Variation in resistance to high hydrostatic pressure of *Escherichia coli*

by

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Abstract

Verotoxigenic *Escherichia coli* (VTEC) pose a risk for foodborne illness that can lead to death. Thermal food preservation to inactivate VTEC also alters food quality. High hydrostatic pressure (HHP) processing has been adopted by the food industry as an alternative to thermal preservation. However, *E. coli* exhibits great variability in resistance to pressure. This research evaluated the pressure resistance of VTEC and non-VTEC *E. coli* in laboratory media and ground beef. A strain cocktail of non-pathogenic *E. coli* was developed to match the pressure resistance of VTEC. The strain cocktail was validated by pressure treatments in ground beef and vegetable products.

Pressure resistance of *E. coli* is affected by the food matrix. Therefore, the effect of the food matrix and food constituents on pressure resistance of *E. coli* was determined. Ground beef showed a baro-protective effect on *E. coli* when compared to treatments in bruschetta and tzatziki at the same pH (5.5). Divalent cations (Ca^{2+}) exerted a baroprotective effect and may partially explain the relative resistance of *E. coli* in ground beef.

To further elucidate mechanisms of pressure resistance, four extremely pressure resistant strains of *E. coli*, including one VTEC, were generated and cross-resistance to other stressors was evaluated. All four strains of *E. coli* evolved as extremely pressure resistant strains when treated with consecutive cycles of increasing pressure. Derivative *E. coli* AW1.7 became sensitive to low pH (2.5) and derivative *E. coli* AW1.3 exhibited increased resistance to heat and osmotic stress. Comparative analysis of the genome sequence of 3 wild type strains to the respective pressure-resistant revealed that derivative strains exhibited deletion of genetic elements. However, deletions were different without apparent consistency among the strains.

In conclusion, the species *E. coli* contains extremely pressure resistant strains that are not readily inactivated by pressure treatment of food. Knowledge on the strain/matrix interaction during and after pressure treatment will facilitate the design, adoption, and/or combination of different intervention methods to warrant food safety.

Preface

A version of chapter 2 of this thesis has been published as Garcia-Hernandez, R., McMullen, L., Gänzle, M.G., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli.* International Journal of Food Microbiology 205, 16-22.

Chapter 4 of this thesis contains experimental work performed by Hui Li under the supervision of Dr. M. Gänzle. A version of this chapter has been submitted for publication to Food Microbiology as: Hui Li, Rigoberto Garcia-Hernandez, Darcy Driedger, Lynn McMullen, Michael Gänzle. Effect of the food matrix and food constituents on pressure resistance of *Escherichia coli*.

The appendix of the thesis has been published in Frontiers in Microbiology as Ryan G. Mercer, Jinshui Zheng, Rigoberto Garcia-Hernandez, Lifang Ruan, Michael G. Gänzle and Lynn M. McMullen. Genetic determinants of heat resistance in *Escherichia coli.* In this study, I was involved on screnning different strains of *E. coli* to determine their heat resistance. Data are shown in Fig A-1 and Table A-2.

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1. Introduction

1.1 High hydrostatic pressure

High hydrostatic pressure (HHP) is a non-thermal food preservation technology that is used for commercial production of an increasing number of food products. HHP causes less deterioration of vitamins, phytochemicals, and aroma compounds compared to thermal treatments (Cheftel, 1995; Heinz and Buckow, 2010; Balasubramaniam et al., 2015). The equipment for high pressure treatment consists of a pressure vessel (thick- wall cylinder), two end closures to cover the cylindrical pressure vessel, yoke (structure for restraining end closures while under pressure), high pressure pump and intensifier for generating target pressures, process control and instrumentation, and a handling system for loading and removing the product (Ting, 2011; Balasubramaniam et al., 2015). Pressure primarily affects the volume of the product that is subjected to high pressure, thus the package must be flexible enough to transmit pressure. Thus, rigid metal containers are not appropriate for pressure treatment (Rastogi et al., 2007; Gupta et al., 2011).

Applications of high pressure are rapidly developing based on the further development of the technology and enhancement of manufacturing capability and facilities. Food laws and regulations also encourage HHP as novel intervention methods for food preservation (Wang et al., 2014). The U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) have approved the use of high pressure as a reliable technological alternative to conventional heat pasteurization of foods, as additional intervention for preheated foods and for commercial sterilization of low-acid foods (Wang et al., 2014; Juliano et al., 2012; Stewart et al., 2015). In Canada, a letter of no objection for the use of HHP to control *L. monocytogenes* in RTE meats and poultry had been issued (Health Canada, 2013). The

worldwide number of high pressure equipment installations in 2010 was around 200, 57% in America, 24% in Europe, 13 % in Asia an d 6 % in Oceania (Wang et al., 2014). At the end of 2007, an average of 120 pressure treated products was reported around the world, with an estimated production of 150,000 tons/year (Hernando-Sáinz et al., 2008). According to Rastogi et al., (2007), the average cost of high-pressure processing is around US$0.05–0.5 per liter or kilogram depending on processing conditions, which is lower than thermal processing costs. However, not all variable costs are included. To date, high pressure represents a $2.5 billion market and is considered as one the most important innovations in food processing during the past 50 years (Balasubramaniam et al., 2015).

Pressure processsing is isostatic, i.e. the pressure is transmitted uniformly and instantly throughout a sample whether the sample is in direct contact with the pressure medium or hermetically sealed in a flexible package (Rastogi et al., 2007). High pressure increases the temperature of the product. The temperature increase in the food and pressure transmitting medium is different, as they depend on food composition, initial temperature, processing temperature, target pressure and the rate of pressurization. During depressurization, the temperature of the food returns back to its initial value (Norton and Sun, 2008; Balasubramaniam et al., 2015). The heat of compression of most of the high-moisture food materials is very similar to that of water: 3°C per 100 MPa at 25°C. However, fatty foods have higher compression heating due to their higher compressibility with long-chain unsaturated fatty acids and lower specific heat (Rasanayagam et al., 2003).

1.1.1 High pressure and bacterial inactivation

High pressure is used to replace traditional thermal pasteurization or in conjunction with

existing techniques. Typically, pressure ranging from 200 to 600 MPa is applied to inactivate some pathogenic and spoilage vegetative bacteria, parasites, yeasts, molds and viruses, thus enhancing the safety and extending the shelf life of the product (Rendueles et al., 2011; Lindsay et al., 2006; Lou et al., 2015; Ye et al., 2014). *E. coli* is one of the main targets for HHP. However, significant variation in pressure resistance has been reported among different strains (Pagan and Mackey, 2000; Liu et al., 2015; Benito et al., 1999), and some strains such as *E. coli* AW1.7 and LMM1030 showed exceptional resistance to pressure (Hauben et al., 1997; Liu et al., 2012). Furthermore, Liu et al., (2015) concluded that pressure alone is not sufficient for the elimination of verotoxigenic *E. coli* (VTEC) in meat or meat products.

The resistance of bacteria to pressure is affected by physiological factors such as growth phase and growth temperature (Casadei et al., 2002) and environmental factors such as the food matrix (Baccus-Taylor et al., 2015; Rodriguez et al., 2005), magnitude of pressure (Benito et al., 1999; Alpas et al., 2000), process temperature (Torres et al., 2015; Sonoike et al., 1992) and the osmotic pressure (Van Opstal et al., 2003). In food systems, inactivation of bacteria under high pressure is greatly influence by water activity (a_w), pH value, and the organic environment (Erkmen and Doğan., 2004). A food system is a complex matrix and reliable quantitative data for the inactivation of pathogens with specific conditions and food systems needs to be generated (Reineke et al., 2015). Furthermore, the effects of such factors that influences the inactivation of pathogens after pressure treatment in food and the inactivation mechanisms needs to be elucidated. This thesis explored the pressure resistance variability of different microorganisms and the interaction with food matrices and the potential synergistic, additive, or antagonistic effects of food additives and/or food ingredients.

1.1.2 Effects of high pressure on foods

High pressure (400–600 MPa) at ambient or chilled temperatures can be useful for pasteurizing a variety of liquid and solid foods, including deli meats, salads, seafood, fruit juices, and vegetable products (Mujica-Paz et al., 2011; Tonello, 2011; Norton and Sun, 2008). The primary structure of food constituents including peptides, lipids, and saccharides is rarely affected by high pressure (Gross and Jaenicke, 1994; Buckow and Heinz, 2008; Heremans and Smeller, 1998). However, food treated with pressure and low or high temperatures for an appropriate period affects the formation of non-covalent bonds of food components, such as hydrogen bonds, electrovalent bonds, and hydrophobic bonds. In aqueous solutions, tertiary and quaternary structures of globular proteins undergo reversible and irreversible changes under high pressure (Knorr et al., 2011). As a result of water penetration into the interior of a protein, pressure leads to conformational transitions resulting in protein unfolding (Saad-Nehme et al., 2001). Enzymes are globular proteins and possess the functional characteristics of globular proteins of binding one or more substrate molecules, thus structural changes can result in a complete loss of enzyme activity (Knorr et al., 2011).

In practical food processing applications, the combined intensity of both thermal and pressure effects can cause various physical, chemical, or biological changes in foods (Balasubramaniam et al., 2015). High-pressure effects on foods are highly dependent on the primary effects of pressure and temperature on the relevant thermodynamic and transport properties of food systems, which, in general, are density, viscosity, thermal conductivity, compressibility, heat capacity, diffusivity, phase transition properties and solubility (Buckow et al., 2013).

1.2 Factors affecting inactivation of bacteria by high pressure

High pressure has the potential to inactivate microorganisms. However, many studies were performed in buffer or specific media systems and inactivation in different substrates (buffer, broth media and food matrix) differs substantially. The inactivation kinetics in buffer and food is influenced by different factors such as species, strain, treatment conditions (pressure/temperature), and substrate composition. Erkmen and Doğan, (2004) found different inactivation rates when the same strain of *E. coli* was treated in broth, milk, peach juice and orange juice. Thus, pressure resistance of different microorganism is multi-factorial and knowledge on the interaction of strain and matrix is critical for the application of high pressure in a specific food systems.

1.3 Pressure resistance variability between species and strains

Variation in pressure resistance has been reported among different species and strains of the same species (Pagan and Mackey, 2000; Wuytack et al., 2002). Significant variation in pressure resistance was observed among different strains of *S. aureus, L. monocytogenes* and *E. coli* O157:H7 (Patterson et al., 1995; Benito et al., 1999; Alpas et al., 1999). Furthermore, VTEC strains had pressure resistance comparable to non-VTEC strains that are considered extremely pressure resistant strain (Liu et al., 2015).

E. coli is more resistant to pressure in the stationary-phase of growth when compared to cells grown to the exponential phase (Robey et al., 2001). Stationary-phase bacteria are generally more resistant to other stresses, such as oxidation and osmotic stress. RpoS changes the specificity of RNA polymerase, allowing it to activate more than 30 genes, some of which are involved in stationary-phase stress survival (Hengge-Aronis, 1996). Casadei et al. (2002)

found that in stationary phase the amount of saturated fatty acids (SFA) decrease and cyclopropane fatty acids (CFA) increase, suggesting that CFA could play a role on pressure resistance during stationary phase of *E. coli*. Differences in pressure resistance among strains may be related to differences in susceptibility to membrane damage (Gänzle and Liu, 2015). High pressure changes the structure of proteins, which are the main components of microorganisms, affecting the cellular functions responsible for reproduction and survival. Govers et al., (2014) observed that high pressure produced a dispersal of protein aggregates (PAs) of *E. coli* and reassembly was a prerequisite to initiate growth. Furthermore increasing pressure treatment increase GFP-labeled aggregates and mild pressure decrease dispersal of PAs and higher probability of survival (Govers and Aertsen, 2015). Biological membranes are one of the most pressure sensitive cellular components. High pressure triggers the phase transformation from liquid to gel phase of lipids bilayer, these changes affect the physiological function of microorganisms (Winter and Jeworrek, 2009). The damage of microbial membranes affects transport of nutrients and the disposal of metabolic products, resulting in sterilization through destruction of the structure of the cytoplasmic membrane (Wang et al., 2014). Furthermore, after depressurization the cell membrane can be injured and/or disrupted, causing leakage of the cell content and then to cell death (Guerrero-Beltran et al., 2005). However, the exact nature of the lethal effect and the role of membrane structure in determining resistance to pressure still have to be clarified (Michiels et al., 2008; Benito et al., 1999).

1.3.1 Growth temperature

Growth temperature is an important factor influencing bacterial resistance to heat and pressure and modification of membrane fluidity (homeoviscous adaptation) is considered to be

one of the contributor mechanisms (Morein et al., 1996). Exponential phase cells are less resistant to pressure than stationary phase cells, and the proportion of unsaturated fatty acids in the membrane lipids decreases with increasing growth temperature in both exponential and stationary phase cells (Casadei et al., 2002). Cells of *Listeria monocytogenes* and *Pseudomonas fluorescens* grown at 8°C to the exponential phase were more resistant to pressure than those grown at 30°C, but for stationary phase cells the reverse was found (McClements et al., 2001). Pressure resistance of exponential-phase cells of *E. coli* was maximal in cells grown at 10°C and decreased with increasing growth temperatures up to 45°C. In contrast, the pressure resistance of stationary-phase cells was lowest in cells grown at 10°C and increased with increasing growth temperature, reaching a maximum at 30 to 37°C. In cells grown to the exponential phase, pressure resistance increased with greater membrane fluidity but in stationary phase, there was apparently no simple relationship between membrane fluidity and pressure resistance (Casadei et al., 2002). The author suggests that membrane fluidity affects resistance in both growth phases but is a dominant factor only in exponentially growing cells. Stationary-phase cells also have a higher protein/lipid ratio in their membranes, which makes them less prone to lateral phase separation (Souzu, 1986), and they have a higher amount of cross linking among membrane proteins (Mirelman and Siegel, 1979. Manas and Mackey, (2004) found that in exponential phase cells the loss of viability is always accompanied by a loss of the physical integrity of the membrane, whereas in stationary-phase cells membranes can remain physically intact, even in dead cells, suggesting that exponential phase cells are inactivated under high pressure by irreversible damage to the cell membrane. In contrast, stationary-phase cells have a cytoplasmic membrane that is robust enough to withstand intense treatments.

1.3.2 Treatment temperature

A temperature increase causes a volume expansion, and an increase in pressure causes a reduction in volume. However, during compression, the sample temperature increases due to adiabatic heating or heat of compression (Delgado et al., 2007; Michiels et al., 2008). For every temperature there is a corresponding pressure and the primary effect of pressure affects the volume of the product being processed. Thus, the combined net effect pressure-temperature during treatment may be synergistic, antagonistic, or additive (Gupta et al., 2011). Therefore, reactions such as phase transitions or molecular reorientation depend on both temperature and pressure and cannot be treated separately (Balasubramaniam et al., 2015). Denaturation kinetics of proteins as a function of temperature and pressure form an elliptic curve when connecting the points of isokineticity (Michiels et al., 2008; Knorr et al., 2006); the same pattern also occurs on inactivation of *E. coli* (Smeller, 2002). High pressure induces unfolding of globular proteins. High pressure leads to a partial or complete inactivation of numerous enzymes and metabolic pathways, which may cause cell death (Knorr et al., 2011).

High pressure is considered a non-thermal food preservation technology. However, for certain application pressure treatment at ambient temperature does not provide adequate microbial inactivation. For example, inactivation of extremely pressure resistant *E. coli* required application of more than 600 MPa (3 min) at room temperature (Liu et al., 2015). For inactivation of *E. coli* O157:H7 and K12 there is a synergistic effect between pressure and moderate temperatures in orange juice (Torres et al., 2015). Van Opstal et al. (2005) found that *E. coli* MG1655 is more pressure resistant (150-600 MPa) at 5 °C when compared to treatments at 20 °C or higher in buffer and carrot juice. The inactivation of *E. coli* O157:H7 treated at 550

MPa in orange juice were higher when the temperature was increased from 20 °C to 30 °C (Linton et al., 1999). Similar, treatment of *L. monocytogenes* at 207 MPa for 5 min at 35 °C reduced cell counts by 2.5 log cycles, whereas treatmentment at 45 °C decreased cell counts by 9 log cycles (Kalchayanand et al., 1998). Pressurization at subzero temperatures without freezing significantly enhances the lethal effect of pressure in *Lactobacillus plantarum* and *Saccharomyces cerevisiae* (Perrier-Cornet et al., 2005). Inactivation of *V. parahaemolyticus* in oyster homogenates at 200 MPa was greatly enhanced by lowering the processing temperature from 15 °C (3.4 log CFU/g) to 5°C (4.6 log CFU/g) or 1.5 °C (6.5 log CFU/g) (Phuvasate and Su, 2015). However, pressure treatment at low temperatures is not consistent. For example *E. coli* and *Staphylococcus aureus* were more resistant to pressure application at 4 °C than to the same pressure at 25 °C (Trujillo et al., 2002). Furthermore, *E. coli* MG1655 is pressure resistant at 5 °C when compared to treatments at 20 °C (Van Opstal et al., 2005). The decrease on pressure resistance at low temperature changes the membrane structure and fluidity through weakening of hydrophobic interactions and crystallization this changes may affect pressure resistance (Kalchayanand et al., 1998; Cheftel, 1995).

1.3.3 Water activity (a_w)

While reduced a_w can inhibit the growth of microorganisms, it can also protect them from other environmental stresses, such as heat (Gould, 1985). Decreasing a_w also increases the resistance of microorganisms to high pressures (Oxen and Knorr, 1993). Increased barotolerance of listeria at elevated osmolarity is partially attributed to the presence of compatible solutes. The percentage of survival of listeria following exposure to 400 MPa for 5 min increased from 0.008 to 0.02% when 5 mM L-carnitine was added and to 0.05% when 5 mM betaine was added

(Smiddy et al., 2004). The addition of 4 M NaCl or 0.5 M sucrose protects *L. lactis* against inactivation at 200 MPa (Molina-Höppner et al., 2004). Moderate sucrose concentrations (<10%) in buffer confer baroprotection to *E. coli* inducing a very small change in the water activity, suggesting that the protective effect is linked to the nature of the solute added and not only a decrease of water activity (Van Opstal et al., 2003). Increased barotolerance may be due to formation of a hydration shell by exclusion of compatible solutes from the immediate surface of proteins, thus protecting essential proteins and enzymes from unfolding. Compatible solutes have previously been suggested to play a role in membrane fluidity, changing fatty acid composition of membrane lipids and that an increase in membrane fluidity may increase resistance to pressure (Smiddy et al., 2004; Molina-Höppner et al., 2004). Reducing a_w stabilizes proteins during high pressure (Moussa et al., 2006; Hayman et al., 2008). For example increasing the water activity of peanut butter (<50%) enhances inactivation of a cocktail of six strains of *Salmonella* after high pressure at 600 MPa for 18 min (D'Souza et al., 2014). Thus food processors should consider a_w of foods before contemplate high pressure as a preservation method of specific substrates.

1.3.4 pH

Another important factor that influences susceptibility to high pressure of different microorganisms is pH. Low pH (3.5-4) alone does not inactivate microorganisms (Pagan et al., 2001) but in combination with high pressure the inactivation is enhanced (Alpas et al., 2000; Pagan et al., 2001; Ogihara et al., 2009). After pressure processing, injured cells cannot recover, and even those that not showed any effect of the treatment may become more sensitive to the high acidity of the medium (Syed et al., 2015). Cellular β-galactosidase is more acid labile in

damaged cells and sensitization to acid may thus involve loss of protective or repair functions (Pagan et al., 2001). Klotz et al., (2010) showed that in a pressure sensitive strain of *E. coli* loss of viability coincided with irreversible loss of membrane integrity. In a pressure resistant strain of *E. coli*, propidium iodide (PI) was taken up during pressure treatment but not after decompression indicating that cells were able to reseal their membranes after pressure treatment. A transient loss of membrane integrity during pressure thus may lead to cell death irrespective of whether cells can reseal their membranes afterwards. High pressure may also inactivate membrane proteins responsible for regulating the trans-membranous flow of protons, leading to inability to maintain homeostasis, disruption of electron transport components leading to oxidative stress loss of critical intracellular components or an irreversible change in the intracellular environment that prevents recovery (Hoover et al., 1989; Klotz et al., 2010). Thus, the suspending matrix may be critical in the survival of transiently permeabilized cells (Hauben et al., 1996). The combination of high pressure and acidic conditions has been studied. For example pressurization in the presence of either citric or lactic acid increased the viability loss of *L. monocytogenes*, *E. coli* O157:H7, *S.* Enteritidis and *S.* Typhimurium by an additional 1.2–3.9 log cycles at pH 4.5 when compared with a pH of 6.5 for both acids at 345 MPa (Alpas et al., 2000). Koseki and Yamamoto, (2006) found that application of 300 MPa in buffered peptone water at pH 7 had not bactericidal effect on *L. monocytogenes*. When the pH was reduced at 4 *L. monocytogenes* was completely inactivated (8 log reduction). Mackey et al., (1995) reported that pressure treatment at 304 MPa resulted in an additional 1.8 log reduction in *L. monocytogenes* when reducing the media pH from 7.1 to 5.3. This combination treatment indicates possible enhancement of microbial inactivation of foods with high a_w and low pH.

1.4 Injured cells after high pressure treatment

During high pressure the microorganism can be inactivated or sublethally injured and microbial cells that survive pressurization also developed sensitivity to physical and chemical environments to which the normal cells are resistant (Kalchayanand et al., 1994; Hauben et al., 1996; Kalchayanand et al., 1998). Survivor cells are likely to have damage to many cell components (Ganzle and Liu, 2015). Outer membrane damage is not believed to be lethal but does allow entry of antimicrobial substances that can enhance lethality of pressure treatments (Garcia-Graells et al., 1999). Damage to the cell membrane is believed to be one of the critical factors leading to the death of pressure-treated bacteria (Ritz et al., 2001; Russell, 2002). Sublethally injured cells are more fastidious in their growth requirements but can repair the damage and grow out if the environmental conditions are suitable (Wuytack et al., 2003). Chilton et al., (2001) found that in *E. coli* K12 pressure treated at 400 MPa for 2 min, more than 99% of the surviving population was sensitive to the presence of bile salts in the recovery medium, but resistance was regained within 1 hr of incubation in TSB (trypticase soy agar). Enhance sensitivity to acid conditions or salt concentration is generally attributed to impairment of cytoplasmic membrane function (Jordan et al., 2001). Bacterial cells are inactivated or injured during high pressure processing, but the damage could be reversible if the medium contains the necessary nutrients under conditions of optimum pH and temperature (Bozoglu et al., 2004). However, unfavorable conditions such as low pH (Fig 1-1) also increase the inactivation of injured bacterial cells during storage time (Alpas et al., 2000).

