al., 2010). Because analysis of only few strains of VTEC may over-estimate the lethal effect of pressure (Hsu et al., 2015), we selected pressure-resistant trains of VTEC for use in the pathogenic cocktail from more than 100 strains of VTEC with known resistance to pressure (Liu et al., 2015). Validation of cocktails was performed in ground meat, and surviving cells as well as sublethally injured cells were enumerated. After pressure treatment of *E. coli,* the difference in cell counts between LB and VRBA is an indication of sublethally injured cells with a damaged outer membrane which are sensitive to bile (Gänzle and Vogel, 2001; Hauben et al., 1996). The cell counts of ground beef that were inoculated with either cocktail were comparable, demonstrating that the 5 strain cocktail composed of non-pathogenic strains reliably indicated the survival of VTEC. The non-VTEC cocktail is thus a suitable surrogate for VTEC strains. Because pressure treatment of ground beef alone does not provide a sufficient reduction of counts of VTEC, further process optimization using this strain cocktail is warranted to ensure food safety.

In conclusion, this study validated a cocktail of non-pathogenic *E. coli* to reliably indicate the survival of VTEC after pressure treatment of food. The VTEC cocktail comprises pressure resistant strains that were identified in a screening of more than 100 strains of VTEC (Liu et al., 2015). This study evaluated the effect of NaCl and temperature on the pressure resistance of several non-pathogenic *E. coli* strains to show that the relative resistance of the two cocktails is not dependent on the process conditions. The use of pressure alone is not a reliable technology to inactivate VTEC in low acid foods (Liu et al., 2015). The availability of a cocktail of surrogate

288 strains will facilitate future studies to increase the bactericidal effect of pressure by combination
289 with additional antimicrobial hurdles.

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440

441 **Figure legends**

442 **Figure 1.** Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 60 °C in LB.
443 AW 1.7 (●), AW 1.3 (○), DM 18.3 (▼), GM 16.6 (Δ), MB 2.1 (■), MB 3.4 (□), GM 9.8 (♦),
444 GM 11.5 (◊), GM 18.3 (▲), GM 11.9, (∇), GGG 10 (-). Cells were grown and treated in LB
445 broth containing 1% NaCl. Data are shown as mean ± standard deviation of triplicate
446 independent experiments. Lines crossing the x axis indicate cell counts at or below the detection
447 limit of 2 log (cfu/ml).

448 **Figure 2**. Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 400 MPa and
449 40 °C. Cells were grown and treated in LB broth containing 1% NaCl. Panel A: AW 1.7 (●), AW
450 1.3 (○), GM 16.6 (▼), DM 18.3 (Δ), and MB 3.4 (■). Panel B: MB 2.1(●), GM 9.8(○), GM 11.5
451 (▼), GM 18.3(Δ), GM 11.9 (■), and GGG10 (□). Data are shown as mean ± standard deviation
452 of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below
453 the detection limit of 2 log (cfu/ml).

454 **Figure 3.** Viable cell counts of non-pathogenic strains of *E. coli* after heat treatment at 60 °C.
455 Cells were grown and treated in LB broth containing the following NaCl concentration: 0 % (●),
456 2 % (○) and 4 % (▼). Panel A: cells were treated from 0 to 40 min. Panel B: cells were treated
457 from 0 to 5 min. Data are shown as mean ± standard deviation of triplicate independent
458 experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log
459 (cfu/ml).

460 **Figure 4.** Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 600 MPa with
461 the following temperatures: 3° (●), 20° (○) and 40 °C (▼). Cells were grown and treated in LB
462 broth containing 1% NaCl. Data are shown as mean ± standard deviation of triplicate

independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/ml).

