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

Rigoberto Garcia-Hernandez, Lynn McMullen, Michael G. Gänzle *

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

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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.

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

* Corresponding author at: 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 address: michael.gaenzle@ualberta.ca (M.G. Gänzle).

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The resistance of *E. coli* to pressure is strongly affected by the food matrix (Huang et al., 2013; Linton et al., 1999; Liu et al., 2012; Morales et al., 2008; Rodriguez et al., 2005), the process temperature (Sonoike et al., 1992) and the osmotic pressure (Van Opstal et al., 2003). Therefore, the validation of novel high pressure processes targeting *E. coli* necessitates in plant challenge studies to verify process efficacy. However, such challenge studies are not possible with pathogenic strains; moreover, biosafety and bioterrorism legislation prevents sharing of strains of VTEC across international borders (Anonymous, 2014). Non-pathogenic strains of *E. coli* are required for use as surrogate organisms that behave similarly to the target pathogen when exposed to processing conditions (Ingham et al., 2010). However, surrogate strains of *E. coli* to match the resistance of VTEC against intervention methods such as heat and pressure remain to be identified (Anonymous, 2006). It was therefore the aim of this study to evaluate heat and pressure resistance of VTEC and non-VTEC in laboratory media and ground beef. The impact of NaCl on the lethality of heat and pressure was determined in LB broth; information on cell 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 16–18 h 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.

2.3. Determination of HP resistance

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

2.4. Effect of NaCl on heat and pressure resistance

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

2.5. Effect of temperature during pressure treatment at 600 MPa

To evaluate the effect of temperature at 600 MPa, overnight cultures were treated at 600 MPa 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 the pressure vessel was monitored continuously during each pressure treatment by internal 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

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.

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