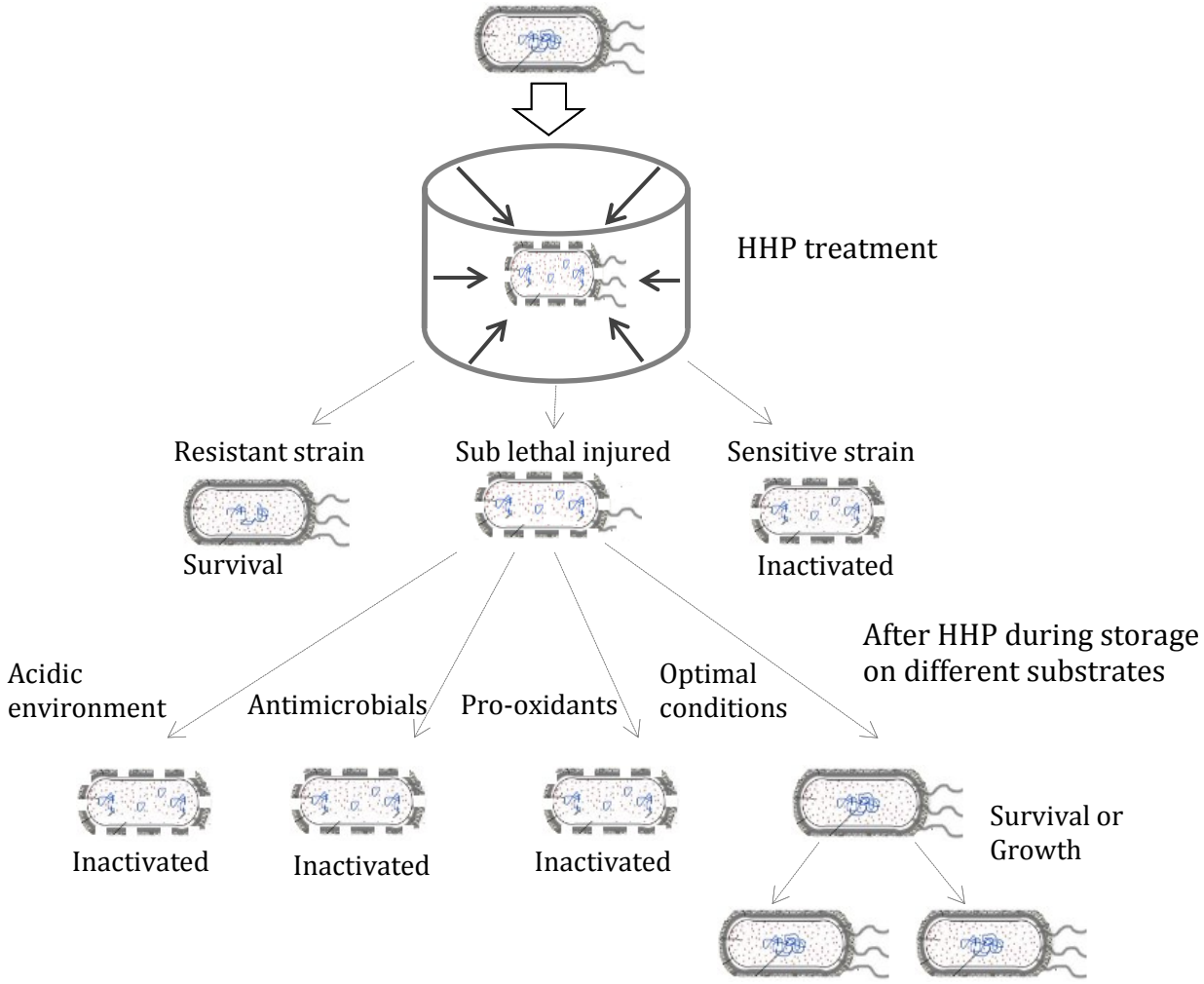

Figure 1-1. Potential impact of storage conditions on the survival or growth of *E. coli* after high pressure treatment.

1.5 Effect of high pressure on survival during the subsequent storage

Product quality and shelf life are also influenced by packing material barrier properties and storage conditions (Balasubramaniam et al., 2015). After pressure treatments of *L. monocytogenes* and *S.* Typhimurium in buffers at pH 7 and 5.6, total bacterial inactivation was achieved but resuscitation was observed for the two microorganisms during storage at 4 and 20 °C (Ritz et al., 2006). Similar results were found on *E. coli* where no colonies were detected on

plate count agar throughout a 120 h incubation at 4 or 37 °C. However, the number of *E. coli* increased during storage at 25 °C from an undetectable level to the level of initial cell counts regardless of the treatment pressure (Koseki and Yamamoto, 2006). During storage for 21 days at 4 °C after HHP at 600 MPa for 3 min *E coli* (O157 cocktail) was able to recover from below detection limit to the level of untreated samples on beef salami samples but cell population remained below detection limit on the Hungarian style salami during the same period of storage (Gill and Ramaswamy, 2008). Pressure reduced *E. coli* O157:H7 in ground beef by 3 log (CFU/g) and caused substantial sublethal injury resulting in further reductions (2 log CFU/g) of bacteria during frozen storage (-20 °C) for 5 days (Black et al., 2010). When *E. coli* was treated in Tris buffer, skim milk and orange juice (600 MPa/ 3 min) the highest recovery of stressed *E. coli* after 24 h of storage at 4 °C was in Tris buffer followed by skimmed milk (1.19 and 0.79 log cfu/ml, respectively). However, samples of orange juice (non favorable environment because of low pH) did not allow stressed cells to recover (Syed et al., 2013). The inactivation of *E. coli* during storage was inversely correlated with pH in juice and HEPES buffer; after pressure at 300 MPa and 20 °C for 15 min cell reduction in apple juice (pH 3.3) was 1.1 log (cfu/mL) followed by 5 log (cfu/mL) reduction during the first 2 days of storage suggesting that treatment caused sublethal injury to a large proportion of cells, resulting in a reduced resistance to low pH during subsequent storage (Garcia-Graells et al., 1998). Huang et al., (2013) found that a cocktail of *E. coli* O157:H7 (5) and *Salmonella* (4) inoculated to strawberry puree (pH 3.6) decreased 2 log after pressure treatment (200 MPa at 20 °C for 15 min) followed by 3 log (cfu/g) during 8 days of storage at -18 °C. Turkey breast and cured ham pressure treated at 400 MPa for 15 min decreased the growth of *Listeria monocytogenes* during subsequent storage at 8 and 12 °C (Pal et al. 2008).

These studies indicate that different incubation conditions and different matrices after pressure treatment may improve recovery, enhance inactivation, and/or maintain the same amount of viable cells. However, more studies are needed in food systems and specific conditions to study the resuscitation and/or further inactivation phenomenon to determine appropriate conditions for storage after HHP and avoid regrowth of microorganisms.

Table 1-1 Effect of high pressure during the subsequent storage at different conditions on the inactivation/recovery of different microorganisms and matrices.

Microorganism (Number of strains)	Treatment GPa/°C/min	Storage °C/days/pH	Matrix	Remarks (+) Growth (-) Inactivation (=) Same	Reference
E. coli (5) *Samonella*(4)	0.2/21/2	-18/8/3.6	Strawberry puree	>-2 log	Huang et al. 2013
E. coli (5)	0.4/20/10	-20/30/nd	Ground beef	>-2 log	Black et al. 2010
E. coli *L. monocytogenes*	0.3/18/8	4/1/4	Buffer/Na nitrite (<1 mmol)	>-3 log	De Alba et al. 2013
E. coli (5)	0.6/RT*/3	15/28/nd	Beef salami Hungarian salami	>+3 log =	Gill and Ramaswamy. 2008
E. coli (5)	0.3/12-34/10	12/60/<5.3	Cheese and bacteriocin-producing/ LAB*	>-2 log	Rodriguez et al. 2005
S. enteritidis *E. coli*	0.45/12/5	22/7/nd	Beef carpaccio/ LPOS*	>-1 log >-2 log	Bravo et al. 2013
L. monocytogenes	0.4/17/10	6/45/nd	Active packaged ham slices (nisin, sakacin and enterocins)	Active package (=) Control (>+3 log)	Jofre et al. 2007

*Lactic acid bacteria (LAB), lactoperoxidase system (LPOS). Room Temp (RT). Not determined (nd)

1.5.1 High pressure and oxidative stress

High pressure treatment induces endogenous intracellular oxidative stress in *E. coli*

suggesting that, at least under some conditions, the inactivation of *E. coli* by high hydrostatic pressure treatment is the consequence of a suicide mechanism involving the induction of an endogenous oxidative burst (Aertsen et al., 2005). Malone et al. (2006) reported that the expression of Fe-S cluster assembly proteins and the fumarate nitrate reductase regulator decreases the resistance to pressure. Yan et al. (2013) reported that intracellular free iron in *E. coli* increased in a pressure-dose-dependent manner, and the addition of an iron chelator protects *E. coli* against high pressure, suggesting that free iron contributed to lethality via production of damaging by-products. The reaction between ferrous iron and oxygen results in the formation of harmful superoxide and hydroxyl radicals, which affect all macromolecules of bacteria such as DNA, lipids and proteins (Cabiscol et al. 2010). Anaerobic incubation after pressure treatment significantly supported the recovery of *E. coli*, suggesting that cell death is prevented by a mechanism involving oxidative stress (Aertsen et al. 2005). Thus anaerobic packaging of the product treated under HHP may allow recovery of *E. coli* if the conditions are appropriate, leading to potential foodborne outbreaks.

1.5.2 High pressure and ions

The composition of food is important, since the presence of minerals such as divalent cations, (Hauben et al., 1998; Lenz and Vogel, 2014), sugars and salts (Molina-Hoppner et al., 2004), and food matrix itself (Baccus-Taylor et al., 2015) serves as a protector and increases microbial resistance toward high pressure. *E. coli* in the presence of divalent cations Ca^{2+} and Mg^{2+} increase pressure resistance in a medium containing EDTA, suggesting that Ca^{2+} and Mg^{2+} can stabilize important cellular targets of high pressure in *E. coli.* (Hauben et al., 1998). Pressure affects ionization equilibria, specially the dissociation of weak acids and bases, as the

equilibrium will shift to minimize the effect of pressure (Le Chatelier principle) inducing pH changes. Water molecules also pack more closely around free ions (electrostriction) resulting in a net reduction in volume of the system (Issacs, 1981). Pressure treatment of *E. coli* and *S. aureus* in buffer with salts causing a large electrostriction effect (Na_2SO_4 and $CaCl_2$) was more bactericidal when compared to treatment in buffer with salts causing a lower electrostriction effect (NaCl and KCl). However, salts with divalent ions (positive reaction volume) were protective at much lower concentrations than salts with monovalent ions (negative reaction volume), suggesting that the effective solute concentration preventing pressure-induced inactivation will differ depending on the solubility of the solute (Gayán et al., 2013). Sahalan et al., (2013) reported that in presence of the divalent cations Ca^{2+} and Mg^{2+}, leakage of enzyme markers and lipopolysaccharide (LPS) released were significantly reduced when *E. coli* was exposed to polymyxin B and the survival cells also increased. This suggests that cations especially Ca^{2+}, help to stabilize and maintain the integrity of the outer membrane by binding between adjacent LPS molecules. Meat is a complex matrix with a high nutrient environment, ground beef showed a great baroprotective effect, protecting *E. coli* O157:H7 from pressure inactivation compared to cells treated in beef gravy and peptone water (Baccus-Taylor et al., 2015). The presence of specific ions such as Ca^{2+} may explain the increase of pressure resistance of particular food substrates such as milk and meat products. The availability of some substrates or the presence of compounds in the matrix such as vitamins and amino acids in the food may allow better recovery of sublethally damaged cells after pressure treatment (Tassou et a., 2007). However, the exact mechanisms of how ions confer baroprotection to different microorganisms needs to be elucidated. Data regarding baroprotective effect of different

ingredients from food systems can be useful to the food industry when considering different matrices and microorganisms in food safety risk assessment.

Table 1-2 Baroprotective effect of different ingredients from food

Microorganism	Treatment GPa/°C/min	Matrix/pH	Compound(s)	Remarks Protective Effect	Reference
L. lactis	0.3/20/20	Milk buffer/<6	4 M of NaCl 0.5 M of sucrose	>2 log >4 log	Molina-Höppner et al. 2004
E. coli	0.27/20/15	Buffer and EDTA/7	$CaCl_2$, $MgCl_2$,	>2 log	Hauben et al. 1998
Penicillium expansum *Saccharomyces cerevisiae* *Fusarium oxysporum*	0.6/20/<2	Buffer/4.2	Sucrose, NaCl and glycerol	>2 log	Goh et al. 2007
E. coli	0.4/30/20	Ground beef/6.5 Peptone water/5.8	Ground beef (matrix)	>2.5 log	Baccus-Taylor et al. 2015
S. enteritidis	0.25/25/30	Buffer/<7	Calcium propionate, potassium sorbate, sodium acetate, sodium lactate, and sucrose myristic acid	>1.5 log	Ogihara et al. 2009

1.6 Synergistic effect between high pressure and different antimicrobials

High pressure technology can be used in combination with additional antimicrobials to more effectively reduce the numbers of microorganisms present in food. Hurdles with synergistic effects are effective and could reduce the intensity of the pressure treatment applied reducing the final cost (Wang et al., 2014). The increase in inactivation rates of different microorganisms in acidic environments may related to a restricted pH range that bacteria can

tolerate under pressure, loss of protective or repair functions, possible because of the inhibition of ATPase-dependent transfer of protons and cations and/or denaturation in the membrane (Michiels et al., 2008; Pagan et al., 2001). High pressure combined with lysozyme or with some bacteriocins exhibited a synergistic antimicrobial effect against pathogens (Hauben et al., 1996). Synergy between HP and LPOS was first reported by Garcia-Graells et al in the late 1990's Synergistic bactericidal interaction of lactoperoxidase (LPOS) and high pressure against *E. coli* and *Listeria Innocua* in Milk (Garcia-Graells et al., 2000) and *S.* Enteritidis and *E. coli* O157:H7 inoculated in beef carpaccio has been reported (Bravo et al., 2014). LPOS oxidizes exposed sulfhydryl groups of enzymes and proteins in the bacterial cell membrane, inhibiting the transport of nutrients as well as DNA and RNA synthesis and the respiratory chain (Pruitt and Reiter, 1985). High pressure and LPOS resistance observed for the baroresistant *E. coli* LMM1010 might be associated to oxidative stress resistance, as the oxidation of sulfhydryl group of enzymes and proteins in the bacterial cell membrane is the main activity of LPOS (García-Graells et al., 2003). Feyaerts et al (2015) reported a synergistic effect of HHP with natural antimicrobial compounds (NACs) such as α,β-unsaturated aldehydes (t-cinnamaldehyde, t-2-hexenal, dimethylfumarate), isothiocyanates (allyl isothio- cyanate, sulforaphane) and other electrophilic compounds (reuterin). Generally the synergistic effect was linked to thiol reactive compounds. High pressure unfolds proteins exposing electrophilic aldehydes groups that react with thiols groups and this reactivity has been linked to its antibacterial activity (Schaefer et al. 2010). However, more studies are needed to determine the cooperative antimicrobial activity of high pressure with LPOS and thiols groups.

1.6.1 High pressure and nitrites

Nitrites and nitrates are used as food additives to inhibit non-spore forming spoilage organisms and pathogens (Honikel, 2008). Nitrous acid breaks down spontaneously to give nitric oxide ($NO^{\bullet}$), a free radical that can react with reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to form a variety of antimicrobial molecular species that are more potent than nitric oxide itself (Brunelli et al., 1995). High pressure damage to membranes, denatured proteins and the generation of oxidative stress has been reported (Mackey et al., 2008; Aertsen et al., 2005). This fact suggests that pressure may enhance the antimicrobial effect of nitrite. Jofre et al. (2009) reported that for *S. aureus* treated with high pressure combined with low pH and nitrite, cell counts progressively decrease. Treatment of *E. coli* at 300 MPa for 8 min reduced cell counts by 1.4 log (CFU/mL), however, combination of the same pressure treatment with 2 mmol/L sodium nitrite enhanced the bactericidal effect of the treatment to 4.9 log (Alba et al., 2013). The exact mechanism of the synergistic effect still unknow but could be due to damage of the protective systems against nitrosamine or the formation of ROS. However more studies are needed to identify the pathway and to define the effect of nitrite on different microorganisms and the effect and amount needed on different food systems.

1.6.2 High pressure and antimicrobials

Combination of pressure and antimicrobials would increase the death rate because cells surviving pressurization also become sublethally injured and are then killed by bacteriocins (Hauben et al., 1996; Garriga et al., 2002). Outer membrane damage is not believed to be lethal but does allow entry of antimicrobial substances that can enhance lethality of pressure

treatments (Hauben et al., 1996; Garcia-Graells et al., 1999). Nisin is a peptide with activity at the cytoplasmic membrane, forming pores that affect stability. Under normal conditions its activity is restricted to Gram-positive bacteria, whose cell wall is not protected by lipopolysaccharides normally present in the outer cell membrane of Gram-negative bacteria. It can also be active in Gram-negative bacteria when the barrier properties of the membrane are removed by high pressure (Kalchayanand et al., 1998; Masschalck et al., 2001). In addition to the synergistic effect of nisin and high pressure, Ogihara et al., (2009), found that different food additives (citric acid, adipic acid, glycerin monocaprylic acid ester (C8), glycerin mono-capric acid ester (C10), tannic acid, wasabi extract, ε-polylysine, or protamine sulfate) had synergistic effects with pressure treatment on the inactivation of *S.* Enteritidis. Another example is essential oils (EOs) or their chemical constituents (+)-limonene, carvacrol, *C. reticulata*, *T. algeriensis* and *C. sinensis* in combination with high pressure enhance inactivation of *E. coli* O157:H7 and *L. monocytogenes* (Espina et al., 2013).

1.6.3 High pressure and chitosan

Chitosan is a collective name for a group of partially and fully deacetylated chitin compounds with a wide spectrum of antimicrobial activity and high killing rate against Gram-positive and Gram-negative bacteria, but lower toxicity toward mammalian cells (Kong et al. 2010). Chitosan acted synergistically with high pressure to enhance inactivation of *E. coli* K-12 in apple juice (Kumar et al., 2009). These results indicate that some food additives are useful for increasing the inactivation ratio of different microorganisms in combination of high pressure treatment (Ogihara et al (2009). Knowledge of the synergistic effect of different food ingredients and high pressure can help to ensure the safety of processed foods. However, the

mechanism of the synergistic effect of these factors (pH, a_w) and food additives with pressure treatment needs to be further studied.

Table 1-3 Synergistic effect of different food ingredients and high pressure

Microorganism	Treatment GPa/°C/min	Matrix/pH	Compound	Remarks Enhancing Effect	Reference
E. coli *L. innocua*	0.4/20/15	Milk/6.7	LP*	<1 log >2 log	Garcia-Graells et al. 2000
E. coli *L. monocytogenes*	<0.4/20/20	Fruit juices/4	Essential oils or their constituents	<≠ log	Espina et al. 2013
L. monocytogenes	0.3/20/ 20	ACES buffer	Carvacrol	<2 log	Karatzas et al. 2001
L. monocytogenes *E. coli*	0.3/4/8/	Citrate Buffer/4	Sodium nitrite (<1 mmol)	>2 logs	De Alba et al. 2013
L. monocytogenes	0.4/10/10	Chicken breast fillets/nd	LF*, AMLF*, PDLF* and ALF*	>3 log	Del Olmo et al. 2012
S. enterica	<0.55/25/10	TSBY broth	nisin	Nd*	Lee and Kaletunç 2010
E. coli	0.27/20/15	Bis- Tris-HCl Buffer/7	$ZnCl_2$, $NiCl_2$, $CuCl_2$ and $CoCl_2$/7	>2 log	Hauben et al. 1998
S. Enteritidis	0.25/25/30	Buffer/<7	Citric acid, adipidic acid, C8*, C10*, tannin, nisin, protamine	>3 log	Ogihara et al. 2009

*Lactoperoxidase (LP), lactoferrin (LF), amidated lactoferrin (AMILF), pepsin digested lactoferrin (PDLF), activated lactoferrin (ALF), glycerin monocaprylic acid ester (C8), glycerin monocapric acid ester (C10), Not determined (Nd).

1.7 Research and objectives

Pathogenic strains of *E. coli* remain a threat of food industry to final consumers and food producers. High pressure has been adopted for the preservation of different food products. Due to the large strain-to-strain variability of the pressure resistance, different pathogenic and non-pathogenic microorganisms survive the application of pressure treatments that matches the

conditions used in food industry (200-600 MPa), making industrial implementation of this technology problematic for specific products. Intrinsic factors influences pressure resistance variability of *E. coli* and environmental factors such as food matrix frequently confer a baroprotective effect. The validation of novel processes (HHP) targeting different microorganisms (species and strains) in specific food systems need to be done to verify the process efficacy. Strain cocktails of non-pathogenic bacteria for use in challenge studies have to be validated with cocktails of pathogenic strains, that behave similarly to the target pathogen when exposed to processing conditions (Ingham et al., 2010). Therefore the objectives of this project were as follows:

1) To develop and validate a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *E. coli.*
2) To determine food constituents that are present in ground beef that may confers protection of *E.coli* toward high hydrostatic pressure.
3) To determine genomic changes in extremely pressure-resistant derivatives of *E. coli* by genome comparison before and after acquisition of pressure resistance.

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

2.1 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 the product (Considine et al., 2008; Hsu et al., 2015; Knorr et al., 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).

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 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.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

Bacterial strains and their origin are listed in Table 2-1. *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.

Table 2-1. 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	-	-	-	

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

2.2.2 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.

2.2.3 Determination of HP resistance

Pressure treatments were carried out as described previously (Liu et al., 2012). 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 sec. 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.

2.2.4 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.

2.2.5 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 were determined by plating serial dilutions on LB agar. Plates were incubated at 37 °C for 24 h. Experiments were performed in triplicate.

2.2.6 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 2.0 log (cfu/g), respectively. Meat (6 g) was inoculated with a fresh 5-strain cocktail (1 mL) to a final cell count of 7.7 ± 0.33 log (cfu/g) for the non-VTEC cocktail and 7.6 ± 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.

2.2.7 Statistical analysis

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

2.3. Results

2.3.1 Heat and pressure resistance of *E. coli*

To determine the heat or pressure resistance of slaughter plant isolates of *E. coli*, eleven strains of *E. coli* were heat treated at 60 °C or pressure treated at 600 MPa in LB broth with 1% NaCl. The heat- and pressure resistant strain *E. coli* AW1.7 was used as reference (Dlusskaya et al., 2011; Liu et al., 2012). Survivor curves are shown in Figure 2-1. Three strains, *E. coli* AW1.3, DM18.3 and GM16.6, showed heat resistance comparable to *E. coli* AW1.7. Cell counts of these strains were reduced by less than 5.0 log (cfu/mL) after 20 min at 60 °C.