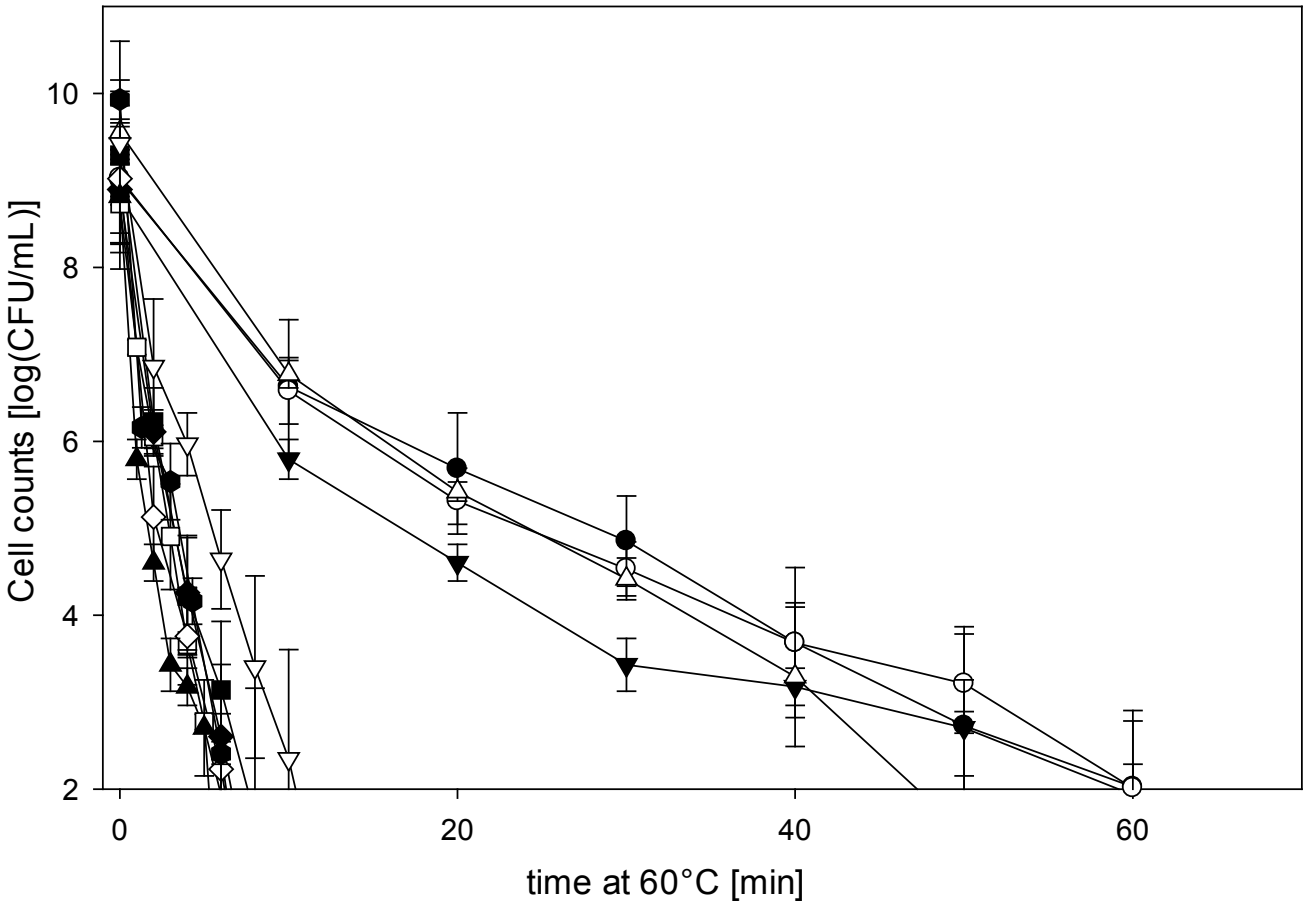
Figure 5. Cell counts of non-VTEC (circles) and VTEC cocktail (triangles) in ground beef after treatment at 600 MPa at 20 °C. Cells counts were enumerated on LB agar (●, ▼) and VRB agar (○, Δ). Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/g).

Table 1. Strains of *E. coli* used in this study

Strain ID	Serotype	Source	stx1	stx2[a)]	eae	Reference
05-6544	O26:H11	Human	+	-	+	Liu et al. (2012)
03-2832	O121:H19	Human	-	+	+	Liu et al. (2012)
03-6430	O145:NM	Human	+	-	+	Liu et al. (2012)
C0283	O157:H7	Cattle feces	+	+	+	Liu et al. (2012)
PARC 449	O145:NM	Unknown	-	-	+	
AW1.7		Slaughter plant	-	-	-	Aslam et al. (2004)
AW1.3		Slaughter plant	-	-	n.d	Aslam et al. (2004)
DM18.3		Slaughter plant	-	-	n.d.	Aslam et al. (2004)
GM16.6		Slaughter plant	-	-	n.d	Aslam et al. (2004)
MB2.1		Slaughter plant	-	-	n.d	Aslam et al. (2004)
MB3.4		Slaughter plant	-	-	n.d	Aslam et al. (2004)
GM9.8		Slaughter plant	-	-	n.d	Aslam et al. (2004)
GM11.5		Slaughter plant	-	-	n.d	Aslam et al. (2004)
GM18.3		Slaughter plant	-	-	n.d	Aslam et al. (2004)
GM11.9		Slaughter plant	-	-	n.d	Aslam et al. (2004)
GGG10		Slaughter plant	-	-	n.d	Dlusskaya et al. (2011)
MG1655	K12	Sensitive reference strain	-	-	-	Hauben et al., 1997