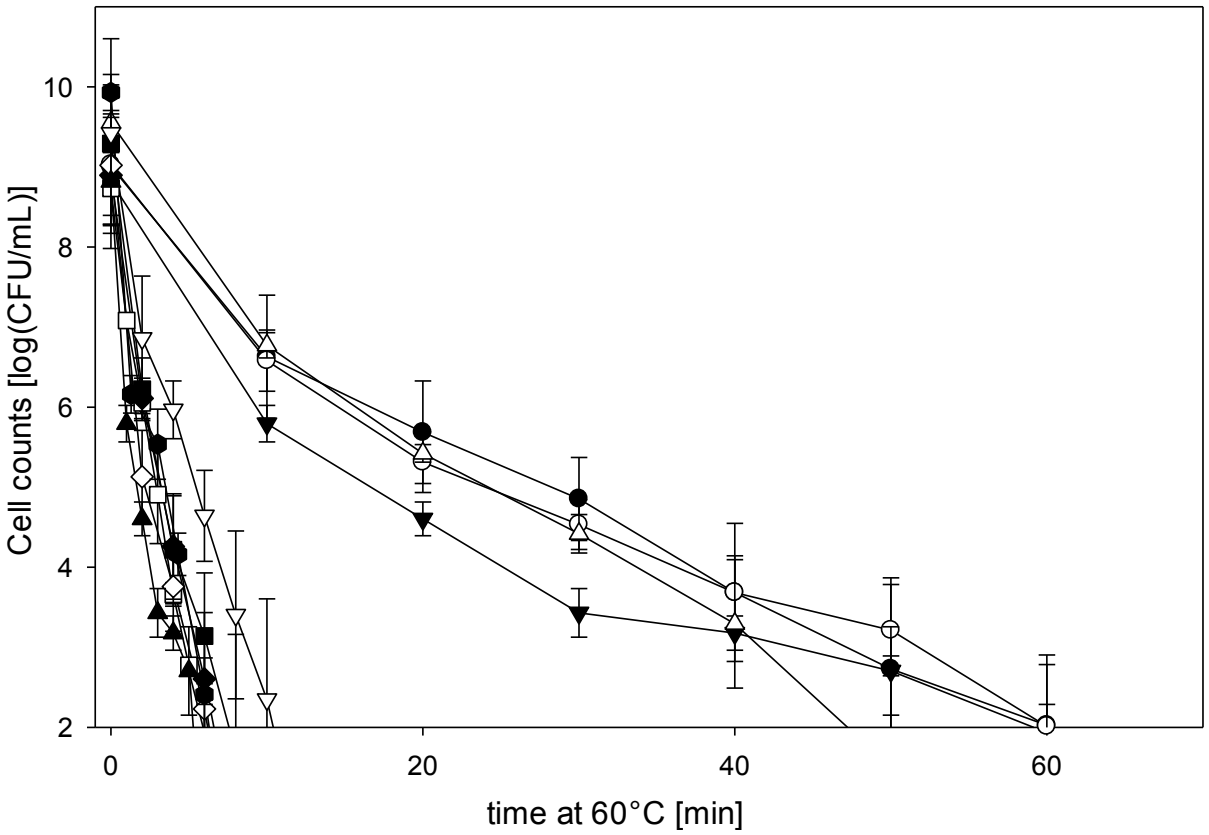

Figure 2-1. Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 60 °C in LB. AW 1.7 (●), AW 1.3 (○), DM 18.3 (▼), GM 16.6 (Δ), MB 2.1 (■), MB 3.4 (□), GM 9.8 (♦), GM 11.5 (◊), GM 18.3 (▲), GM 11.9, (∇), GGG 10 (-). Cells were grown and treated in LB broth containing 1% NaCl. 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).

The pressure resistance of ten strains of *E. coli* was comparable to *E. coli* AW1.7, corresponding to a reduction of cell counts of less than 6.0 log (cfu/mL) after 15 min at 400 MPa and 40 °C. *E. coli* GGG10 was sensitive to pressure (Figure 2-2). Four heat resistant strains, *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6, and three heat sensitive strains, *E. coli* GM18.3, GM11.5 and GGG10, were selected for further experiments. *E. coli* MG1655 was added as a reference strain.

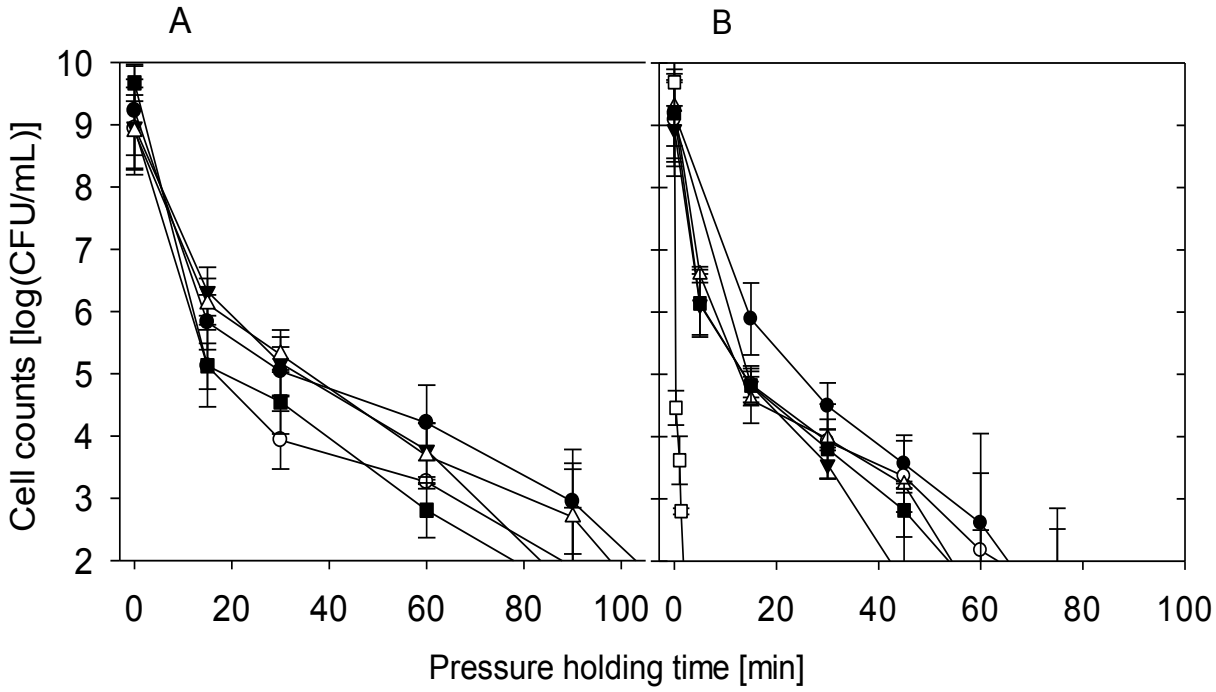

Figure 2-2. Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 400 MPa and 40 °C. Cells were grown and treated in LB broth containing 1% NaCl. Panel A: AW 1.7 (●), AW 1.3 (○), GM 16.6 (▼), DM 18.3 (Δ), and MB 3.4 (■). Panel B: MB 2.1(●), GM 9.8(○), GM 11.5 (▼), GM 18.3(Δ), GM 11.9 (■), and GGG10 (□). 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).

2.3.2. Effect of NaCl on heat and pressure resistance

Supplementation of media with NaCl increased the heat resistance of *E. coli* AW1.7 (Pleitner et al., 2012). To determine whether NaCl has a comparable effect on the resistance of other strains of *E. coli,* the heat and pressure resistance was determined after addition of 0 to 4% NaCl to LB broth. The addition of NaCl increased the heat resistance of *E. coli* AW1.3, DM18.3, GM16.6, GM18.3, GM11.5 and MG1655, comparable to the effect of NaCl on the heat resistance of *E. coli* AW1.7 and GGG10 (Figure 2-3). Omission of NaCl in the growth and treatment medium reduced the heat resistance of all *E. coli* strains. For example, cell counts of

E. coli AW1.7 decreased about 5.5 log (cfu/ml) in the absence of NaCl and about 2.2 log (cfu/ml) in the presence of 2 or 4 % NaCl after treatment at 60 °C for 40 min. Interestingly, the addition of 2 and 4 % NaCl did not affect the resistance of *E. coli* AW1.3, DM18.3, GM16.6, GM18.3, GM11.5 and MG1655, to treatment at 400 MPa at 40 °C, or to treatment at 600 MPa and 20°C (data not shown).

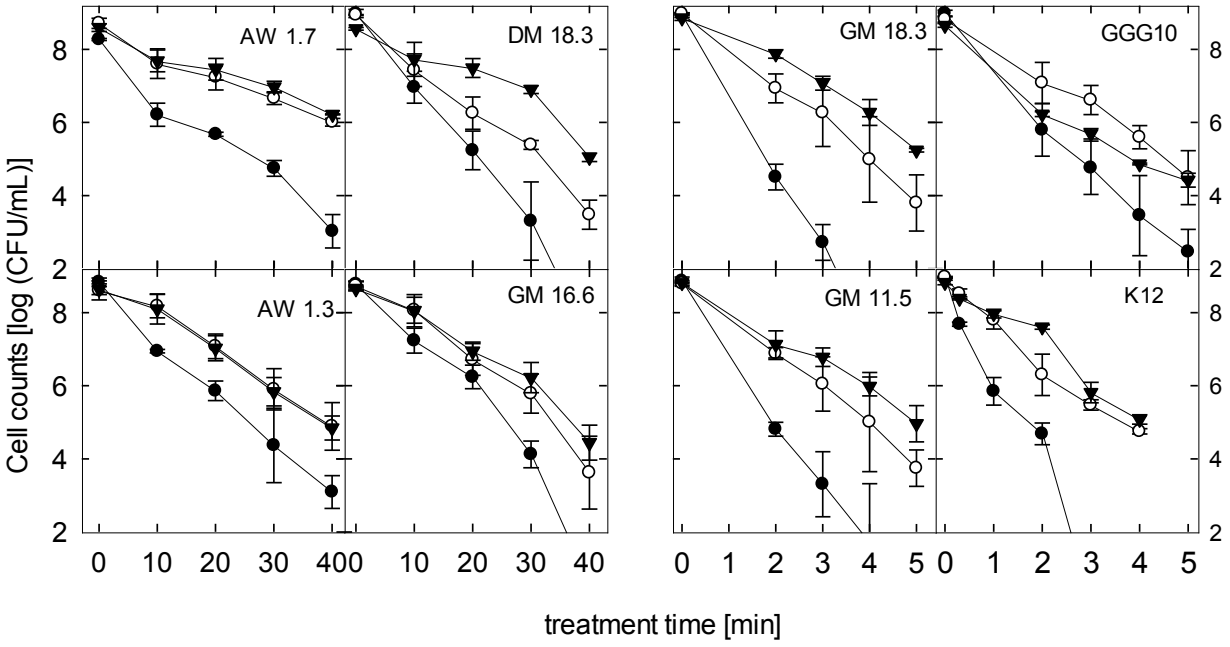

Figure 2-3. Viable cell counts of non-pathogenic strains of *E. coli* after heat treatment at 60 °C. Cells were grown and treated in LB broth containing the following NaCl concentration: 0 % (●), 2 % (○) and 4 % (▼). Panel A: cells were treated from 0 to 40 min. Panel B: cells were treated from 0 to 5 min. 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).

2.3.3 Effect of temperature during HP treatment at 600 MPa

To determine the effect of temperature during pressure inactivation, the resistance of *E. coli* to treatment at 600 MPa was determined at 3, 20 and 40 °C in LB broth. Pressure death time data are shown for *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 at each temperature in Figure 2-4. All strains of *E. coli* were least resistant to pressure at 40 °C and most resistant to pressure at 3 °C (Figure 2-4). After 5 min of treatment at 40 °C and 600 MPa, cell counts of all strains were reduced to less than 2.0 log (cfu/ml). Pronounced tailing was observed when samples were treated at 3 °C and 600 MPa. Cell counts of all four strains of *E. coli* remained higher than 3.0 log (cfu/mL) after treatment at 3 °C and 600 MPa for up to 30 min (Figure 2-4).

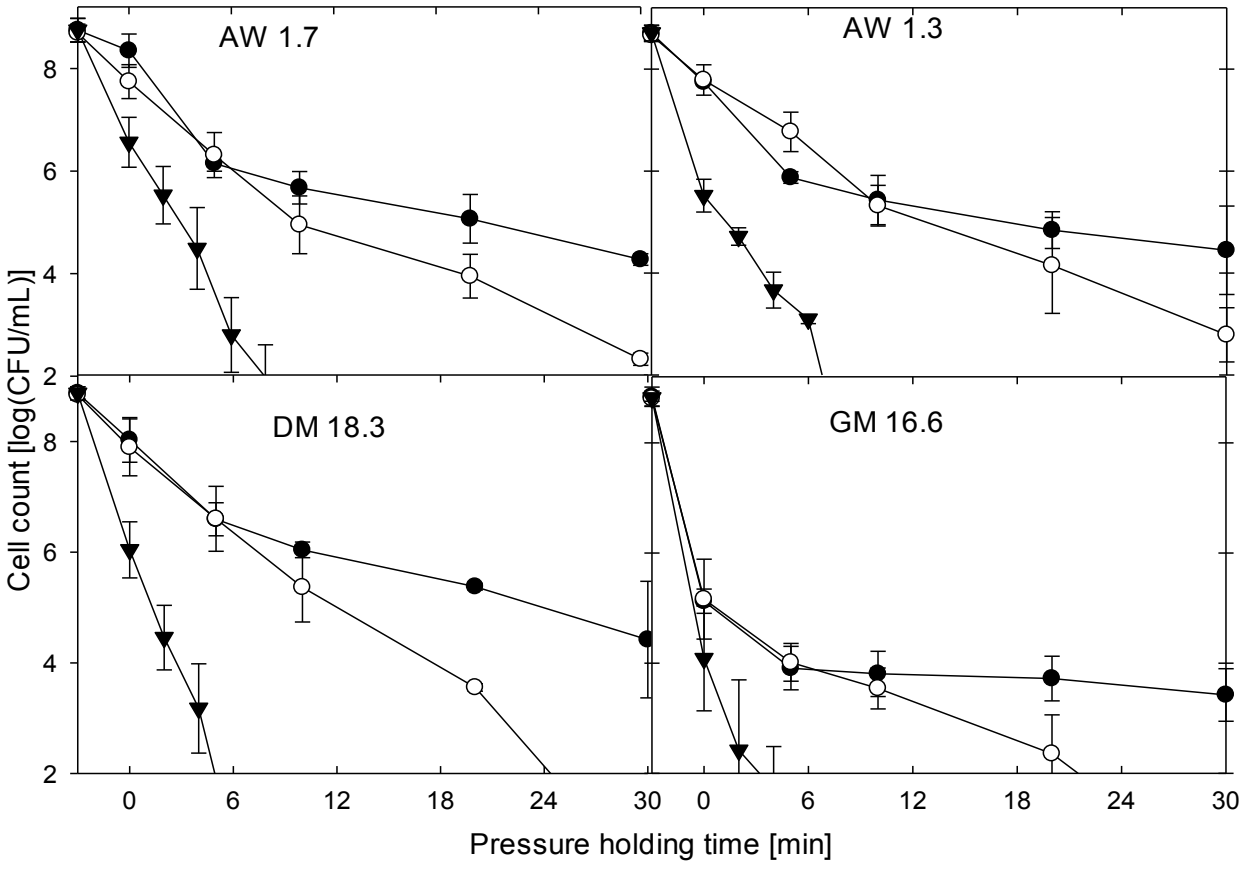

Figure 2-4. Viable cell counts of non-pathogenic strains of *E. coli* after treatment at 600 MPa with the following temperatures: 3° (●), 20° (○) and 40 °C (▼). Cells were grown and treated in LB broth containing 1% NaCl. Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/ml).

2.3.4 Pressure inactivation of VTEC and Non-VTEC on ground beef

To validate pressure resistance data in a food model system, and to compare the pressure resistance of meat isolates with VTEC, treatments at 600 MPa and 20 °C were performed with two five-strain cocktails in ground beef. The VTEC strain cocktail contained five pressure resistant strains of VTEC that were identified after screening of 102 VTEC (Liu et al., 2015). Surviving cells were enumerated on LB agar to quantify total viable cells; the low initial cell counts of the meat used (less than 400 cfu/g) allowed the accurate quantification of the inoculum without interference of indigenous microbiota. Surviving cells were also enumerated on VRBA, which inhibits growth of sublethally injured cells with a permeabilized outer membrane (Hauben et al., 1996). Survival of both strain cocktails was generally equivalent (Figure 2-5); a significant difference between total cell counts of the two cocktails was observed after 2 min of treatment but cell counts at other treatment times or cell counts on VRBA were not significantly different. Both strain cocktails exhibited a substantial resistance to pressure. The reduction of cell counts was about 2.0 and 5.0 log (cfu/g) after 5 and 30 min, respectively. Cell counts on VRBA were reduced below 2.0 log (cfu/g) after 15 min, indicating that surviving cells were sublethally injured.

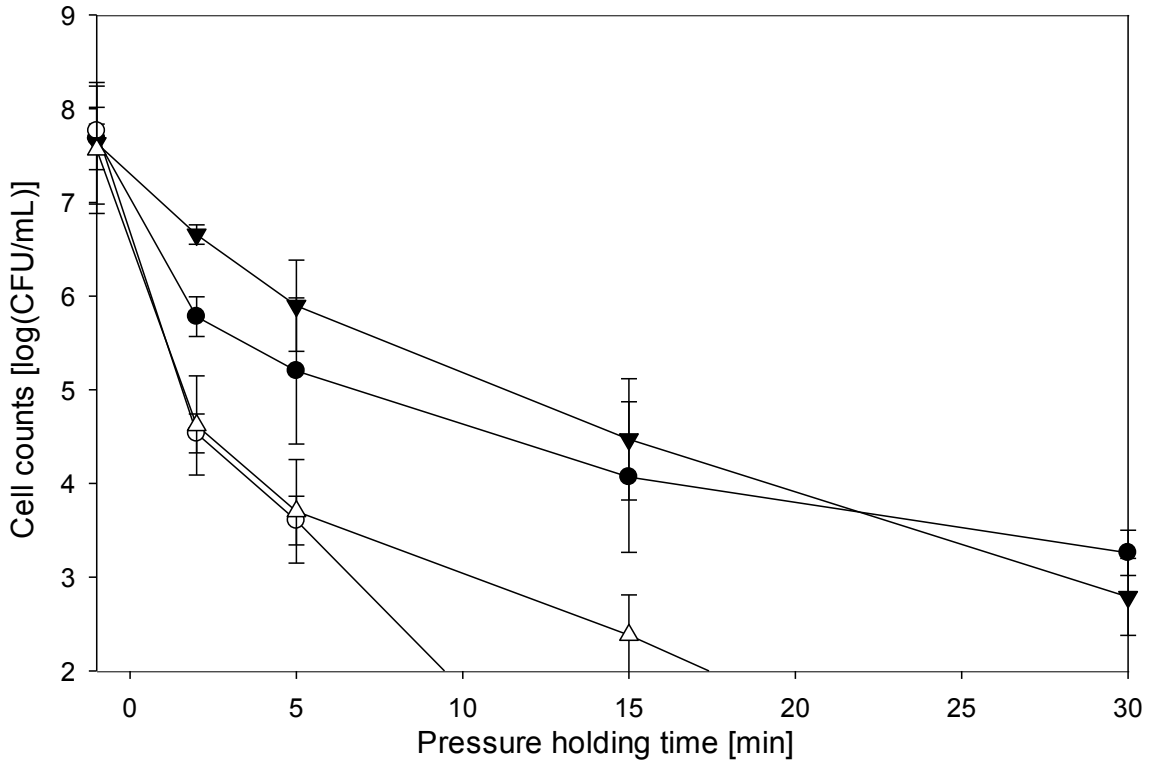

Figure 2-5. Cell counts of non-VTEC (circles) and VTEC cocktail (triangles) in ground beef after treatment at 600 MPa at 20 °C. Cells counts were enumerated on LB agar (●, ▼) and VRB agar (○, Δ). Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis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

2.4. Discussion

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

different process temperatures and NaCl levels to encompass a variety of different process parameters, and compared to heat resistance.

E. coli AW1.7 was described as an exceptionally heat resistant strain; its cell counts are reduced by only 2.0 and 4.0 log (cfu/g) when inoculated into ground beef patties cooked to a core temperature of 63 and 71°C, respectively (Dlusskaya et al., 2011, Liu et al., 2015). The current study demonstrated that the heat resistance of this strain is not exceptional, but was matched by 3 of the 11 tested strains of *E. coli*. The pressure resistance of *E. coli* AW1.7 was matched by 10 additional strains of *E. coli*. The direct comparison of the pressure resistance of mutant strains generated by multiple cycles of sublethal pressure treatment and sub-culturing of surviving cells (Hauben et al., 1997; Vanlint et al., 2011) to the pressure resistance of *E. coli* AW1.7 demonstrated that the pressure resistance of the wild type *E. coli* AW1.7 in poultry meat or beef matches or exceeds the resistance of pressure-resistant mutant strains (Liu et al., 2012; Liu et al., 2015). The heat- and pressure resistance of *E. coli* strains isolated from meat or a meat processing plant suggests that beef may be contaminated with *E. coli* strains that are resistant to heat and pressure. The screening of 100 strains of STEC revealed that about 30% of STEC are pressure resistant while heat resistant strains of STEC were less frequent (Liu et al., 2015). This study also observed a higher prevalence of pressure resistant strains among non-pathogenic *E. coli*. Pressure resistant mutant strains of *E. coli* have a marginal cross-resistance to heat (Hauben et al., 1996; Vanlint et al., 2011) and *E. coli* AW1.7 is both heat- and pressure resistant. The σ^H mediated heat shock response and the σ^S mediated general stress response contribute to both pressure and heat resistance (Aertsen et al., 2004; Robey et al., 2001). Exposure to pressure selects for increased σ^S activity and also increases thermotolerance in *E. coli* O157:H7 (Vanlint et al., 2013). However, sequential exposure to sublethal pressure, followed by cultivation of

surviving cells readily generates pressure resistant mutants of *E. coli* while the same strategy failed to produce heat-resistant derivatives (Vanlint et al., 2012). Taken together, pressure resistant strains of *E. coli* occur relatively frequently and mechanisms of resistance are likely multi-factorial while resistance to heat (60 °C) is a less frequent trait.

The heat resistance of *E. coli* AW1.7 is linked to ribosome stability and accumulation of compatible solutes (Pleitner et al., 2012; Ruan et al., 2011). Accumulation of disaccharides in response to a high external osmolarity also protects vegetative bacteria against pressure-mediated cell death (Lange and Hengge-Aronis, 1994; Molina-Höppner et al., 2004; Van Opstal et al., 2003). *E. coli* AW1.7 accumulates higher levels of amino acids and trehalose in response to 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 or 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 strains will facilitate future studies to increase the bactericidal effect of pressure by combination with additional antimicrobial hurdles.

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*3. Effect of the food matrix and food constituents on pressure resistance of *Escherichia coli*

Astericks (*) indicates contribution by Hui Li

3.1 Introduction

High hydrostatic pressure is an alternative to thermal preservation; the technology experiences worldwide commercial growth (Balasubramaniam et al., 2015; Georget et al., 2015). Pressure in the range of 400 – 600 MPa eliminates most spoilage organisms and pressure-sensitive pathogens (Patterson et al.,. 1995, Balasubramaniam et al., 2015; Georget et al., 2015); however, some foodborne pathogens including strains of *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). STEC, also referred to as verotoxigenic *E. coli* causes severe foodborne disease and even death (Frenzen et al., 2005; Karch et al., 2005). STEC are primarily associated with ruminants but plant foods including fruit juice and produce are also recognized as major vectors for foodborne outbreaks of STEC (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 highly variable (Hauben et al., 1997; Liu et al., 2015). The lethality of 600 MPa towards 100 strains of STEC differed by more than 5.0 log(cfu/mL) for different strains; the cell counts of approximately 30% of strains of STEC were reduced by less than 2.0 log(cfu/mL) after treatment at 600 MPa for 3 min (Liu et al., 2015). Moreover, the food matrix, the process temperature, and pH strongly influence the pressure resistance of *E. coli* (Gänzle and Lui, 2015). For example, strains of *E. coli* O104:H4 that were isolated in the 2011 outbreak of STEC in Germany were reduced by 3.0 log(cfu/mL) after

treatment at 400 MPa and 40°C in carrot juice with a pH of 5.1 (Reineke et al., 2015); however, a derivative of the same strain was reduced by 1.0 log (cfu/mL) only after treatment at 600 MPa at 20 °C for 3 min in LB broth (Liu et al., 2015). The pressure resistance of several strains of *E. coli,* e.g. *E. coli* AW1.7, LMM1010 or ATCC 25922, was assessed in different food products; however, the comparison of literature data is confounded by the choice 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).