[a)] Data from Liu et al., (2015). n.d. not determined

473 Garcia Hernandez et al., Figure 1.



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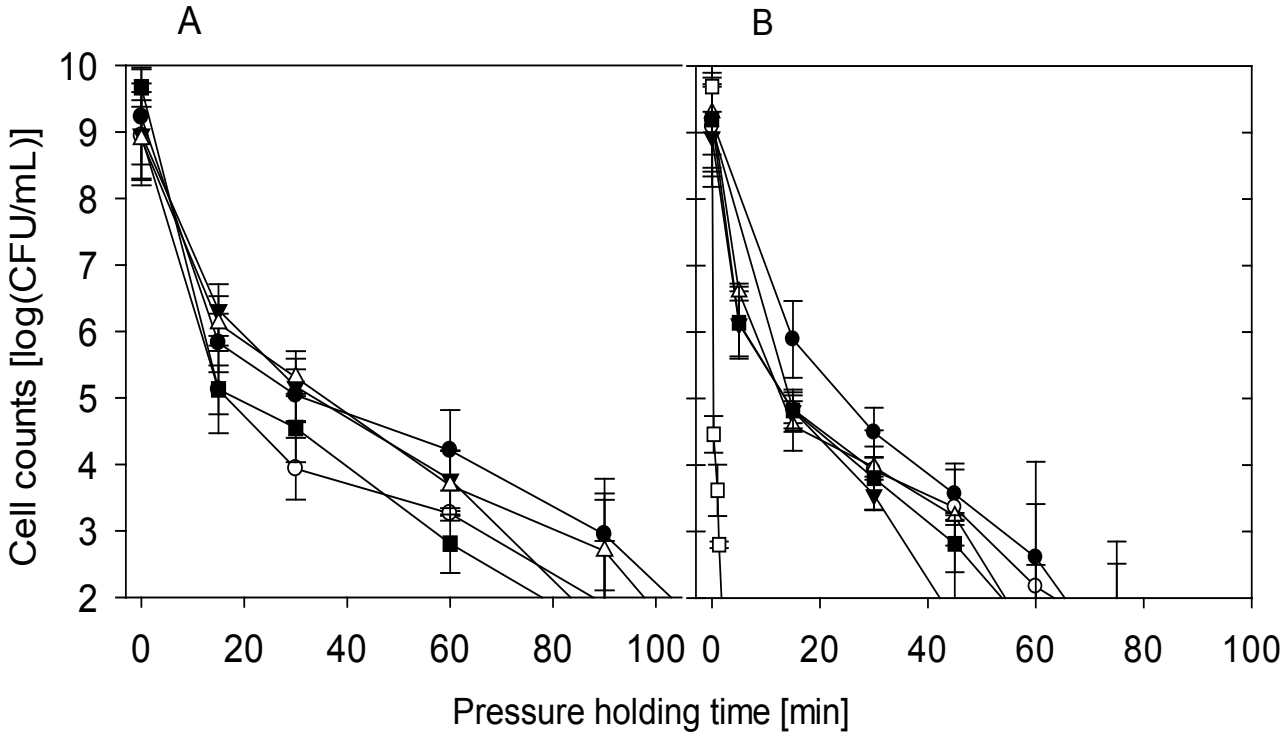
477 Garcia Hernandez et al., Figure 2.



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481 Garcia Hernandez et al., Figure 3.