If pressure processing alone does sufficiently inactivate target pathogens, the use of additional antimicrobial hurdles is necessary. The targeted design of improved pressure processes is greatly facilitated by improved understanding of the mechanisms of pressure-mediated cell death and the role of matrix constituents that promote sensitivity or resistance. Multiple pressure-sensitive targets have been described in *E. coli*. Pressure permeabilises the outer membrane in *E. coli* and related Gram-negatives, resulting in synergistic activity with outer membrane impermeant inhibitors (Gänzle and Vogel, 2001; Ritz et al., 2000). Pressure also induces a phase transition in the cytoplasmic membrane (Casadei et al., 2002), resulting in inhibition of membrane bound enzymes, the dissipation of the proton motive force (Wouters et al., 1998; Winter, 2002; Kilimann et al., 2005), and the elimination of acid resistance (Garcia-Graells et al., 1998). Ribosomes, protein folding, and the disposal of misfolded proteins were also identified as pressure-sensitive targets in *E. coli* (Niven et al, 1999; Aertsen et al., 2004; Govers et al., 2014). Moreover, pressure induces endogenous oxidative stress in *E. coli* which enhances pressure-mediated inactivation (Aertsen et al., 2005). In keeping with pressure-induced oxidative stress as "suicide mechanism" in *E.* coli, thiol reactive antimicrobials

exhibited synergistic bactericidal activity with pressure while the effect of other antimicrobials was additive or antagonistic (Feyaerts et al., 2015).

The successful application of hurdle technology in food was demonstrated for combinations of pressure with high temperature (40 – 60 °C) (Liu et al., 2012, Reineke et al., 2015). However, even moderately elevated temperatures in the range of 40 – 60 °C may substantially alter sensory properties of food when combined with high pressure (Omama et al., 2011). The combination of pressure with low pH also accelerates the elimination of *E. coli* after pressure treatment (Alpas et al., 2000; Garcia-Graells et al., 1998) but not all food products can be acidified. The synergistic activity of other antimicrobial compounds, including thiol-reactive antimicrobials and bacteriocins, was demonstrated in model systems but limited data document their effect in food. It was therefore the aim of this study to compare the pressure resistance of *E. coli* in different food products to directly assess the matrix effect on pressure resistance. Moreover, model studies were carried out to evaluate the synergistic or antagonistic effect of different food constituents and antimicrobial compounds with pressure. Experiments were performed with a cocktail of 5 STEC and EPEC strains, a cocktail of non-pathogenic surrogate strains (Garcia-Hernandez et al., 2015; Chapter 2 of this thesis), and the heat- and pressure resistant model organism *E. coli* AW1.7 (Dlusskaya et al., 2011; Liu et al., 2012).

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

This study employed two cocktails each containing five strains of *E. coli* (Garcia-Hernandez et al., 2015; Chapter 2 of this thesis). One strain cocktail was composed of four strains of STEC (05-6544, 03-2832, 03-6430 and C0283) and the enteropathogenic *E. coli*

PARC 449; 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 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.

3.2.2 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, Alberta, Canada. The formulation of the products is shown in Table 3-1. 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 noninoculated products were quantified by surface plating onto LB agar; all cell counts were less than 2.6 log(cfu/g). Strain cocktails or the pressure resistant strain *E. coli* AW1.7 (1.5 ml) were inoculated into the food products (10 ml or g) to an initial population of around 10^7-10^8 cfu/ml. Therefore, interference of of the native microorganisms was not a concern. The inoculated food products were homogenized for 2 min. Subsamples of 250 µL or µg were packed into 3-cm R3603 tygon tubes (Akron, PA, USA) and heat-sealed after exclusion of air. Prior to pressure treatment, tubes were placed into a 2-ml Cryovial (Wheaton, Millville, NJ) filled with 10% bleach.

***Table 3-1.** Product composition of bruschetta and tzatziki

Bruschetta	%	Tzatziki	%
Tomato	94.821	Cucumber	24.093
Balsamic Vinegar (6% acidic acid)	1.546	Sour Cream (14%)	34.36
Olive Oil	1.288	Plain Yoghurt	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

*3.2.3 Pressure treatments of food samples

Pressure treatments were carried out as described previously (Liu et al., 2012). Samples were treated in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) at 600 MPa and 20 °C for 3 min. After the pressure treatment, the cell counts were determined by serial 10-fold dilution and surface plating on LB agar. Lactic acid bacteria in untreated or pressure treated yoghurt were enumerated by surface plating on modified de Man Rogosa Sharpe medium (Reuter, 1985). Samples were stored at 4 °C for 16 days and cell counts were determined during storage. Cell counts of uninoculated and untreated as well as uninoculated and pressure-treated samples were used as controls. During enumeration of the colonies, the colony morphology was noted to determine whether it matched the colony morphology of the *E. coli* inoculum. All experiments were performed in triplicate.

3.2.4. Effect of food constituents on pressure resistance of *E. coli

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

***3.2.5 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 4.0 or 5.5. Ethanol and 2-phenylethanol (Sigma-Aldrich) 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.

3.2.6 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, followed by refrigerated storage at 4 °C over 12 days. Cell counts were obtained as described in 3.2.5 section. Experiments were performed in triplicate.

3.2.7 Effects of calcium on permeability of cell membrane

Outer membrane permeability was determined with the probe 1-*N*-phenylnaphtylamine (NPN) (Helander and Matila-Sandholm, 2000). In brief, a solution of 10 mmol/L NPN in ethanol was diluted to 20 μmol/L in imidazole (IM) buffer. *E. coli* AW1.7 cultures suspended in IM buffer (pH 5.5) supplemented with 10 mmol/L calcium, or not, were treated with 100, 300, or 500 MPa for 3 min at 20 °C. Aliquots of 100 μL of pressure treated samples were mixed with 100 μL of the NPN solution and the fluorescence intensity was measured using a fluorescence spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada) at an excitation and emission wavelength of 340 and 420 nm, respectively. Each assay was performed in triplicate. Results were 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, and reported as NPN uptake factor.

3.2.8 Statistical analysis

Significant differences among cell counts were determined by two way analysis of variance in SAS. Student Newman Keuls multiple range test was used to determine differences among means. Significance was accessed at an error probability of 5% ($p<0.05$).

3.3 Results

*3.3.1 The effects of food matrix on pressure resistance

We were initially interested in the survival of two pressure resistant strain cocktails of *E. coli* in bruschetta, a tomato-based sauce, and tzatziki, a sauce containing yoghurt, cucumbers, and garlic. The products were inoculated with both cocktails and treated at 600 MPa and 20 °C, conditions matching current industrial practice for pressure treatment of food. Cells counts of both of *E. coli* cocktails in bruschetta and tzatziki after pressure treatment were reduced by more than 5.0 log(cfu/ml) (Figure 3-1). Similar cell counts were observed in products inoculated with cocktail composed of pathogenic strains and the cocktail composed of surrogate strains. Cell counts in both products were maintained at less than 2.5 log(cfu/ml) over 16 days of refrigerated storage. Cell counts after pressure treatment were not different from the uninoculated control. Moreover, surviving cells cultured after pressure treatment and during storage exhibited a colony morphology which was very distinct from the colony morphology of *E. coli* strains, demonstrating that the background originates from product microbiota rather than surviving *E. coli*.

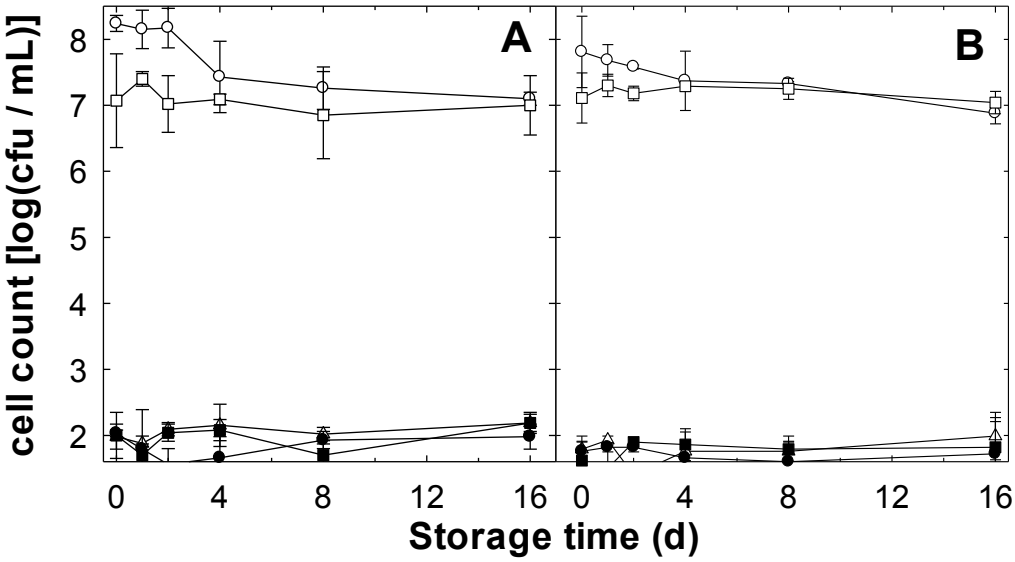

***Figure 3-1.** Cell counts of bruschetta **(Panel A)** and tzatziki **(Panel B)** during storage at 4 °C. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was used as control (△). 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.

The sensitivity to pressure of the two strain cocktails in bruschetta and tzatziki was strikingly different from the resistance of the same cocktails that was previously observed in beef (Garcia-Hernandez et al., 2015; Chapter 2 of this thesis). To determine whether the low pH accounted for this difference, the pH of bruschetta and tzatziki was adjusted to 5.5, equivalent to the pH of ground beef. Samples were inoculated with the two strain cocktails, and subjected to the same pressure treatment, followed by refrigerated storage (Figure 3-2). Treatment in ground beef was performed for comparison (Figure 3-2C). Increasing the pH increased pressure resistance of *E. coli* slightly (bruschetta, Fig. 3-2A) or substantially (tzatziki, Figure 3-2B). The lethality of pressure treatment in tzatziki was similar to that of pressure treatment in ground beef; however, cell counts of *E. coli* in tzatziki were reduced to levels below the detection limit

after 4 d of refrigerated storage while cell counts of *E. coli* in ground beef were reduced by less than 1.0 log(cfu/g) in the same period. These results demonstrate that the pH is not the only factor governing survival of *E. coli* during pressure treatment, and that the food matrix differentially affects survival during pressure treatment and survival during post-pressure refrigerated storage.

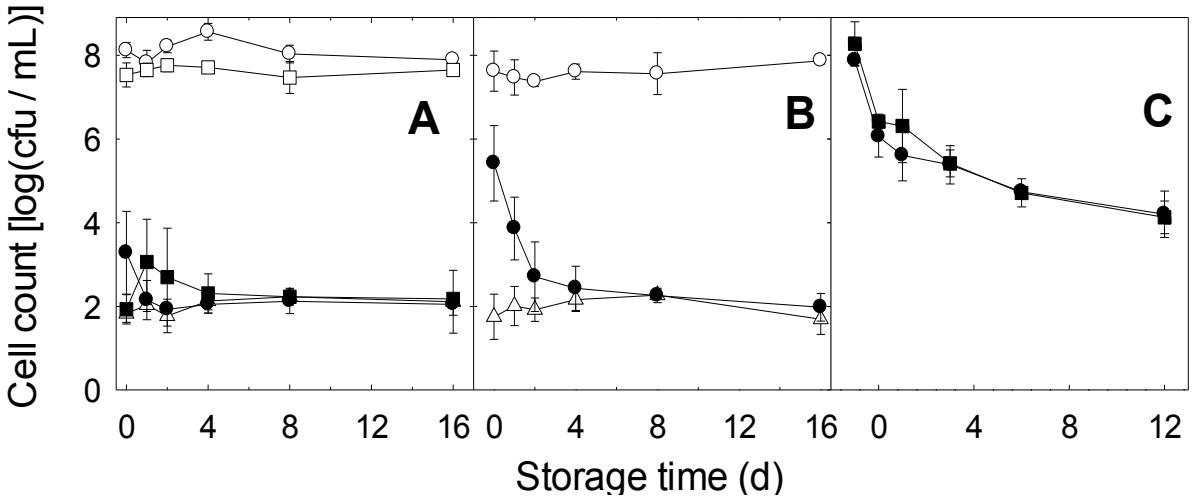

***Figure 3-2.** Cell counts of bruschetta **(Panel A)**, tzatziki **(Panel B)** and ground beef **(Panel C)** during storage at 4°C. The pH of the products was adjusted to 5.5 prior to inoculation and treatment. The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains 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.

To further confirm the role of pH on survival of *E. coli*, treatments were performed with bruschetta and tzatziki at a pH of 7.5 (Figure 3-3), and with plain yoghurt after adjustment to pH 4.0 (unadjusted), 5.5, and 7.5. The two strain cocktails composed of pathogenic and surrogate

strains exhibited similar survival during and after pressure treatment in previous experiments, therefore, subsequent experiments were carried out only with the surrogate cocktail. Adjusting the pH of bruschetta and tzatziki to 7.5 did not substantially alter the lethality of pressure treatment (Fig. 3-2 and 3-3) but virtually eliminated the reduction of cell counts during refrigerated storage (Figure 3-3).

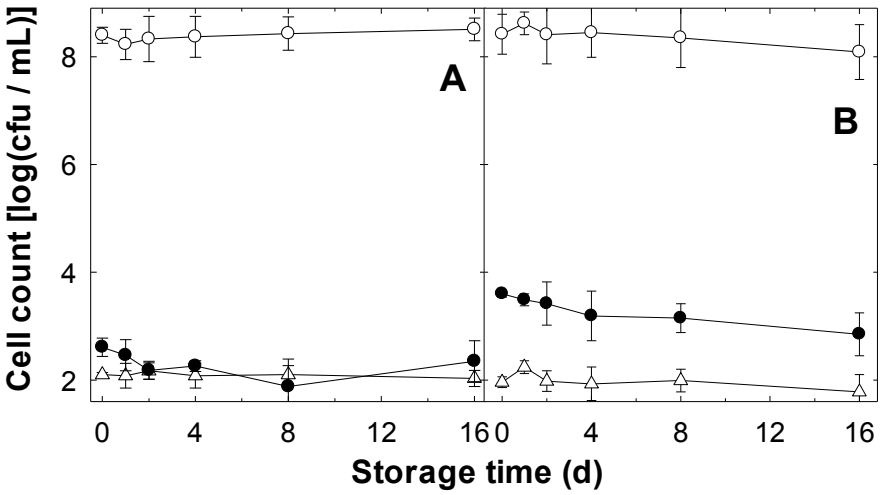

***Figure 3-3.** Cell counts of bruschetta (**Panel A)** and tzatziki (**Panel B)** after pressure treatment and during storage at 4°C. The pH of the products was adjusted to 7.5 prior to inoculation and treatment. Products were inoculated with a surrogate cocktail consisting of 5 strains of *E. coli* (●). Uninoculated product was used as a control (Δ). Prior to storage, products were treated for 3 min at 600 MPa and 20 °C (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.

Pressure treatments in yoghurt further confirmed the effect pH on the lethality of pressure and post-pressure refrigerated storage (Figure 3-4). At pH 4.0, pressure treatment reduced cell counts of *E. coli* by more than 5.0 log(cfu/mL). At pH 5.5, the resistance of *E. coli* to pressure was substantially increased but cell counts were reduced by more than 2.0 log(cfu/mL) after 4 d of refrigerated storage. Treatments in yoghurt at pH 7.5 did not change the

lethality of pressure when compared to treatments at pH 5.5, however, cell counts remained unchanged over 4 days of refrigerated storage (Fig 3-4). Of note, cell counts of lactic acid bacteria were below the detection limit after pressure treatment at any pH (data not shown), indicating that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* are substantially more pressure sensitive than *E. coli*.

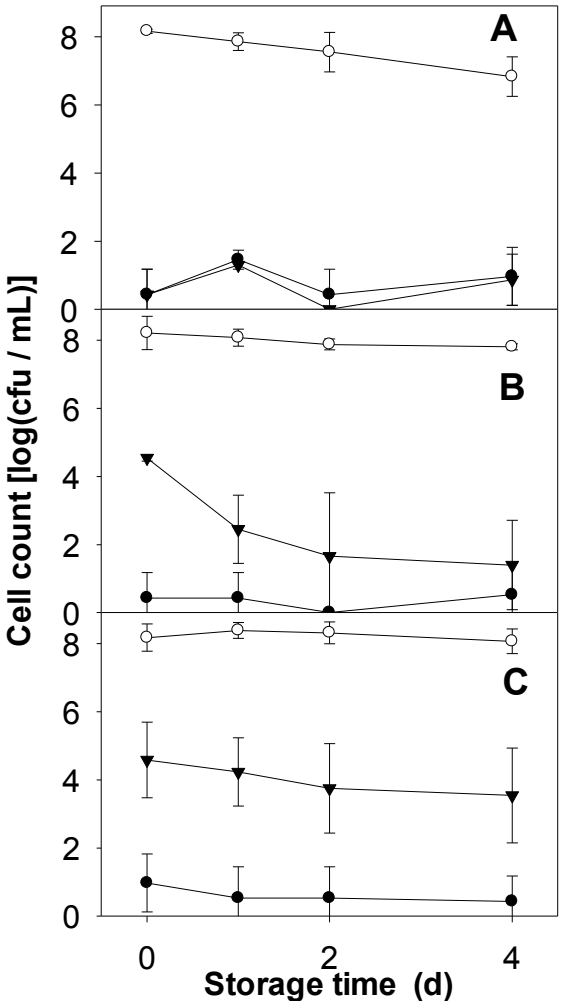

***Figure 3-4.** Cell counts of yoghurt during storage at 4°C. The initial pH of yoghurt was 4.0 **(Panel A)**; the pH was also adjusted to 5.5 **(Panel B)** or 7.5 **(Panel C)** prior to inoculation and treatment. Products were inoculated with *E. coli* AW 1.7. Uninoculated product was used as control (●). 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).

*3.3.2 Effect of food constituents on pressure resistance of *E. coli*

The above data demonstrate that food constituents other than the pH affect survival of *E. coli* after pressure treatment and refrigerated storage. To identify food constituents that account for these effects, we designed model experiments with or without addition of individual compounds. Calcium, magnesium, and glutamate were chosen because they have protective effects (Hauben et al., 1998; Niven et al., 1999; Kilimann et al., 2005) and occur in meat or dairy products; acetic and caffeic acids were selected as antimicrobial organic acids with a potential synergistic effect (Sanchez-Maldonado et al., 2011). Addition of magnesium, or glutamate protected *E. coli* AW 1.7 against pressure-induced inactivation; this effect was modest (~ 2 log(cfu/mL)) but significant ($p<0.05$) after 16 min of treatment in presence of glutamate but quite substantial (~ 4 log(cfu/mL), $p < 0.05$) for magnesium (Figure 3-5). Surprisingly, caffeic acid and acetic acid also protected *E. coli* after 16 min of treatment when compared to the control without additives ($p<0.05$) (Figure 3-5).

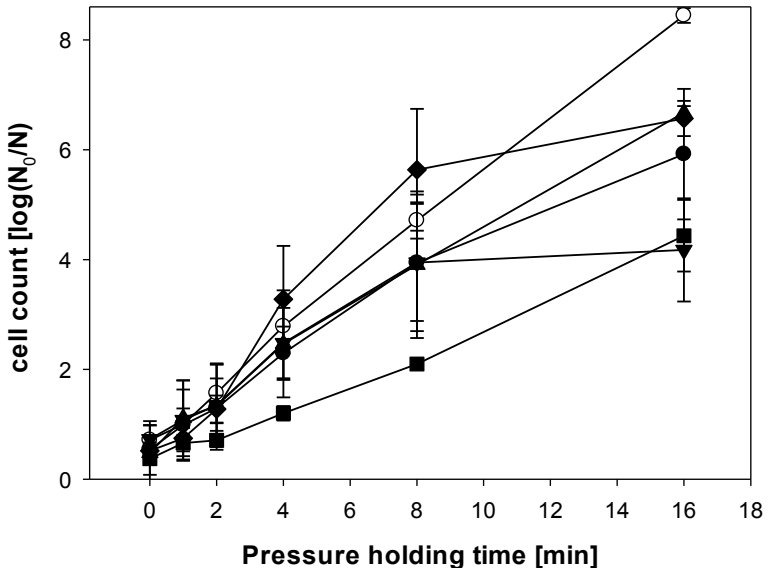

***Figure 3-5.** Cell counts of *E. coli* AW1.7 after pressure treatment in MES buffer (pH 5.5) with or without additions of food constituents. The following compounds were added: 10 mmol/L calcium (●), 10 mmol/L magnesium (▼), 10 mmol/L glutamate (▲), 1 g/L acetic acid (■) or 1 g/L caffeic acid (♦). Samples were treated with at 600 MPa and 20 °C. Inoculated buffer without added any compound was used as control (○). 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

The biophysical properties of the membrane play a decisive role in the pressure resistance of *E. coli* (Casadei et al., 2002; Charoenwong et al., 2011), therefore, further experimentation manipulated membrane properties of *E. coli* by addition of ethanol or phenylethanol. Ethanol and phenylethanol strongly enhanced the lethal effect of pressure on *E. coli* AW1.7 although the concentrations used, 2% and 20 mmol/L, are not lethal or inhibitory to *E. coli*. The effect was observed at pH 4.0 as well as pH 5.5 (Figure 3-6A and 3-6B) and was of approximately equal magnitude for phenylethanol or ethanol.

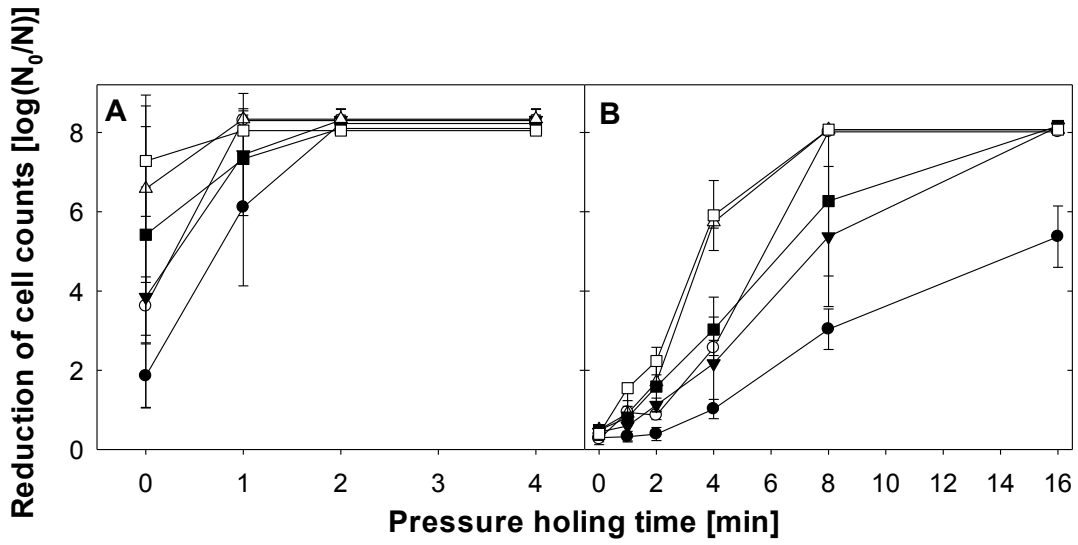

***Figure 3-6.** Cell counts of *E. coli* AW1.7 after treatment in buffer at a of pH 4.0 **(Panel A)** or pH 5.5 (**Panel B**). Ethanol (2%, ▲) or phenylethanol (20mmol/L, ■) were added to the buffer prior to inoculation and treatment at 600 MPa and 20 °C. Inoculated buffer without addition was used as control (●). Cell counts were determined by plating on LB agar (closed symbols) and VRB agar (open symbols). 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.

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

Prior experiments established that individual food constituents strongly influenced survival of *E. coli* during pressure treatment. Because individual food products differentially affected the resistance of *E. coli* during pressure treatment and post-pressure refrigerated storage, we additionally explored the role of selected food constituents on post-pressure survival. The selection of compounds focused on potentially protective compounds that occur in meat, i.e. calcium, magnesium, glutamine, glutamate, and glutathione. In keeping with prior data obtained with a different buffer system, none of these compounds affected survival of *E. coli* after 3 min at 600 MPa (compare Fig. 3-5 and 3-7). However, cell counts of *E. coli* in buffer at

pH 5.5 were reduced by more than 5.0 log(cfu/mL) over 12 days of post-pressure refrigerated storage (Fig. 3-7A and 3-7B). This reduction of cell counts was strongly reduced by addition of calcium or magnesium (Fig. 3-7A); in contrast, glutamine, glutamate, or glutathione had no effect on the survival of *E. coli* after pressure treatment (Fig. 3-7B).

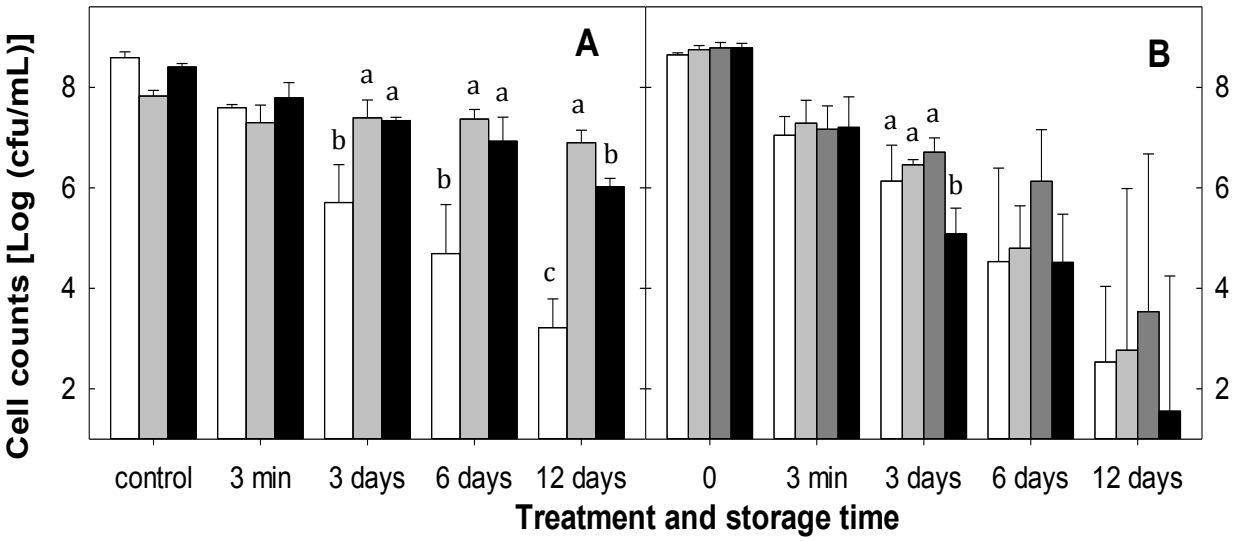

Figure 3-7. Cell counts of *E. coli* AW1.7 after treatment at 600 MPa for 3 min at 20 °C in imidazole buffer (pH 5.5). Treatments were performed in buffer without additives (white bars) or with addition of 10 mM calcium (grey bars), magnesium (black bars) (**Panel A**), or with addition of glutamine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

3.3.4 Effects of calcium on the integrity of the outer membrane

Divalent cations interact with multiple cellular components, including ribosomes, the cytoplasmic membrane, and the outer membrane. The outer membrane is a pressure sensitive target in *E. coli* that is perturbed by less than 300 MPa (Gänzle and Vogel, 2001). To determine

whether the protective effect of calcium related to stabilization of the outer membrane, we used NPN to probe the integrity of the outer membrane of *E. coli* AW1.7 that was pressure treated in presence or absence of 10 mmol/L calcium (Table 3-2). Pressure fully permeabilised outer membrane of *E. coli* after treatment with 300 MPa or higher (Table 3-2). The addition of calcium did not influence the permeability of the outer membrane of pressure treated cells.

Table 3-2. 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.

3.4 Discussion

The resistance of *E. coli* to pressure is strain-, pH-, and matrix-dependent (Garcia-Graells et al., 1998; Alpas et al., 2000; Liu et al., 2015; Reineke, et al., 2015). Based on the substantial variation of pressure resistance within the species *E. coli*, the use of strain cocktails is recommended for validation of pressure processes aiming to establish food safety (Garcia-Hernandez et al., 2015; Chapter 2, this thesis). This study demonstrated that treatment with 600

MPa for 3 min in bruschetta or tzatziki reduce cell counts of two strain cocktails by more than 5.0 log(cfu/mL). The pathogenic and surrogate strain cocktails exhibited a comparable resistance to pressure in bruschetta and dairy products; in keeping with prior results that were obtained in ground beef (Garcia-Hernandez et al., 2015; Chapter 2, this thesis). The strain cocktail composed of surrogate non-pathogenic strain is thus useful for validation of pressure processes in a wider range of products. However, we also demonstrated that the lethality of the same pressure treatment on the same strains can differ by up to 4.0 log(cfu/mL) when applied to different foods or at different pH values.

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

Glutathione contributes to redox homeostasis in *E. coli* (Carmel-Harel and Storz, 2000), and may thus counteract the pressure mediated "oxidative suicide" of *E. coli* (Aertsen et al., 2005, Malone et al., 2006) or the effect of thiol-reactive antimicrobials (Feyaerts et al., 2015). Meat but not dairy products or tomatoes are rich in low-molecular weight thiol compounds.

However, glutathione did not change the pressure resistance or the post-pressure survival of *E. coli*. Caffeic and acetic acids exhibited a modest protective effect on pressure resistance of *E. coli*. Organic acid affect the survival of *E. coli* by changing the pressure-induced pH shift but cosmotropic and specific ion effects additionally play a role, making the effect of ions difficult to interpret (Gayán et al, 2013, Molina-Gutierrez et al., 2002). The protective effect of caffeic acid is nevertheless remarkable because caffeic acid was used at 1 g/L, a level well above the MIC against *E. coli* AW1.7 (0.2 g/L, Sánchez-Maldonado et al., 2011). Acidification of the cytoplasm by undissociated and membrane permeant caffeic acid is thought to contribute to its antimicrobial activity (Choi and Gu, 2001; Cueva et al., 2010; Sánchez-Maldonado et al., 2011); such activity would support the pressure-mediated acidification of the cytoplasm. However, caffeic acid also influences the fluidity of the cytoplasmic membrane (Keweloh et al., 1991) and this interaction may account for its protective effect during pressure treatment. The divergent effect of the antimicrobial compounds nisin and reutericyclin on pressure-assisted inactivation of *Bacillus* and *Clostridium* endospores has been related to their divergent effects on spore membrane fluidity (Hofstetter et al., 2013).

Glutamate decarboxylation is the most effective system for pH homeostasis of acid challenged *E. coli*. Depending on the extracellular pH, glutamate mediated acid resistance consumes an intracellular proton, exports negative charges and thus contributes to generation of the pmf, and / or neutralizes the extracellular pH (Foster 2004; Feehily and Karatzas, 2012; Teixeira et al., 2014). Glutamate decarboxylation was more pressure resistant than glucose-mediated acid resistance and thus improved survival during post-pressure acid challenge (Kilimann et al., 2005). In food, glutamate dependent acid resistance is complemented by glutamine deamination, which provides the substrate for glutamate decarboxylase but also

consumes an intracellular proton and thus directly contributes to acid resistance in *E. coli* (Lu et al., 2013). Surprisingly, glutamate addition did not have a significant effect on the post-pressure survival of *E. coli*. Refrigerated storage of *E. coli* may have reduced the rate of glutamate decarboxylation to; prior studies incubated *E. coli* at a temperature permitting growth and metabolism (Kilimann et al., 2005).

The conversion of unsaturated membrane fatty acids to cyclopropane fatty acids in *E. coli* decreases the fluidity of the membrane and increases its pressure resistance (Casadei et al., 2002; Charoenwong et al., 2011). Ethanol and phenylethanol fluidize the membrane and thus directly antagonize pressure effects on bacterial membranes (Welch and Bartlett, 1998; Huffer et al, 2011). Ulmer et al. (2002) argued that membrane-bound proteins are more sensitive to pressure-mediated denaturation when embedded in a liquid crystalline membrane. The strong synergistic effect of even modestly elevated temperatures on the pressure-inactivation of *E. coli* (Liu et al., 2012, Reineke et al., 2015) and our data on the synergistic activity of ethanol or phenylethanol and pressure are consistent with this hypothesis.

Divalent cations such as calcium and magnesium protect *E. coli* against pressure inactivation (Hauben et al., 1998; Gayán et al., 2013). For example, Ca^{2+} in concentrations ranging from 0.5 to 80 mmol/L increased the pressure resistance of *E. coli* at 300 MPa, and this effect increased proportional to the calcium concentration (Hauben et al., 1998). The protective effect of divalent cations may be explained by stabilization of the outer membrane (Gänzle and Vogel, 2001), by stabilization of the ribosome (Niven et al., 1999), or by stabilization of the cytoplasmic membrane or other pressure sensitive targets (Hauben et al., 1998). Our data confirm with Hauben et al., (1998) who concluded that the protective effect of Ca^{2+} is not related to the stabilization of the outer membrane. We extend prior data by demonstrating that

the effect of Ca^{2+} and Mg^{2+} on the post-pressure survival is more pronounced than the effect on survival during pressure treatment (Fig. 3-5 and 3-7). The protective effect of Ca^{2+} and Mg^{2+} may thus partially explain the relative resistance of *E. coli* in meat (rich in magnesium), dairy products (rich in calcium and magnesium) and bruschetta (low levels of divalent cations).

In conclusion, this study demonstrated that the food matrix strongly influences the pressure-mediated inactivation of STEC and EPEC. The product pH influenced both the survival of *E. coli* during pressure treatment and the survival during refrigerated storage after pressure treatment. However, differences in the product pH failed to account for the product-specific effect on pressure resistance of *E. coli*. Remarkably, divalent cations exhibited a protective effect on *E. coli* during post-pressure refrigerated storage. In combination with pH effect, the presence of divalent cations in dairy and meat products accounts for the higher resistance of *E. coli*. Membrane-active antimicrobial compounds that increase the membrane fluidity exhibit synergistic activity with pressure-mediated elimination of *E. coli* in food.

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4. Acquisition of pressure resistance by strains of *Escherichia coli*: cross resistance to other stressors and genomic comparison to parent strains

4.1 Introduction

High hydrostatic pressure (HHP) is used commercially for food preservation (Balasubramaniam et al., 2015). Pressure in the range of 200 to 600 MPa inactivates some pathogens and spoilage bacteria (Rendueles et al., 2011; Lou et al., 2015). However, some strains of *E. coli* including verotoxigenic *E. coli* (VTEC) are highly resistant to pressure (Liu et al., 2015). The pressure resistance and the mechanism of inactivation by pressure are not fully elucidated.

Microorganisms growing at elevated pressures exist in the deep-sea and deep-subsurface sediments (Meersman et al., 2013). The effect of high pressure on *E. coli* has been widely studied. Marietou et al. (2014) reported that after 500 hundred generations of selection *E. coli* K12 acquired the ability to grow at pressure non-permissive for the parental strain (60 MPa). Furthermore, extremely pressure-resistant mutants of *E. coli* were obtained during consecutive cycles of increasingly severe pressure shocks with intermittent resuscitation and outgrowth of the surviving population (Vanlint et al., 2011; Hauben et al., 1997).

The acquisition of new genes through horizontal gene transfer (HGT) provides bacteria with a variety of new traits (Croxen et al., 2013). Phylogenetic comparison of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) pathovars suggests that they have a common genetic background except for virulence factors such as shiga toxin (Stx) acquired by EHEC strains through horizontal gene transfer, which increases virulence and severity of the disease (Beutin et al., 2005; Turner et al., 2006). The acquisition of the locus of heat resistance (LHR) increases heat resistance of heat sensitive strains of *E. coli* strains (Mercer et al., 2015).

However, gene loss can also increase the fitness or adaptation of a pathogen in a particular niche that allows the survival of the pathogen in adverse environments (Croxen et al., 2013). Loss of lysine decarboxylase (*cad*) activity has been shown in EPEC, STEC, and enteroaggregative *E. coli* (EAEC) and may enhance virulence in STEC and EAEC (Jores et al., 2006; Hwang et al., 2010; Vazquez Juarez et al., 2008). After a random transposon knock-out library of *E. coli,* 3 transposon mutants (*rssB, crp* and *cyaA*) strongly increased pressure resistance, demonstrating that the loss of the negative regulator (transcription) of *rpoS*, cAMP/CRP, significantly increased resistance to pressure in *E. coli*. However, pressure-resistant mutants of *E. coli* ATCC 43888 isolated previously did not have any mutations in *crp* or *cyaA*, indicating that other loci also increase pressure resistance in *E. coli* (Vanlint et al., 2013a). The acquisition of extreme pressure resistance was observed to coincide with increased expression of heat shock proteins in three previously evolved mutants of *E. coli* MG1655 (Aertsen et al., 2004). High pressure stress can rapidly select for strongly increased RpoS activity in a hypersensitive *E. coli* O157:H7, leading to a simultaneous increase in pressure resistance (Vanlint et al., 2013b).

Knowledge on mechanisms of pressure resistance in *E. coli* can be used to increase the lethality of high pressure processing. Understanding the development of high levels of barotolerance is important for the practical application of pressure technology in food and determination of the mechanism will facilitate the adoption and/or design of novel intervention methods to enhance food safety. Pressure resistant strains that were derived from sensitive parent strains may provide novel insights by studying of genetically closely related organisms. In this study 4 extremely pressure resistance strains of *E. coli* were generated by successive rounds of pressure treatment and regrowth of the surviving cells. The genome sequence of 3 wild type strains of *E. coli* and the respective pressure resistant strains was compared. In

addition, the resistance of the pressure-resistant derivatives to other stresses (heat, low pH and osmotic stress) was determined.

4.2 Material and Methods

4.2.1 Bacterial strains and growth conditions

The following strains were used as parental strains: *E. coli* AW1.7 and *E. coli* AW1.3, which are heat and pressure resistance strains (Dlusskaya et al., 2011; Liu et al., 2015); *E. coli* AW1.7ΔpHR1, a heat sensitive strain derivative of *E. coli* AW1.7 (Pleitner et al., 2012); and *E. coli* CO6CE1943 (O157:H7) (Liu et al., 2015). Stock cultures were stored at −80 °C, subcultured by streaking onto Luria–Bertani (LB) agar (Difco; BD, Sparks, MD, USA) followed by a second subculture in LB broth containing 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl unless otherwise noted. Cultures were incubated separately at 37 °C for 18–23 h with agitation (200 rpm) in 7 mL of LB broth in 15 mL conical tubes.

4.2.2 Determination of pressure resistance

Cells from stationary-phase 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, placed in the pressure vessel, and treated at 20 °C for 15 min in a U111 Multivessel Apparatus (Unipress Equipment, Warsaw, Poland). The vessel was compressed to a pressure of 300 to 800 MPa in 1-3 min and decompressed in less than 1 min. The temperature of the unit was maintained by a thermostat jacket coupled to an external water bath and changes during compression and decompression were less than 4 °C. Polyethylene glycol was used as pressure transferring fluid.

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% 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.

4.2.3 Selection of pressure resistant strains of *E. coli*

Extremely pressure resistant strains of *E. coli* were obtained as described previously (Hauben et al., 1997). In brief, stationary-phase cultures were pressure treated in LB broth. Treated cultures were diluted 1000 fold into 15 mL tubes with 7 mL of fresh LB broth and grown for 23 h at 37 °C and 200 rpm prior to the next round of pressurization. Initial treatments were performed at 300 MPa for 15 min and the pressure was increased by 25 MPa every round throughout the selection procedure until it reached 800 MPa after 21 cycles of pressure treatment and re-growth. Sample preparation and surface plating were performed as described previously. After the last selection cycle, a single colony from each culture was selected for further studies. To determine the stability of pressure-resistant derivatives, cultures from stationary-phase pressure resistance variants was inoculated with a 0.1% inoculum into fresh LB and regrown every 24 h for 5 consecutive days before pressure treatment at 600 MPa at 20 °C for 15 min.

4.2.4 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 (GeneAMP PCR System 9700, Applied Biosystems, Streetsville, Canada) at 60 °C for 5, 15, 30 and 60 min. Treated/untreated samples were surface plated and incubated as described previously. Experiments were performed in triplicate.

4.2.5 Determination of acid resistance

To determine acid resistance, overnight cultures were diluted to 8.0 log (cfu/mL) in 15 mL tubes with 7 mL LB broth adjusted to pH of 2.5 with HCL and incubated for 2 and 4 h at 37 °C and 200 rpm. Treated/untreated samples were surface plated and incubated as described previously. Experiments were performed in triplicate.

4.2.6 Determination of salt stress

To determine the effect of osmotic stress, overnight cultures were diluted to 8.0 log (cfu/mL) in 15 mL tubes with 7 mL LB broth supplemented with 5, 10, and 20 % NaCl (w/v) and incubated for 24 and 48 h at 37 °C and 200 rpm. Treated/untreated samples were surface plated and incubated as described previously. Experiments were performed in triplicate.

4.2.7 DNA isolation and genome sequencing, assembly, annotation and comparison

DNA was extracted from overnight cultures of *E. coli* grown in 5 ml of LB broth. Genomic DNA was isolated using the Wizard ® Genomic DNA Purification Kit (Promega, Madisson, Wisconsin, USA). The quality and quantity of each sample was assessed using a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). DNA samples were sequenced by Axeq Technologies (Seoul, South Korea) using Illumina HiSeq2000 with an insert size of 300 bp. The quality of the 100-bp paired-end reads was assessed using the FastQC tool (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and low quality reads were filtered by Quake (Kelley et al., 2010). Assemblies were obtained using ABySS 1.3.4. Genomes were annotated automatically by the RAST server. Mauve (http://gel.ahabs.wisc.edu/mauve) was applied to align, compare and identify rearrangement and deletions of pressure resistant

derivative strains. To confirm sequence identity, rearrangement, deletion, and open reading frames (ORFs) Geneious (Biomatters, Auckland, New Zealand) was used.

4.2.8 Confirmation of deletion of genomic elements

PCR was used for the confirmation of the deletion of genomic elements. Primers targeting missing elements were designed in Geneious. DNA was extracted from overnight cultures of *E. coli* grown in 5 ml of LB broth using the QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, Canada). The quality and quantity of each sample was assessed using a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Primer designed for the assays are listed in Table 4-1. Genomic DNA from the high pressure mutant strains was used as template in the PCR reaction; the pressure sensitive parental strains were used as a positive control.

Table 4-1. Primer sequences for genome elements deletion confirmation

Strain (Marker)	Primer	Primer sequence (5'-3')	Annealing Temp	Product (bp)
AW1.7 (A)	Forward	CCAGAAACTCTGCAGACGGT	57 °C	937
	Reverse	ACAGTCAGTCGGCATTTCGT		
AW1.7 (B)	Forward	TGAAAGGCTGAGCGTTTTGC	57 °C	1496
	Reverse	AGTGATAGCAGCCATCGAGC		
AW1.7 (C) p1	Forward	GCTCCGATTCGTTTAGTTCC	57 °C	1164
	Reverse	CGATCTGGCAAACATCGCTG		
AW1.7 (C) p2	Forward	GAAACTGAAAGCGCGTGGTT	57 °C	1311
	Reverse	GAAAAGCAGCTTTGACGCCA		
AW1.7 (C) p3	Forward	TAGCCACGACCAGTTTCACC	57 °C	519
	Reverse	GCGGCAATCTGCTTAAGCTC		
ΔpHR1 (A)	Forward	GGTGGTAGCCTCTTGTGCAT	57 °C	525
	Reverse	CTGAACTTCCTGACGGCCTT		
ΔpHR1 (B) p1	Forward	GTGGTGCAACAACGACAGTC	57 °C	2436
	Reverse	GCATCGATTACACGTGGTGC		
ΔpHR1 (B) p2	Forward	CCGCAACAGATAGCAATGCC	57 °C	3077
	Reverse	CGCCCACTATTTTACGCTGC		
ΔpHR1 (B) p3	Forward	GTTTCTCATGCCGATCGTGC	57 °C	552
	Reverse	CCGTTAGTGATCCGATGCGA		
ΔpHR1 (C) p1	Forward	GATGCGGTGGTGATTAACGC	57 °C	1709
	Reverse	CGAAAGGGTTAAGCATCGCG		
ΔpHR1 (C) p2	Forward	CGGAGGTATTGACGAGGCTC	57 °C	210
	Reverse	ACTACGGCCTCCTTCCTGAT		
ΔpHR1 (D)	Forward	ACCAGACGGTACTCAGACGA	57 °C	385
	Reverse	AGCTGCTCGACTCAAAACGA		
ΔpHR1 (E)	Forward	CATCGCATTGCTCGACACAG	57 °C	738
	Reverse	GATTGCGTATTGTGGCGTCC		
AW1.3 (A)	Forward	AAAAACGTTCTGGCGCTCG	57 °C	934
	Reverse	CAAAGAAACGCGGCACAGAA		

E coli AW1.7 (AW1.7), *E. coli* AW1.7ΔpHR1 (ΔpHR1) and *E. coli* AW1.3 (AW1.3) Conting identification/marker (A), (B), (C), and (D). Primers pair when more than one region was amplified on the same contig 1, 2 and 3 (p1, p2 and p3).

4.3 Results

4.3.1 Selection of pressure resistant strains of *E. coli*

Pressure resistant strains of *E. coli* were selected by repetitive rounds of pressure treatment with a subsequent regrowth of surviving cells from all 4 cultures of *E. coli* AW1.7,

AW1.7ΔpHR1, AW1.3 and CO6CE1943. Cell counts of the parental strains decreased to levels below the detection limit after treatment at 800 MPa (Fig 4-1). *E. coli* AW1.7ΔpHR1 is a heat sensitive derivative of *E. coli* AW1.7; its cell counts were reduced to levels below detection limit after treatment with 600 MPa at 20 °C for 15 min while cell counts of *E. coli* AW1.7 remained at 5.0 log (cfu/mL) when treated with the same conditions (Fig 4-1). The pressure resistance of all cultures increased after each cycle of selection. After 21 cycles, all four strains of *E. coli* survived treatment with 800 MPa (Fig 4-1). The pressure resistant strains that were obtained after 21 rounds of selection were designated *E. coli* AW1.7-PR, AW1.7ΔpHR1-PR, AW1.3-PR and CO6CE1943-PR. To ensure that pressure resistance is a stable trait, pressure-resistant derivatives were subcultured 5 days (400 generations) prior to treatment at 600 MPa and 20 °C for 15 min. Cell counts of the pressure-resistant derivative strains remained consistent at 8 log (cfu/mL) while cell counts of the parental strains were reduced to less than 5 log (cfu/mL) (Fig 4-1).