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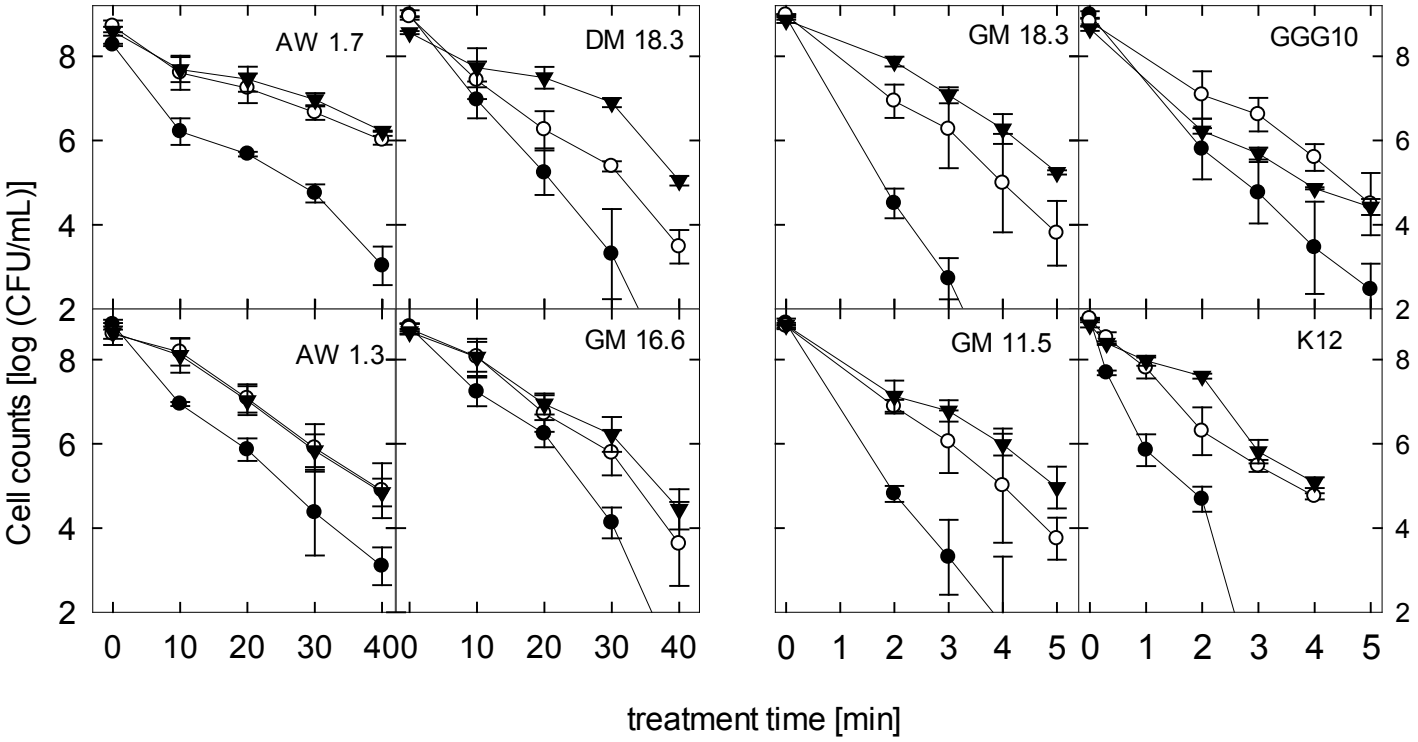
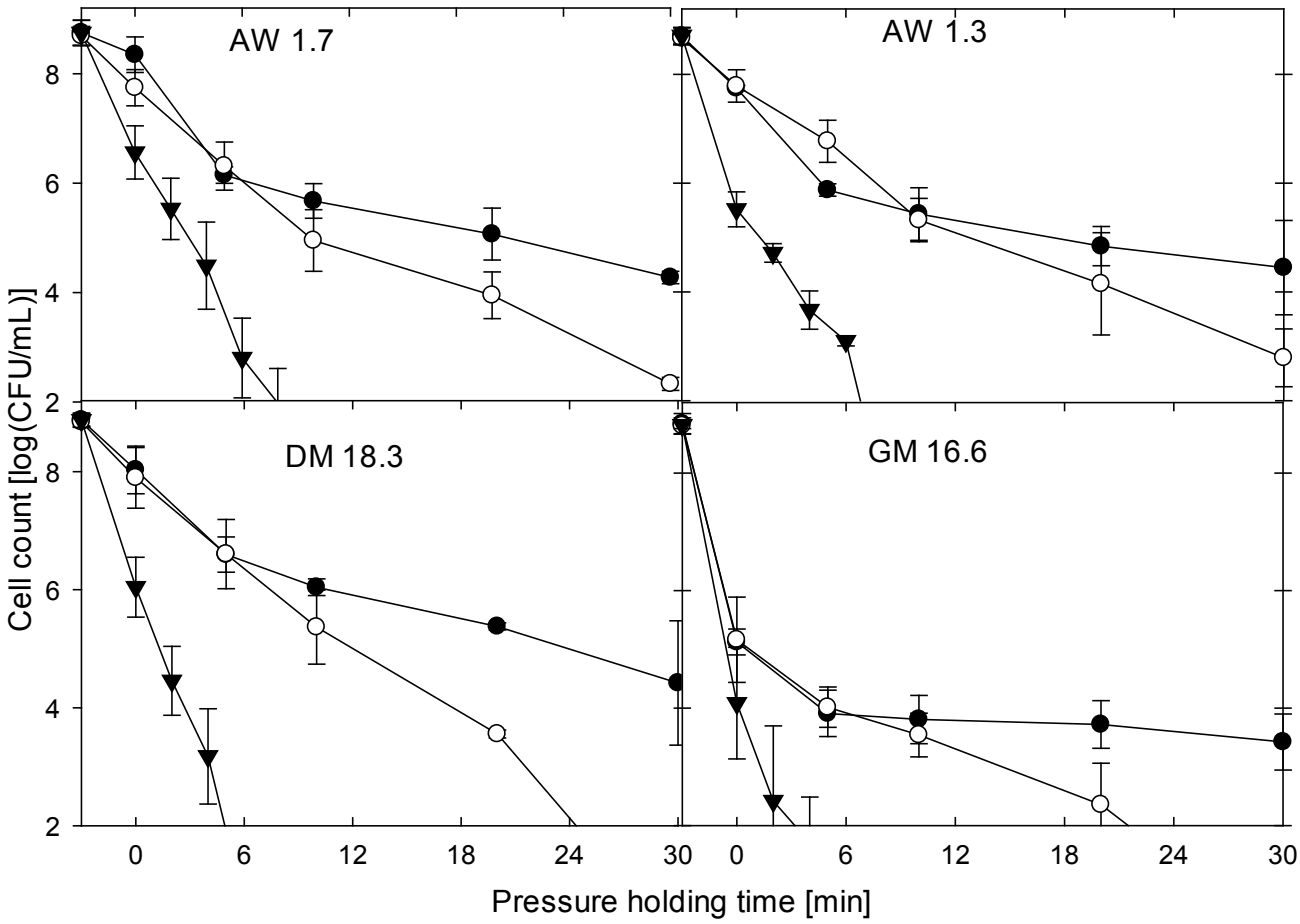


484 Garcia Hernandez et al., Figure 4.



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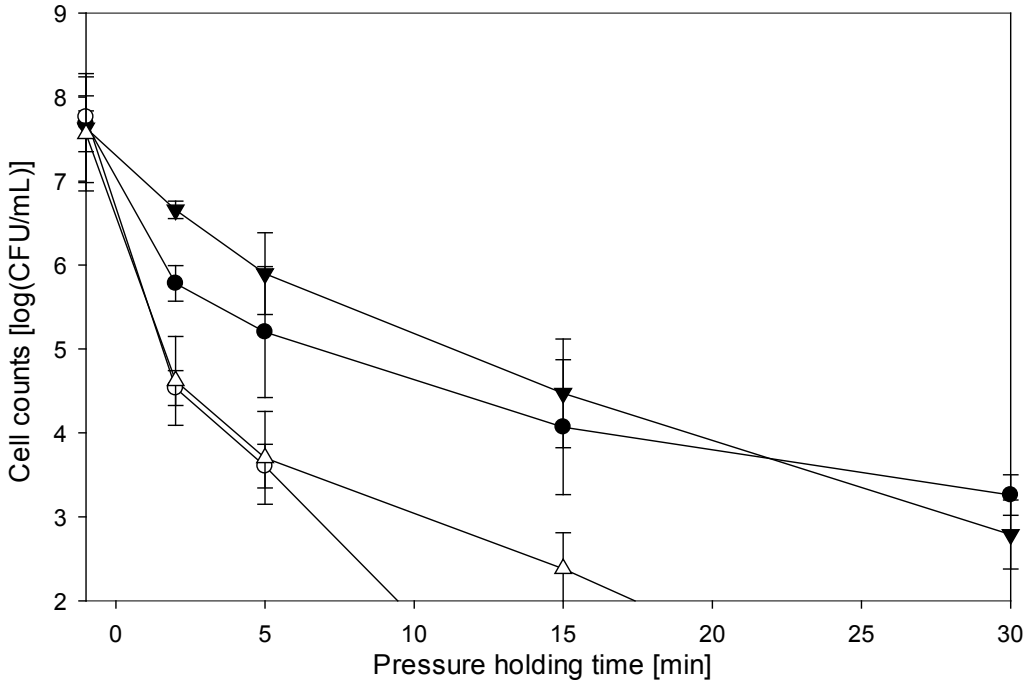
487 Garcia Hernandez et al., Figure 5



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