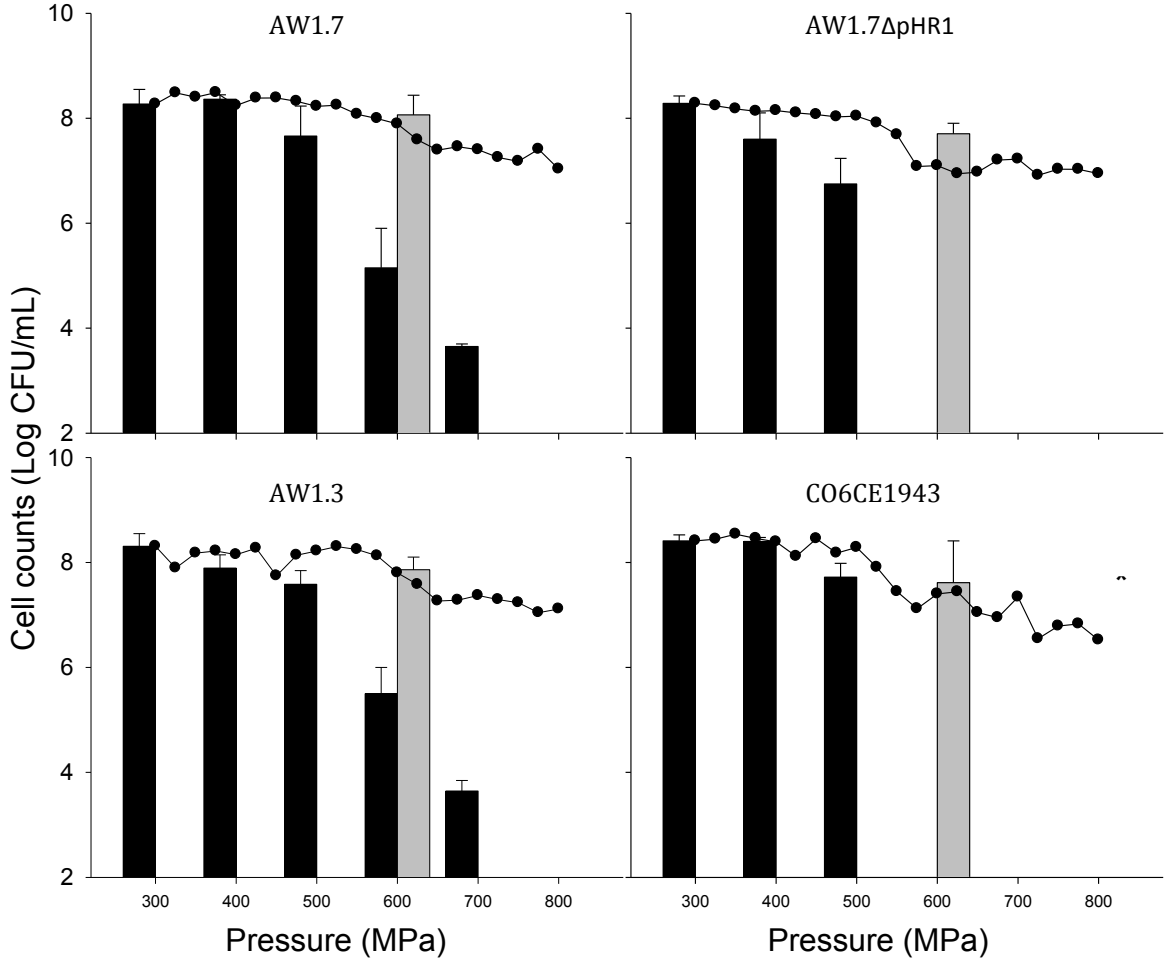

Figure 4-1. Generation of pressure resistant derivatives of four strain of *E. coli* and pressure resistance of parental and derivative strains. *E. coli* AW1.7, AW1.7ΔpHR1, AW1.3, and CO6CE1943 were treated at 300 MPa for 15 min. The treated cultures were subcultured with 1% inoculum. This sub-culture was treated with 325 MPa for 15 min and sub-cultured as before. This procedure was repeated 21 times with 25 mPA increments until a pressure of 800 MPa was reached. (●) indicates cell counts after the incremental pressure treatment of the culture; black bars present the cell counts of the parental strains after treatment for 15 min at 300 – 800 MPa; gray bars represent the cell counts of the derivative strains after treatment at 600 MPa.

4.3.2 Determination of heat resistance

For the following experiments only non-VTEC strains were used. Heat resistance of parental and derivative strains of *E. coli* was determined by heating at 60 °C for 5, 15, 30 and 60 min. Survivor curves are shown in Fig 4-2. Cell counts of *E. coli* AW1.7 and AW1.7-PR

decreased about 2.0 log (cfu/mL) after 30 min of treatment. Cell counts of *E. coli* AW1.7ΔpHR1 and AW1.7ΔpHR1-HP were below detection limit before 15 min. Interestingly AW1.3-HP increased heat resistance (Fig 4-2).

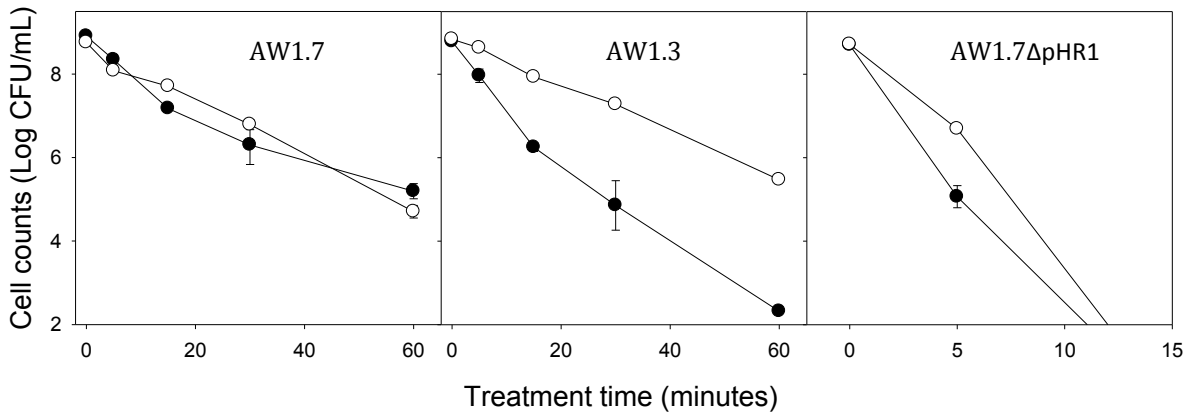

Figure 4-2. Viable cell counts of parental (●) and derivative strains (○) of *E. coli* after heat treatment at 60 °C. Cells were grown overnight in LB broth and treated for 0, 5, 15, 30 and 60 min. Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/ml).

4.3.3 Determination of acid resistance

Acid resistance of parental and derivative strains of *E. coli* was compared after incubation in LB broth acidified to pH 2.5. Survivor curves of *E. coli* are shown in Fig 4-3. Cell counts of *E. coli* AW1.3 and AW1.3-PR decreased about 1.0 log (cfu/mL) after 4 h of incubation (Fig 4-3). Likewise, the acid resistance of *E. coli* AW1.7ΔpHR1 and *E. coli* AW1.7ΔpHR1-PR was comparable. Remarkably, exposure to acid conditions reduced cell counts of *E. coli* AW1.7-PR to levels below the detection limit although cell counts of the parent strain *E. coli* AW1.7 remained higher than 7.0 log (cfu/mL) after 4 h of acid challenge (Fig 4-3).

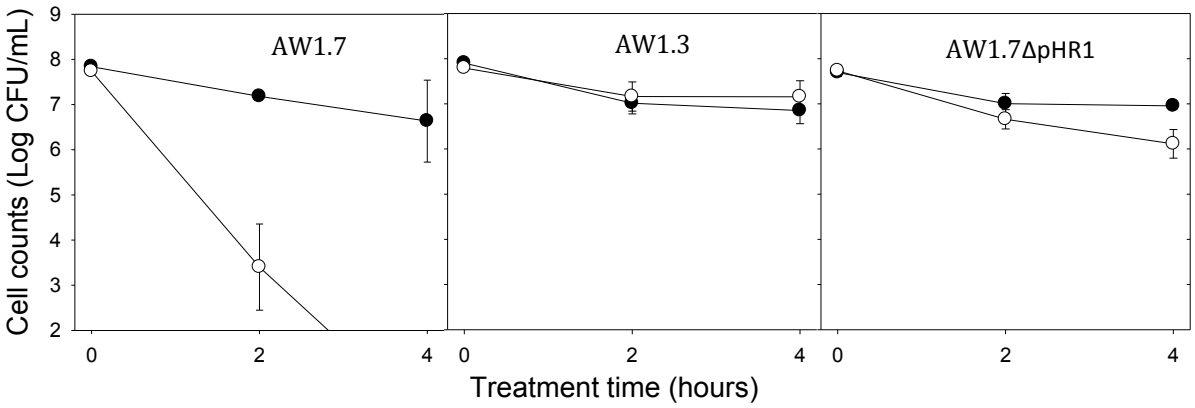

Figure 4-3. Viable cell counts of parental (●) and derivative strains (○) of *E. coli* after low pH treatment. Cells were grown overnight in LB broth (1% NaCl) and diluted to 8 log (cfu) on fresh LB broth (pH 2.5) and incubated for 0, 2 and 4 h. Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/ml).

4.3.4 Determination of osmotic stress (NaCl) resistance

Osmotic stress resistance was determined by challenge of parental and derivatives strain of *E. coli* with NaCl. Survivor curves of *E. coli* incubated with 20% NaCl are shown in Fig 4-4. Cell counts of all cultures decrease gradually over the treatment time (Fig 4-4). Cell counts were reduced about 3.0 log (cfu/mL) after 48 h of treatment. The resistance to osmotic stress was higher in *E. coli* AW1.3-PR when compared *E. coli* AW1.3.

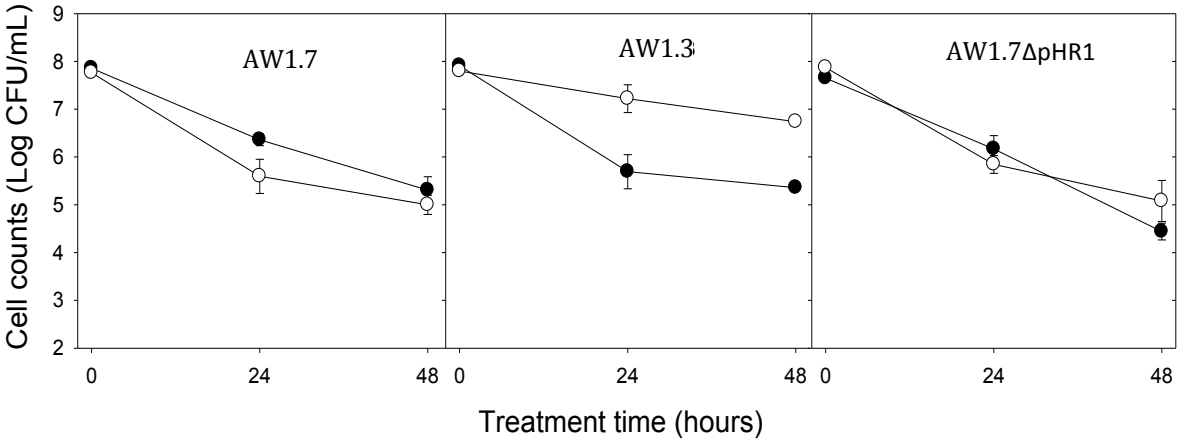

Figure 4-4. Viable cell counts of parental (●) and derivative strains (○) of *E. coli* after osmotic treatment. Cells were grown overnight in LB broth and diluted to 8 log (cfu/mL)) on fresh LB broth (20% NaCl) and incubated for 0, 24 and 48 h. Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2 log (cfu/ml).

4.3.5 Comparative genome analysis of parental strains and pressure-resistant derivatives

To identify differences in gene content and identify potential candidates that may play a role in the acquisition of pressure resistance of *E. coli*, genome sequences of *E. coli* AW1.7, AW1.7ΔpHR1, and AW1.3 were compared to the genome sequences of the respective derivative strains of *E. coli* AW1.7-PR, AW1.7ΔpHR1-PR, AW1.3-PR (Tables 4-2, 4-3 and 4-4). The genomes of *E. coli* AW1.7ΔpHR1 and *E. coli* AW1.7 were virtually identical with exception for 3 deletions in *E. coli* AW1.7ΔpHR1 of 14469, corresponding to the LHR, LHR), 21768 and 16248 bp (Mercer et al., 2015). All derivative strains had deletion of genetic elements coding for transport system, metabolic activity, aerobic respiration, fitness factors, or mobile genetic elements. Many of these ORFs in the strains can be grouped as coding for hypothetical protein, and/or unknown gene products, without apparent consistence among strains. The number of deletions from derivative strains when compared with parental strains is

shown in Table 4-2 for *E. coli* AW1.7, Table 4-3 for *E. coli* AW1.7ΔpHR1 and Table 4-4 for *E. coli* AW1.3 respectively. *E. coli* AW1.7-PR showed three deletions. The size of these deletions is 8577, 10737 and 39154 bp, coding for 7, 6, 34 putative proteins respectively. *E. coli* AW1.7ΔpHR1-PR showed five deletions with the following sizes; 4660, 20234, 8450, 1479 and 8410 which code for 1, 10, 8, 3 and 7 putative proteins. *E. coli* AW1.3-PR showed only one deletions of 1026 bp, which code for 1 putative protein. Pressure resistant derivative strains exhibited deletions of mobile genetic elements when compared to parent strains. Two and ten mobile elements were deleted in *E. coli* AW1.7-PR and AW1.7ΔpHR1-PR respectively and a prophage related protein was deleted in *E. coli* AW1.3-PR.

Table 4-2. List of deletions in the genome of *E. coli* AW1.7-PR compared to the genome of *E. coli* AW1.7, and list of putative proteins encoded in these regions.

Contig size	ORFs	# of Bp	Potential Product
8,577	1	570	Mobile element protein (which?)
	2	1173	N-acetylgalactosamine 6-sulfate sulfatase (GALNS)
	3	762	HTH-type transcriptional regulator ydeO
	4	183	Hypothetical protein
	5	1563	Putative formate dehydrogenase oxidoreductase protein
	6	2061	1 fimbriae anchoring protein FimD
	7	711	Chaperone FimC
10,737	1	114	Hypothetical protein
	2	2784	Probable zinc protease pqqL
	3	2373	Hypothetical protein
	4	1659	Hypothetical ABC transporter ATP-binding protein yddA
	5	1158	GALNS arylsulfatase regulator (Fe-S oxidoreductase)
	6	540	N-acetylgalactosamine 6-sulfate sulfatase (GALNS)
39,154	1	234	Probable tautomerase ydcE
	2	570	NAD(P)H-flavin oxidoreductase
	3	846	N-hydroxyarylamine O-acetyltransferase
	4	894	Phenazine biosynthesis protein PhzF
	5	681	Respiratory nitrate reductase gamma chain
	6	696	Respiratory nitrate reductase delta chain
	7	1545	Respiratory nitrate reductase beta chain
	8	3741	Respiratory nitrate reductase alpha chain
	9	1389	Nitrate/nitrite transporter
	10	1155	Internalin, putative
	11	189	Outer membrane porin protein NmpC precursor
	12	825	Permease of the drug/metabolite transporter (DMT) superfamily
	13	3048	Formate dehydrogenase N alpha subunit (selenocysteine-containing)
	14	885	Formate dehydrogenase O beta subunit
	15	654	Formate dehydrogenase N gamma subunit
	16	285	HigA protein (antitoxin to HigB)
	17	1011	Alcohol dehydrogenase
	18	1725	NAD-dependent malic enzyme
	19	138	Stationary-phase-induced ribosome-associated protein
	20	216	Bdm protein
	21	432	Osmotically inducible protein
	22	927	Dipeptide transport ATP-binding protein DppF
	23	987	Dipeptide transport system permease protein DppC; Putative hemine transporter ATP-binding subunit
	24	897	Dipeptide transport system permease protein DppB
	25	1023	Dipeptide-binding ABC transporter, periplasmic substrate-binding component; Putative hemin-binding lipoprotein
	26	1110	Dipeptide-binding ABC transporter, periplasmic substrate-binding component; Putative hemin-binding lipoprotein
	27	171	Dipeptide-binding ABC transporter, periplasmic substrate-binding component; Putative hemin-binding lipoprotein
	28	582	D-alanyl-D-alanine dipeptidase; Putative- metaloprotease associated with hemin utilization

	29	2424	Heme-regulated cyclic AMP phosphodiesterase
	30	1383	Putative Heme-regulated two-component response regulator
	31	1155	COG1649 predicted glycoside hydrolase
	32	126	Hypothetical protein
	33	1536	Probable glutamate/gamma-aminobutyrate antiporter
	34	1095	Glutamate decarboxylase

Table 4-3. List of deletions in the genome of *E. coli* AW1.7ΔpHR1-HP compared to the genome of *E. coli* AW1.7ΔpHR1, and list of putative proteins encoded in these regions.

Contig size	ORFs	# of bp	Potential Product
4,660	1	864	Outer membrane protein A precursor
20,234	1	10233	Hypothetical protein
	2	1458	Hypothetical protein
	3	2133	Putative ATP-binding component of a transport system
	4	1191	HlyD family secretion protein
	5	1209	Putative transposase
	6	693	Mobile element protein
	7	288	Mobile element protein
	8	318	Mobile element protein
	9	789	Mobile element protein
	10	267	Mobile element protein
8,450	1	267	Mobile element protein
	2	369	Methionine ABC transporter substrate-binding protein
	3	3066	Tetrathionate reductase subunit A
	4	1023	Tetrathionate reductase subunit C
	5	735	Tetrathionate reductase subunit B
	6	1782	Tetrathionate reductase sensory transduction histidine kinase
	7	585	Tetrathionate reductase two-component response regulator
	8	204	Hypothetical protein
1,479	1	210	RecD-like DNA helicase YrrC
	2	438	Hypothetical protein
	3	531	Alpha/beta hydrolase fold
8,410	1	462	Mobile element protein
	2	249	Mobile element protein
	3	405	Mobile element protein
	4	1038	Mobile element protein
	5	2157	Putative endonuclease
	6	1308	McrBC 5-methylcytosine restriction system component
	7	996	Degenerate transposase

Table 4-4. List of deletions in the genome of *E. coli* AW1.3 -HP compared to the genome of *E. coli* AW1.3, and list of putative proteins encoded in these regions**.**

1,026	1	117	Phage DNA binding protein

4.3.6 Confirmation of deletion of genomic elements

To confirm deletions of genetic elements, these regions were amplified in the parental strains by standard PCR with the 14 sets of primers confirmed their absence in the pressure resistant derivative strains. Fig 4-5 shows the nine regions that were analysed in the three strains, and indicates the amplicons that were generated by PCR with genomic DNA of the parental strains as template. The absence of genetic elements as predicted by bioinformatic analyses was confirmed by PCR amplification in all cases (Figure 4-5).

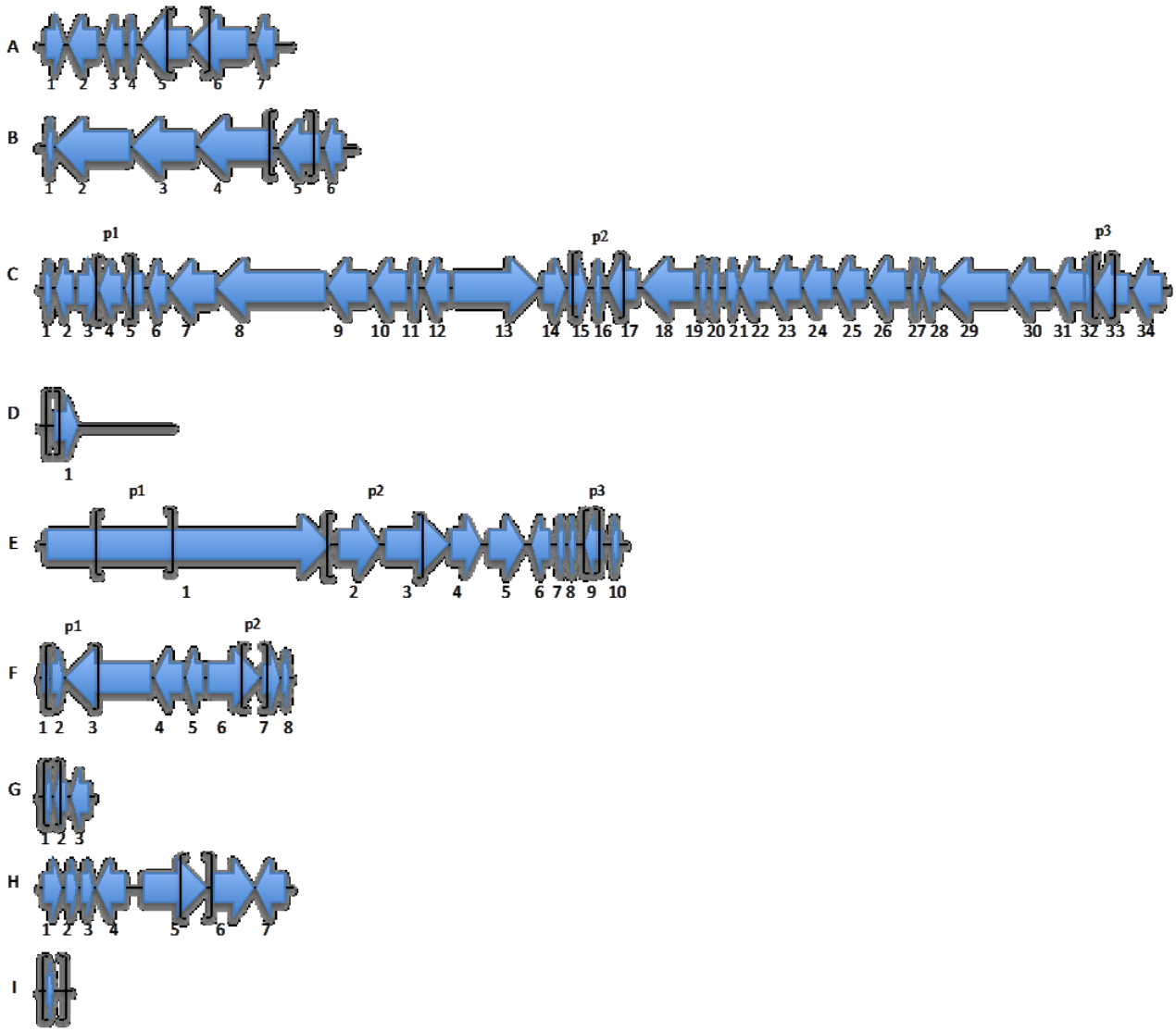

Figure 4-5. PCR products (brackets) obtained in the parental strains and abcent in the derivative strains. A, B, and C are deleted elements of AW1.7. D, E, F, G, and H of AW1.7ΔpHR1. I of AW1.3. Primers pairs forward (open brackets) and reverse (closed brackets) for the amplification of specific targets are label as p1, p2, and p3. Primer characteristics and product sizes are specified in Table 4-1. Genetic elements and their potential products are numbered to correspond to ORFs listed in Table 4-2, 4-3, and 4-4, that were present in parental strains and absent on derivative strains.

4.4 Discussion

This study provides the first comparative genomic analysis of pressure resistant derivative strains with their parental strains. Four strains of *E. coli* including one strain of STEC acquired pressure resistance, indicating that the development of pressure resistance in *E. coli* is highly reproducible. Evolution of a pressure resistant phenotype after several rounds of sublethal pressure treatment and re-growth is in agreement with previous studies (Vanlint et al., 2011; Hauben et al., 1997). However, Vanlint et al. (2012), found that not all strains of *E. coli* are able to acquire HHP resistance, suggesting that during evolution (stress adaptation) some strains may not hold the genetic predisposition to become HHP resistant.

E. coli have a flexible genome. The high variability in gene content among different *E. coli* strains is mainly due to the acquisition/deletion of genetic information (Hayashi et al., 2001; Dobrindt et al., 2003). Genome rearrangement or deletions may result in resistance to food preservation methods such as high pressure (Malone et al., 2006). Remarkably, deletions of single genes confer protection of *E. coli* towards high pressure (Malone et al., 2006; Vanlint et al., 2013a). In this study, derivative strains disclosed deletion of genetic elements coding for transport systems, metabolic activity, aerobic respiration, and fitness factors. Mobile elements in bacteria constitute a flexible gene pool that can be exchanged between cells, inducing DNA breaks and rearrangement (Foster, 2007; Hacker and Carniel, 2001). *E. coli* AW1.7ΔpHR1 is a heat sensitive derivative of heat resistant *E. coli* AW1.7. According to Mercer et al., (2015) the presence of the LHR is required for acquisition of extreme heat resistance in strains of *E. coli*, including AW1.7ΔpHR1. *E. coli* AW1.7 and AW1.3, which are LHR positive, survived 700 MPa for 15 min while cell counts of *E. coli* AW1.7ΔpHR1, were below detection limit (2.0 log cfu/mL) after only 600 MPa for 15 min, suggesting that LHR may improve natural pressure

resistance of the wild-type *E. coli* AW1.7 strain. In this study both strains with/out the LHR were able to acquire extreme pressure resistance, suggesting that the LHR is not required for pressure resistance.

Remarkably *E. coli* AW1.7-PR became sensitive to low pH (2.5). *E. coli* has developed sophisticated acid resistance systems; one involves conversion of glutamine to glutamate which can be further decarboxylated by GadA/GadB to generate GABA, expelling a cytoplasmic proton to the extracellular environment, thus increasing intracellular pH promoting survival during acidic environment (Lu et al., 2013). Richard and Foster (2004) reported that the presence of glutamate enhanced survival of *E. coli* at low pH (2.5). Glutamate decarboxylase was deleted in the *E. coli* AW1.7-PR strain, which may explain the sensitivity of the pressure derivative strain to acidic conditions.

E. coli AW1.3-PR exhibited increased resistance to heat and osmotic stress (Fig 4-2 and 3-4). *E. coli* exposed to high pressure can rapidly and reproducibly select for increased RpoS activity and concomitant cross-resistance to pressure and heat without any mutations in the *rpoS* locus of the mutant strain, suggesting that genes related to RpoS in response to pressure, could provide cross resistance to other stresses (Vanlint et al., 2013a; Battesti et al., 2001), such as heat and osmotic stress.

In this study, Fe-S oxidoreductase and nitrate reductase genes (Table 4-2) were deleted in *E. coli* AW 1.7-PR. Expression of Fe-S cluster proteins and a nitrate reductase regulator decreased the resistance of *E. coli* to pressure (Malone et al., 2006). This may partly explain the acquisition of pressure resistance by *E. coli* AW1.7-PR. However, deletions in *E. coli* AW1.7ΔpHR1 were different without apparent consistency between the two nearly isogenic strains (Tables 4-2 and 4-3). Interestingly *E. coli* AW1.3 showed only a mobile element (phage)

deleted (Table 4-4). The inconsistent loss of specific genes during acquisition of pressure resistance is in partial agreement with previous studies (Vanlint et al., 2013b; Malone et al., 2006). This suggests that several alternative routes to development of pressure resistance exist in *E. coli*. Mechanisms of pressure resistance do not necessarily depend on one specific pathway and/or potential changes in gene expression rather gene content may dramatically increase acquisition of pressure resistance in *E. coli*. If during acquisition of pressure resistance in *E. coli,* random mutations appear after 5 (Vanlint et al., 2013b) to 21 pressure cycles, wild type strains with comparable pressure resistance likely exist in nature. This phenomenon could be a key factor of the tremendous variability of *E. coli* against different intervention methods. The variability of pressure resistance in *E. coli* and other pathogenic organisms implies that high pressure is not sufficient as a pathogen intervention technology when used alone. To ensure food safety, it must be used in combination with other treatments (Liu et al., 2015).

The effect of high pressure exposure on the evolution of foodborne pathogens and spoilage microorganism needs to be determined to enhance food safety. Antimicrobial efficacy of pressure is enhanced in combination with other treatments (Feyaerts et al., 2015). But the additive combination must have beneficial application to enhance microbial inactivation in different food systems. Therefore the mechanisms of potential synergistic activity between treatments must be determined to prevent cross protection of pressure against other antimicrobial hurdles. Knowledge on the potential factors that confer pressure resistance of *E. coli* will lead to the design, combination and/or adoption new intervention methods on the food industry.

4.5 References

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5. General discussion and conclusion

Verotoxigenic *E coli* (VTEC) remain an unsolved problem in food safety. Ruminants especially cattle represent the major reservoir of VTEC (Orden et al., 2002). Meat can be contaminated during slaughter and dressing operations. Beef and dairy products including undercooked beef products, have been linked to approximately 75% of *E. coli* O157 outbreaks in the USA (Duffy et al., 2014) However, VTEC outbreaks resulting in enteric and systemic disease have been also associated with the consumption of other products such as water, juices, and fresh produce (Tuttle et al., 1999; Vojdani et al., 2008; Karch et al., 2012; Beuchat, 1996; EFSA, 2013; Bavaro, 2012). Thermal and non-thermal intervention methods are applied to inactivate pathogens and spoilage microorganisms; however, pathogen intervention methods often also decrease food quality. Moreover, some strains of *E. coli* exhibit a substantial resistance to thermal or non-thermal intervention technologies. For example, some strains of VTEC survive the application of 600 MPa in ground beef with minimal reduction of cell counts (Liu et al., 2015). The great variability of *E. coli* toward high pressure is affected by physiological factors including the growth temperature and the stage of growth, and by extrinsic factor such as treatment conditions (pressure, temperature and treatment time) and substrate composition. In addition, extremely pressure-resistant mutants of *E. coli* can be easily generated (Vanlint et al., 2011; Hauben et al., 1997).

Resistance of *E. coli* to pressure is related to protein denaturation and aggregation. GFP-labeled proteins aggregates (PAs) of *E. coli* were dispersed under pressure treatment and reassembly was a prerequisite to initiate growth. Pressure treatment at low intensity corresponded to a lower dispersal of PAs and higher probability of survival (Govers et al., 2014; Govers and Aertsen, 2015). The acquisition of extreme HHP resistance was observed to

coincide with increased expression of heat shock proteins (Aertsen et al., 2004). Sigma factor (RpoE), lipoprotein (NlpI), thioredoxin (TrxA), thioredoxin reductase (TrxB), a trehalose synthesis protein (OtsA), and a DNA-binding protein (Dps) promoted barotolerance of *E. coli* (Malone et al., 2006). The function of many of these proteins related to protein folding and turnover, and may thus relate to the formation and stability of protein aggregates.

Loss of RpoS activity was found to increase inactivation of *E. coli* treated with high pressure (Charoenwong et al., 2011; Robey et al., 2001). Loss of cAMP/CRP regulation increased resistance to HHP in some *E. coli* strains (Vanlint et al., 2013). In this study, 4 strains of *E. coli* including one strains of VTEC acquired pressure resistance, suggesting that selection of pressure resistant strains of *E. coli* can be relatively easy generated during high-pressure food processing. Genome comparison of *E. coli* AW1.7-PR and *E. coli* AW1.7ΔpHR1-PR showed deletion of various genetic elements coding for transport system, metabolic activity, aerobic respiration, fitness factors, hypothetical protein, and/or unknown gene products, without apparent consistency (association) among strains. Interestingly *E. coli* AW1.3-HP had only one genetic element (phage) deleted suggesting that different mechanisms to evolve extreme HHP resistance in the cell and/or potential changes in gene expression rather than gene content may dramatically increase pressure resistance. The development of high levels of barotolerance of *E. coli,* after consecutive cycles of HHP provides an opportunity to study factors influencing pressure resistance of *E. coli*. Combination of pressure with other antimicrobials may optimize the killing effect and avoid the potential development of pressure resistance of bacteria.

To validate new intervention methods, challenge studies are required to verify process efficacy. The use of pathogenic strains is not possible for in-plant challenge studies. Thus, is necessary to use microbial surrogates in specific food matrices and under desired conditions

(target pressure, treatment time and temperature). *E. coli* exhibits great variability in pressure resistance; thus, the analysis of only pressure sensitive or a few strains of VTEC may over-estimate the lethal effect of pressure (Hsu et al., 2015). It is necessary to develop cocktails of non-pathogenic *E. coli* for use in challenge studies that are validated with cocktails of pathogenic strains (Ingham et al., 2010). This study evaluated the pressure resistance of non-pathogenic strains of *E. coli* to validate a cocktail of surrogate strains with equal resistance to pressure when compared to pressure-resistant VTEC using ground beef as a food matrix. The VTEC cocktail comprises pressure resistant strains that were identified in a screening of more than 100 strains of VTEC (Liu et al., 2015). Ground beef inoculated with either cocktail exhibited comparable cell counts after pressure treatment, with cell counts of 2 and 5 log (cfu/g) after 5 and 30 min of treatment at 600 MPa and 20 °C. The 5 strain cocktail composed of non-pathogenic strains thus reliably indicates the survival of VTEC. However, according to Health Canada guidelines pressure is approved for use with ground beef for only 3 min and 600 MPa (Health Canada, 2013); this treatment intensity is not sufficient to reduce resistant strains of VTEC by more than 2 log(cfu/g). Additional antimicrobial treatments are required to enhance inactivation. To combine different intervention methods with high pressure, it is necessary to determine the potential cross protection between different stresses. *E. coli* AW1.3-PR exhibited increased resistance to heat and osmotic stress, and *E. coli* AW1.7-PR became sensitive to low pH (2.5), indicating that acquisition of pressure resistance also affects resistance to other stresses such as heat, low pH and osmotic stress. These genetic changes during evolution may explain the tremendous variability of *E. coli* against different intervention methods treated on different substrates.

In addition to the natural variability of pressure resistance of *E. coli*, the water activity, pH, and the composition of the food matrix greatly influences the inactivation of bacteria subjected to high pressure. Food matrices are complex environments that may offer shelter to microorganisms, even under harsh treatment conditions including high pressure. Molina-Höppner et al, (2004) reported that the presence of disaccharides or compatible solutes could protect bacteria against pressure-mediated cell death in high concentration sodium chloride or sucrose solution. However, complex food systems may lead to unexpected results of bacteria inactivation after pressure treatment due to the matrix composition and not only the influence of a_w, pH, and solutes. Ground beef is a complex matrix with a high nutrient environment. Baccus-Taylor et al. (2015) found that ground beef had a great baroprotective effect on *E. coli* from pressure inactivation compared to cells treated in beef gravy and peptone water. In the current study, cell counts of two strain cocktails inoculated in ground beef were 4 log(cfu/mL) higher compared to bruschetta and tzatziki after treatment at 600 MPa for 5 and 3 min (pH 5.5). This study demonstrated that divalent cautions (Ca^{2+}) protected *E. coli* against pressure inactivation, which is in agreement with previous studies (Hauben et al., 1998; Gayán et al., 2013). Interestingly, Ca^{2+} and Mg^{2+} exhibited a major protective effect on *E. coli* during post-pressure refrigerated storage. The protective effect of calcium is related to stabilization of the outer membrane (Sahalan et al., 2013). However, in this study, the addition of calcium did not influence the permeability of the outer membrane of pressure treated cells, suggesting that Ca^{2+} can stabilize other important cellular targets such as ribosome and cytoplasmic membrane of *E. coli* during and/or after pressure treatment. Furthermore, antimicrobial compounds that increase the membrane fluidity exhibit synergistic activity with pressure-mediated elimination of *E. coli* in food. Thus, understanding strain/matrix complex interaction during and after pressure

treatment will facilitate the design, adoption, and/or combination of different intervention methods to warrant food safety.

In conclusion, both pressure sensitive and pressure resistance of *E. coli* treated with consecutive cycles of increasingly pressure shocks with intermittent resuscitation evolved as extremely pressure-resistant strains affecting the resistance (increasing/decreasing) to other stresses such as heat, low pH and osmotic stress, deletion/rearrangement of genetics elements may explain the observed affect. Furthermore, food constituents greatly increase pressure resistance of *E. coli.* Thus, when pressure alone or combined with other antimicrobial does not eliminate *E. coli* in different food matrices, the result could be improved pressure resistance of *E. coli.* This may also confer cross-resistance to other intervention methods including heat the most traditional intervention method in food processing. Thus, more studies with specific food and allowed/desired pressure treatment conditions combined with different antimicrobial must be performed to ensure enhanced inactivation of *E. coli* after pressure and during storage time post-pressure to avoid creation of unwanted side effects.

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APPENDIX

A. Genetic determinants of heat resistance in *Escherichia coli*

A.1 Introduction

Escherichia coli are commensals in the human and animal gut but the species also comprises intestinal and extraintestinal pathogens. The ecological versatility of *E. coli* is reflected in its genome plasticity. The average *E. coli* genome is approximately 5.16 Mb, encoding an average of 5190 genes. The core genome of *E. coli* comprises about 1700 genes (Kaas et al., 2012); however, the pan-genome of *E. coli* contains more than 18,000 genes and is still considered to be open (Kaas et al., 2012; Rasko et al., 2008; Touchon et al., 2009).

Lateral gene transfer promotes the evolution and diversity of *E. coli*, and allows acquisition of virulence factors (Croxen et al., 2013; Dobrindt et al., 2004; Gordienko et al., 2013). Genes responsible for colonization, toxin production and antibiotic resistance are encoded on mobile genetic elements and are transmitted between strains of *E. coli* (Croxen et al., 2013). One prominent example is the Shiga toxin (*stx1* or *stx2*), carried on the genomes of lambdoid prophages (O'Brien et al., 1984). The horizontal transfer of large gene clusters, called genomic islands, also provides accessory genes for niche adaptation and pathogenicity (reviewed in Rasko et al., 2008; Croxen et al., 2013; Schmidt and Hensel, 2004). The locus of enterocyte effacement (LEE) is a 35-kb genomic island coding for virulence genes for attachment and effacement of intestinal epithelial cells and other pathogenic traits (McDaniel et al., 1995). Novel combinations of accessory genes present significant challenges to public health. Transduction of an *E. coli* by a Shiga toxin-converting phage resulted in a new pathovar, enteroaggregative hemorrhagic *E. coli*, which caused a large foodborne outbreak in summer 2011 (Bielaszewska et al., 2011).

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Pathovars of *E. coli* are characterized by their virulence gene profile, mechanisms for cellular adhesions, and site of colonization, and include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroaggregative hemorrhagic *E. coli* (EAHEC), enterotoxigenic *E. coli* (ETEC) and uropathogenic *E. coli* (UPEC) (Agarwal et al., 2012; Croxen et al., 2013). Due to the severity of infections and the low infectious dose, EHEC and EAHEC are particularly of concern for both public health and the food industry (Croxen et al., 2013; Bielaszewska et al., 2011; Scallan et al., 2011). EHEC carry *stx* genes and are also referred to as Shiga toxin-producing *E. coli* (STEC) (Croxen et al., 2013). STEC causes an estimated 175000 food-borne infections per year in the United States (Scallan et al., 2011). The most frequent serotype of STEC in North America is O157:H7, but other serotypes have also been implicated in STEC infections and are food adulterants in the U.S. (USDA, 2012).

Ruminants including cattle are the primary reservoir for STEC (Croxen et al., 2013; Lainhart et al., 2009). The beef processing industry applies thermal intervention methods such as steam pasteurization and hot water washes to reduce STEC contamination of meat. However, heat resistance in *E. coli* is highly variable and some strains exhibit a stable thermotolerant phenotype (Rudolph and Gebendorfer, 2010). While most strains of *E. coli* have D_{60} values of less than 1 min, moderately or exceptionally heat resistant strains exhibit D_{60} values of more than 1 and more than 10 minutes, respectively (Hauben et al., 1997; Dlusskaya et al., 2011; Liu et al., 2015). The beef isolate *E. coli* AW1.7 has a D_{60} value of more than 60 min and survives in beef patties grilled to a core temperature of 71°C or "well done" (Dlusskaya et al., 2011). Heat resistance in *E. coli* AW1.7 has been attributed to the accumulation of compatible solutes (Pleitner et al., 2012) and membrane transport proteins including the outer membrane porin

NmpC (Ruan et al., 2011); however, the genetic determinants of heat resistance remain unknown. This study aimed to identify genetic determinants of heat resistance in *E. coli* by comparative genomic analysis of heat sensitive, moderately heat resistant, and extremely heat resistant strains of *E. coli*. Analyses focused on food and clinical isolates of *E. coli* and included Shiga-toxin producing strains.

A.2 Materials and Methods

A.2.1 Strain selection and heat treatments

The 29 strains of *E. coli* used in this study included previously characterized heat resistant and sensitive food and clinical isolates (Liu et al., 2015, Ruan et al., 2011). Strains were selected to include isolates differing in their heat resistance, and to include the phylogenetic variability in the species *E. coli*. All strains were grown overnight in Luria-Bertani (LB) broth at 37 ˚C with 200 rpm agitation. To determine the heat resistance, *E. coli* strains were treated at 60 ˚C for 5 min as previously described (Dlusskaya et al., 2011). After heating, the cultures were serially diluted, plated onto LB agar and incubated aerobically overnight at 37 ˚C. Strains were classified into phenotypic groups based on their survival after heating. Strains with a reduction in cell counts of more than 5 log (cfu mL) after 5 minutes at 60 ˚C were classified as heat sensitive. Strains demonstrating a reduction in cell counts of 1 to 5 log (cfu mL) were classified as moderately heat resistant while strains with reductions less than 1 log (cfu mL) designated as highly heat resistant.

A.2.2 Genomic DNA isolation, genome sequencing, assembly, and annotation

DNA for genome sequencing was isolated from overnight cultures of *E. coli* grown in 5 ml of LB broth. Genomic DNA was isolated using the Wizard ® Genomic DNA Purification Kit (Promega, Madisson, Wisconsin, USA) following the manufacturer's guidelines. The quality

and quantity of each sample was assessed using gel electrophoresis and a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). DNA samples were sequenced using Illumina HiSeq2000 with an insert size of 300 bp by Axeq Technologies (Seoul, South Korea). The quality of the 100-bp paired-end reads was assessed using the FastQC tool (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and low quality reads were filtered by Quake (Kelley et al., 2010). Assemblies were obtained using ABySS 1.3.4 (Assembly By Short Sequence; Simpson et al., 2009) with the most optimal k-mer value for each genome. Genomes were annotated automatically by the RAST server. For O157:H7 strains, the genomes assemblies were improved by scaffolding the contigs using the reference genomes of strains EDL933 (Accession: NC002655) and Sakai (Accession: NC002695). All genomic sequences of the 29 strains used in this current study are deposited to the NCBI wgs database under BioProject PRJNA277539.

A.2.3 Core genome phylogenetic analysis and identification of orthologous genes unique to different phenotypes

To construct a core-genome phylogenetic tree, the 28 sequenced genomes obtained in this study were combined with 48 reference genomes obtained from NCBI Genbank (ftp://ftp.ncbi.nlm.nih.gov/genome) for a total of 76 *E. coli* and *Shigella* genomes. Reference genomes were selected to prioritize closed genomes over whole genome shotgun sequences, and to represent the phylogroups A, B1, B2, D, E, and *Shigella*. Construction of the core genome phylogenetic tree employed the previously described workflow (Touchon et al., 2009; Hazen et al., 2013) including genome alignment to identify the core genome, extraction of nucleotide sequences of the core genome, and calculation of a maximum likelihood phylogenetic tree. The genomes were aligned with Mugsy with default parameters (Angiuoli and Salzberg, 2011).

Homologous blocks present in each genome were extracted and concatenated using an in-house Perl script. The most disordered regions were eliminated using Gblocks with default parameters (Talavera and Castresana, 2007). The core genome size of the 76 genomes was approximately 2.7 Mbp. A maximum likelihood phylogenetic tree was constructed by RaxML with default parameters (Stamatakis, 2014) using bootstrapping for 1000 replicates.

To identify orthologous genes unique to the different phenotypic groups, protein sequences from all 29 genomes were combined and searched using all-against-all BLAST. The protein sequences with identities and coverage above 70% were clustered into families using the program OrthoMCL (Li et al., 2003). The inflation value of 2 was used for the MCL clustering. Sequence identity and comparisons of open reading frames (ORFs) were analyzed in Geneious (Biomatters, Auckland, New Zealand).

For phylogenetic analysis of the locus of heat resistance, genomes containing homologous sequences with >80% coverage of the ~14 -kb LHR nucleotide sequence from *E. coli* AW1.7 were retrieved from NCBI. Sequences with homology to the LHR of *E. coli* AW1.7 were manually extracted and aligned with ClustalW implemented in MEGA6 (Tamura et al., 2013). The MEGA6 oftware package was used to construct a maximum-likelihood tree with default parameters. Bootstrap support values were calculated from 100 replicates.

To assess frequency of the locus of heat resistance in *E. coli*, a BLAST search of both the NCBI Genomes and whole-genome shotgun assemblies (wgs) database was performed. For each study, the number of strains containing sequence corresponding to >80% coverage was counted and totaled to give an approximate percentage of strains that were positive for the locus. Bioinformatic characterization of the genetic island was completed using BPROM (Solovyev

and Salamov, 2013) and ARNold (Gautheret and Lambert, 2001) to identify putative promoters and rho-independent terminator sequences, respectively.

A.2.4 Genetic complementation of the LHR

To construct a plasmid-borne copy of the LHR, primers were designed in Geneious to selectively amplify the entire genomic island in 3 separate fragments. All primers and plasmids used in this study are listed in Table A-1. PCR reactions were carried out using Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to manufacturer guidelines. F1 (~6 kb) was amplified using primer pair HR-R1/HR-R1 and included the native putative promoter sequence. F2 (~3.3 kb) and F3 (~7 kb) were amplified by primer pairs HR-F2.1/HR-R2 and HR-F3/HR-R3, respectively. F1 and F2 were cloned separately into pUC19 as KpnI/XbaI inserts, while F3 was inserted as a XbaI/HindIII fragment, yielding recombinant vectors pUCF1, pUCF2 and pUCF3 (Table A-1). All 3 fragments were sequenced and subsequently sub-cloned into the low-copy plasmid, pRK767 (Gill and Warren, 1988), generating pRF1, pRF2 and pRF3 (Table A-1). To construct the entire LHR on a plasmid, F1 was ligated into pUCF2 as a KpnI/SmaI fragment, resulting in pUCF1-2. The 8.3 kb insert, F1-2, was sub-cloned to pRK767 as a KpnI/XbaI fragment. The new recombinant vector, pRF1-2, and F3 were digested with BglII and HindIII and ligated to form pRF1-2-3, or simply, pLHR. The plasmids pRF1, pRF2, pRF3 and pLHR were electroporated into *E. coli* AW1.7ΔpHR1, a heat sensitive derivative of AW1.7 (Pleitner et al., 2012). The resulting strains, as well as the DH5α strains used for cloning, were assayed for heat resistance as previously described (Liu et al., 2015). All transformants carrying either pUC19- or pRK767-based recombinant vectors were plated on LB media containing 50 mg l ampicillin or 15 mg l tetracycline, respectively.

A.2.5 PCR screening of beef isolates for heat resistance

A set of 55 strains of *E. coli* that were previously isolated from a beef processing plant (Dlusskaya et al., 2011) was screened for heat resistance with primers targeting the locus of heat resistance. *E. coli* AW1.7 and its heat sensitive derivative *E. coli* AW1.7ΔpHR1 (Pleitner et al., 2012) were used as positive and negative controls, respectively. Primers (Table A-1) were designed and used to selectively target 3 separate regions (size ranging 1.8-2.8 kb) of the locus of heat resistance. The primer pairs HR-F1/HS-R1 and HR-F2.2/HR-R2 amplified regions A (~1.8 kb) and B (~2.8 kb), respectively. Primers HS-F1 and HR-R3 were used to amplify region C (~2.8 kb). Recombinant Taq ® DNA polymerase (Invitrogen, Burlington, Ontario) was used to amplify products in a standard colony PCR reaction mixture and amplicons were visualized by staining with SYBRsafe (Invitrogen, Burlington, Ontario) after agarose gel electrophoresis. Heat resistance for strains *E. coli* MB1.8, DM19.2, AW1.1, GM12.6, MB 16.6, MB 3.5, GM9.1 and AW 12.2 (Dlusskaya et al., 2011) was determined by incubation at 60°C for 5 min and enumeration of surviving cells as described above.

Table A-1. Primers and plasmids used in this study

Primer	Sequence (5' → 3')	Reference
HR-F1	TTAGGTACCGCTGTCCATTGCCTGA	This study
HS-R1	AGACCAATCAGGAAATGCTCTGGACC	This study
HR-R1	TATCTAGAGTCGCGTGCCAATACCAGTTC	This study
HR-F2.1	AGGGTACCAGCGATATCCGTCAATTGACT	This study
HR-F2.2	GAGGTACCTGTCTTGCCTGACAACGTTG	This study
HR-R2	TATCTAGAATGTCATTTCTATGGAGGCATGAATCG	This study
HR-F3	TATCTAGAGATGGTCAGCGCAGCG	This study
HS-F1	GCAATCCTTTGCCGCAGCTATT	This study
HR-R3	GTCAAGCTTCTAGGGCTCGTAGTTCG	This study
Plasmids	**Description**	**Reference**
pUC19	High-copy cloning vector	Sigma
pRK767	Low-copy cloning vector	(Gill and Warren, 1988)
pUCF1	LHR fragment 1 in pUC19	This study
pUCF2	LHR fragment 2 in pUC19	This study
pUCF3	LHR fragment 3 in pUC19	This study
pUCF1-2	LHR fragments 1-2 in pUC19	This study
pRF1	LHR fragment 1 in pRK767	This study
pRF2	LHR fragment 2 in pRK767	This study
pRF3	LHR fragment 3 in pRK767	This study
pRF1-2	LHR fragments F1-2 in pRK767	This study
pLHR	The entire LHR sequence, F1-F2-F3, in pRK767	This study

A.3 Results

A.3.1 Heat resistance of *E. coli*

Strains of *E. coli* were selected for genome sequencing to obtain a wide range of heat resistance despite the limited number of strains (Figure A-1). In Figure A-1, strains are grouped based on their virulence profiles. O157:H7 STEC and non-O157 STEC were grouped based on serotype and the presence of *stx1* or *stx2* coding for Shiga toxins (Liu et al., 2015). Strains from four groups included heat sensitive strains (Figure A-1), in agreement with the heat sensitivity of a majority of strains of *E. coli* (Hauben et al., 1997; Dlusskaya et al., 2011; Liu et al., 2015). Both O157:H7 STEC and non-O157:H7 STEC included moderately heat resistant strains (Figure A-1). Four non-pathogenic strains of *E. coli* including *E. coli* AW1.7 were highly heat

resistant. All of these strains were obtained from a beef processing facility (Dlusskaya et al., 2011).

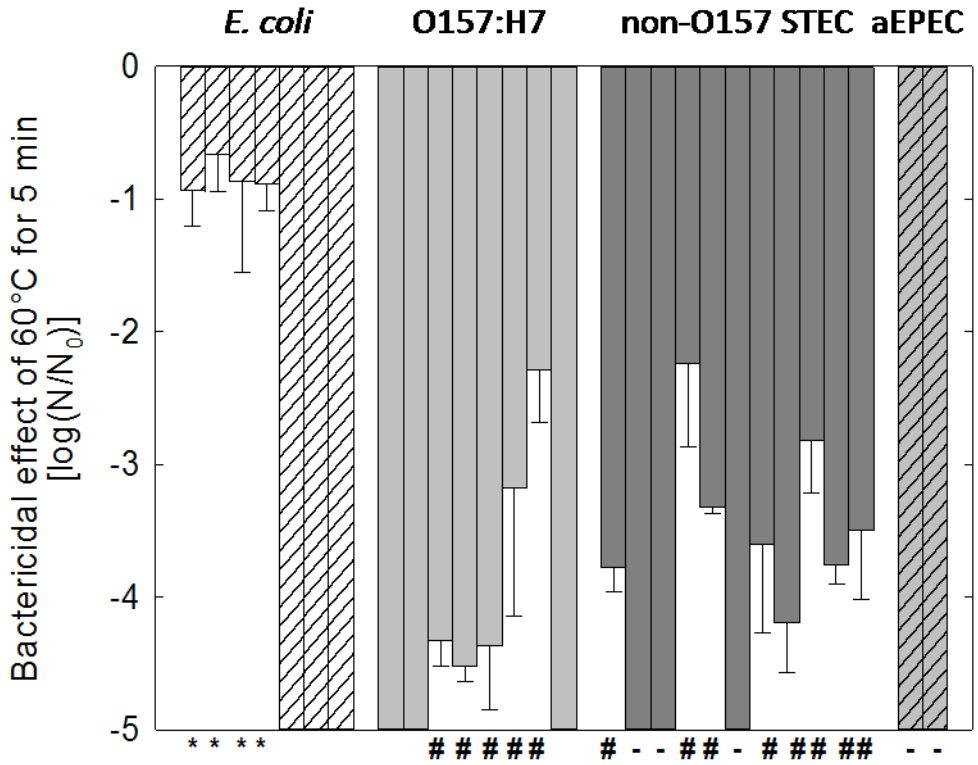


Figure A-1. Reduction of cell counts of strains of *E. coli* after heating at 60 °C for 5 min in LB broth. Strains are separated based on pathotypes: no virulence factors (*E. coli*); O157:H7 STEC and non-O157 STEC); eae^{+} stx^{-} (atypical EPEC). **-** indicates strains that were designated as "heat sensitive" because cell counts were reduced by more than 5 log (cfu mL). # indicates strains that were designated as "moderately heat resistant" because the reduction of cell counts was less than 5 log (cfu mL). * indicates strains that were designated as "highly heat resistant" because the reduction of cell counts was less than 1 log (cfu mL). The figure includes data from Liu et al., 2015

A.3.2 Genome sequences and characteristics

The 29 *E. coli* genomes sequences obtained in this study included genomes from 4 highly heat resistant strains, 13 moderately resistant strains, and 12 heat sensitive strains (Figure A-1 and Table A-2). Genebank accession numbers of the 29 genomes sequenced in this study

are indicated in Table A-2. The number of contigs larger than 500 bp per genome ranged from 95-277, with max sequence lengths ranging from 204263-435416 bp (Table A-2).

Genome sequence data confirmed the presence or absence of *stx1/stx2*and *eae* that was determined earlier by PCR (Liu et al., 2015, Table A-2). The atypical EPEC (aEPEC) carried the *eae* gene, but no pEAF-encoded *bfp* (bundle-forming pilus) genes (Trabulsi et al., 2002). Other strains of *E. coli* were negative for *eae*, *stx1/2* and *bfp*. None of the highly heat resistant strains of *E. coli* carried any virulence factors (Table A-2). The genomes of *E. coli* AW1.7 and its heat-sensitive derivative *E. coli* AW1.7ΔpHR1 (Pleitner et al., 2012) were virtually identical; however, in addition to the loss of the 4842 bp plasmid pHR1, two additional deletions of 21768 and 16248 bp were identified in the heat sensitive *E. coli* AW1.7ΔpHR1. Of the 19 STEC, 16 possessed the *eae* gene/LEE pathogenicity island; the remaining 3 STEC were categorized as LEE negative STECs (Table A-2), which still have the ability to cause disease (Newton et al., 2009). The 19 STEC included moderately heat resistant and heat sensitive strains (Table A-2). A moderately heat resistant STEC isolate from the 2011 Germany outbreak, O104:H4 11-3088, carried *stx2*, as well as a gene encoding a β-lactamase from the EHEC plasmid, pHUSEC2011. This plasmid also encodes EAEC virulence factors such as the *aaf* and *agg* genes (Estrada-Garcia and Navarro-Garcia, 2012).

Table A-2. *E. coli* strain used in this study and features of their genome sequences.

Accession Numbers	Strain (reference); phylogenetic group	Coverage (X)[1]	Number of contigs assembled	Max contig size (bp)	Number of putative proteins[2]	Heat Resistance	EHEC Virulence Factors	Origin
LDYI00000000	AW1.3 (1); A	579.12	184	317424	5041	High	n.d.[3)]	Beef
LDYL00000000	DM18-3 (2); A	469.26	111	353387	4700	High	n.d.	Beef
LDYM00000000	GM16-6 (2); A	442.89	164	209077	4678	High	n.d.	Beef
LDYJ00000000	AW1.7 (1); A	494.63	165	245564	4971	High (1)	n.d.	Beef
LDYK00000000	AW1.7ΔpHR1; A	519.61	152	246187	4952	Sensitive (3)	n.d.	AW1.7 mutant
LECO00000000	O103:H2 PARC444 (2); B1	471.64	98	356993	4864	Sensitive (2)	n.d.	Unknown
LECG00000000	O103:H2 PARC445 (2) ; B1	584.81	158	327869	5096	Sensitive (2)	n.d.	Unknown
LECL00000000	O44:H18 PARC450 (2); E	458.44	146	343811	4951	Sensitive (2)	n.d.	Unknown
LEAF00000000	O157:H7 CO6CE1353 (2); D	484.64	205	376588	5572	Moderate (2)	*stx1 stx2 eae*	Clinical
LEAG00000000	O157:H7 CO6CE1943 (2); D	477.76	185	374853	5436	Moderate (2)	*stx1 stx2 eae*	Clinical
LEAH00000000	O157:H7 CO6CE2940 (2); D	475.57	197	376618	5537	Moderate (2)	*stx2 eae*	Clinical
LEAE00000000	O157:H7 CO6CE900 (2); D	470.23	225	376513	5554	Moderate (2)	*stx2 eae*	Clinical
LEAJ00000000	O157:H7 E0122 (2); D	480.56	189	399998	5478	Moderate (2)	*stx2 eae*	Cattle
LEAD00000000	O157:H7 1935 (2); D	502.88	194	393069	5523	Sensitive (2)	*stx1 stx2 eae*	Human
LEAI00000000	O157:H7 CO283 (2); D	531.16	184	376583	5296	Sensitive (2)	*stx1 stx2 eae*	Cattle
LEAK00000000	O157:H7 LCDC7236 (2); D	492.65	181	376583	5461	Sensitive (2)	*stx1 stx2 eae*	Human
LDYN00000000	O26:H11 05-6544 (2)	426.65	280	219684	5691	Moderate (2)	*stx1 eae*	Human
LECF00000000	O103:H25 338 (2); B1	439.31	218	376897	5321	Moderate (2)	*stx1 eae*	Clinical
LECH00000000	O104:H4 11-3088 (2); B1	515.77	173	320350	5254	Moderate (2)	*stx2* [4]	Human
LECI00000000	O111:NM 583 (2); B1	492.35	185	323305	5067	Moderate (2)	*stx1 eae*	Clinical
LECK00000000	O113:H4 09-0525 (2); A	475.86	165	254878	5275	Moderate (2)	*stx1 stx2*	Unknown
LDZZ00000000	O121:H19 03-2832 (2); B1	457.58	213	434838	5272	Moderate (2)	*stx2 eae*	Human
LEAA00000000	O121:NM 03-4064 (2); B1	568.02	221	435416	5298	Moderate (2)	*stx2 eae*	Human
LEAB00000000	O145:NM 03-6430 (2); n.a.	528.20	210	359240	5371	Moderate (2)	*stx1 eae*	Human
LECM00000000	O45:H2 05-6545 (2); B1	508.74	263	261384	5352	Sensitive (2)	*stx1 eae*	Human
LECN00000000	O76:H19 09-0523 (2); B1	456.09	191	404223	5432	Sensitive (2)	*stx1 stx2*	Unknown
LECJ00000000	O111:NM PARC447 (2); B1	544.42	200	376589	5672	Sensitive (2)	*stx1 stx2 eae*	Unknown
LDYO00000000	O26:H11 PARC448; B1	489.45	240	204263	5429	Sensitive (2)	*eae*	Unknown
LEAC00000000	O145:NM PARC449 (2); n.a.	502.50	181	328848	5390	Sensitive (2)	*eae*	Unknown

n.a., not assigned

1 Based on the Lander-Waterman equation using an average size of *E. coli* genome (5.16 Mb)

2 Based on OrthoMCL analysis of all annotated genes; 3. n.d., not detected

4 Carries at least the beta lactamase gene present on pHUSEC2011-2 present in EAEC. Other genes on this plasmid includes factors for adhesion

References: (1) Dlusskaya et al., 2011; (2), Liu et al., 2015; (3), Pleitner et al., 2012.

A.3.3 Phylogenetic distribution of heat resistant isolates

To assess the phylogenetic relationships of the heat resistant and sensitive strains, a core genome phylogenetic tree was constructed with the genomes from this study, and 48 obtained from the NCBI database. The *E. coli* phylogenetic groups A, B1, B2, D and E (Touchon et al., 2009; Jaureguy et al., 2008) were well supported by our core genome tree (Figure A-2).

Moderately heat resistant strains were found in the phylogenetic groups A, B1 and E (Figure A-2). Resistant and sensitive strains of the serotype O157H7 and O26:H11, 05-6544 and PARC448, respectively, group together near NCBI strains of similar serotypes. This grouping of heat resistant and sensitive isolates occurs with O145:NM isolates as well, however, these strains are found distinctly separate from other phylogenetic groups (Figure A-2). Some moderately resistant, non-O157 STEC are located on branches with pathogenic *E. coli* including O104:H4 11-3088 (Figure A-2). The overall genomic similarity of sensitive and resistant strains may illustrate the ease of acquiring genetic variations to become moderately heat resistant. Particularly strains within phylogenetic group E, comprising O157:H7 STEC (Figure A-2), are highly related and therefore the differences in the accessory genes, content or sequence, accounts for differences in heat resistance.

All four highly resistant strains were assigned to group A. The highly heat resistant strains *E. coli* AW1.7 and GM16.6 are located in divergent branches separate from other *E. coli* in this group (Figure A-2). *E. coli* AW1.3 shares a high degree of sequence similarity to *E. coli* P12b, a model strain for flagellar studies (Ratiner et al., 2010), while *E. coli* DM18.3 is closely related to the commensal *E. coli* strain HS (Levine et al., 1978). The phylogenetic diversity of highly heat resistant strains indicates that these strains do not share a common ancestor (Figure A-2).

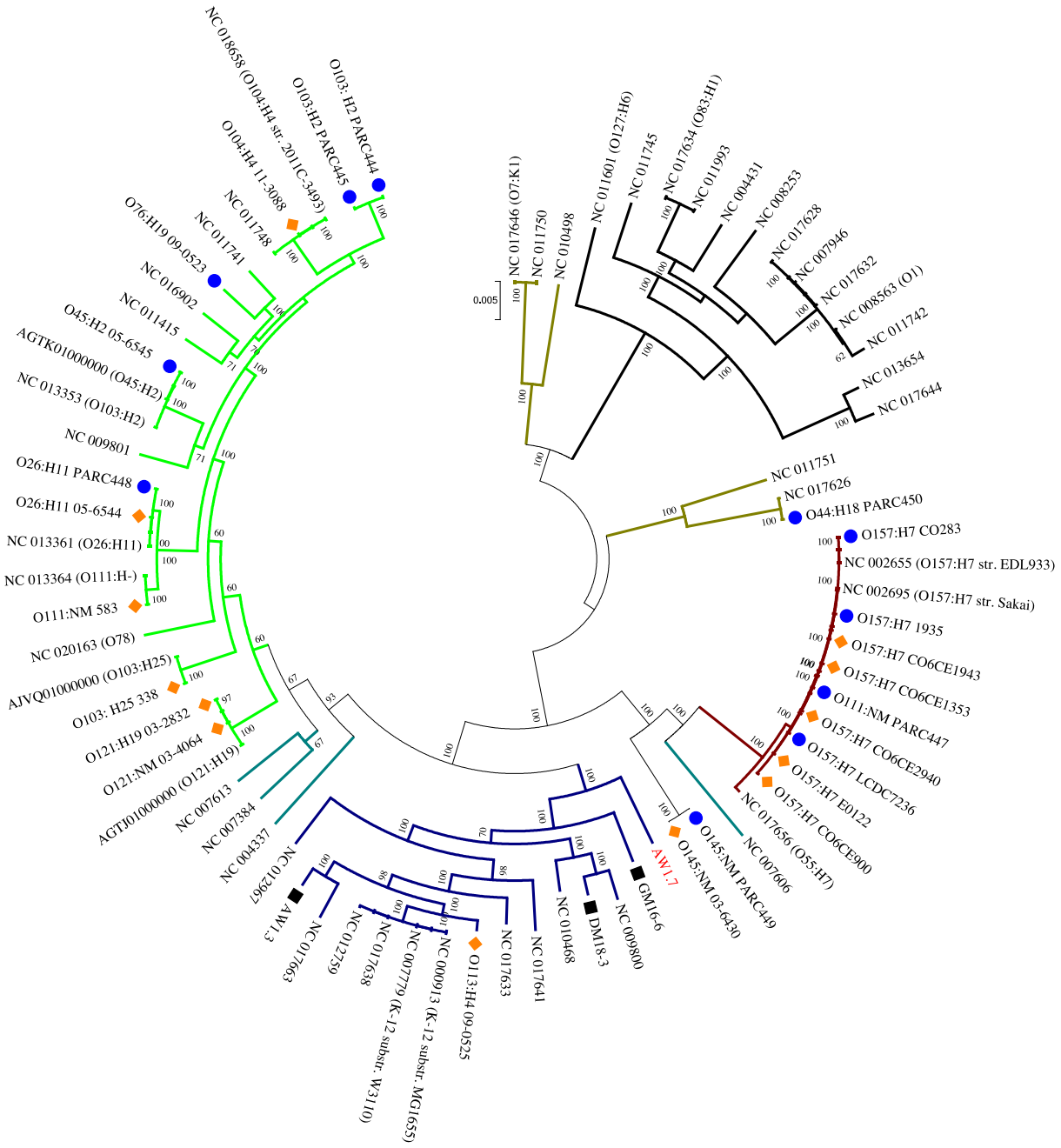


Figure A-2. Phylogenomic distribution of strains of *E. coli* and *Shigella* spp. A core genome phylogenetic tree was constructed using the 28 sequenced genomes from this project, indicated by strain numbers and serotype as applicable, and 48 genome sequences from NCBI, indicated by serotype and Accession numbers. The phylogenetic groups of *E. coli* are colour coded: A – Blue, B1 – Green, B2 – Black, D – Brown and E - Maroon; *Shigella* spp. (indicated by teal coloured branches) were included in the phylogenetic tree because this genus is considered a host-adapted pathovar of *E. coli*. Bootstrapping values are indicated for each branch. The sequenced genomes from this project are coded by blue circles and orange diamonds indicating heat sensitive and moderately heat resistant strains, respectively. Black squares represent highly heat resistant strains.

A.3.4 Identification the locus of heat resistance (LHR)

To identify differences in gene content conferring high heat resistance, the genomes were separated into their phenotypic groups: highly resistant; moderately resistant; and sensitive. An OrthoMCL analysis found 3147 orthologs shared among all 28 genomes, however, none of these were unique to heat sensitive or moderately heat resistant strains (Figure A-3). A set of 6 genes was unique to the highly heat resistant strains (Figure A-3); all of these genes are located on a 14,469 bp genomic island present in *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 (Figure A-4).

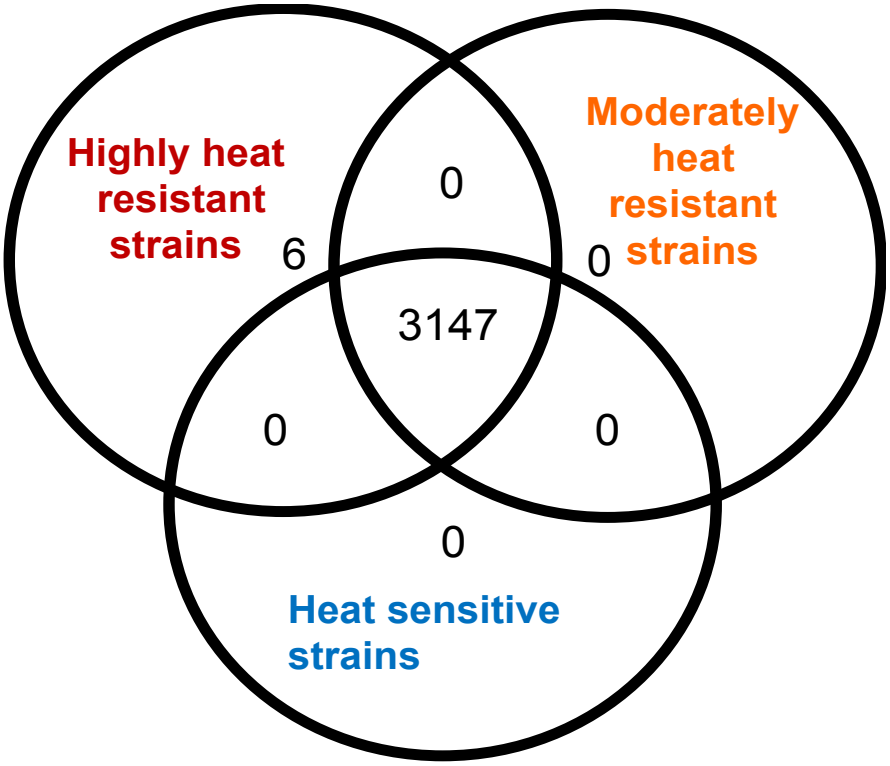
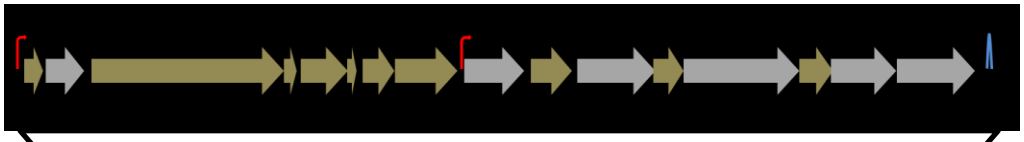


Figure A-3. Analysis of orthologous protein coding sequences identified in highly heat resistant, moderately heat resistant and heat sensitive *E. coli* strains by OrthoMCL. The Venn diagram indicates the number of protein coding sequences that are shared by all strains analysed in this study, the number of protein coding sequences that were shared between any two of the phenotypic groups, and the number of protein coding sequences that were found only in one of the three phenotypic groups.

The 6 genes specific to the highly heat resistant group are scattered among an additional 10 ORFs in this genomic island (Figure A-4). Remarkably, this genomic island was absent in *E. coli* AW1.7ΔpHR1. The plasmid curing protocol used to generate *E. coli* AW1.7ΔpHR1 (Pleitner et al., 2012) thus also resulted in a 16,248 bp deletion encompassing the genomic island and the flanking mobile genetic elements. This operon was previously identified in heat resistant strains of *Cronobacter sakazakii* (Gajdosova et al., 2011) and *Klebsiella pneumoniae* (Bojer et al., 2010). Due to its presence in highly heat resistant *E. coli,* the genomic island was named the locus of heat resistance (LHR).

A



B

Strain #	# of 5' mobile elements	Size of open reading frame (bp)															# of 3' mobile elements
		orf1	*orf2*	*orf3*	*orf4*	*orf5*	*orf6*	*orf7*	*orf8*	*orf9*	*orf10*	*orf11*	*orf12*	*orf13*	*orf14*	*orf15*	*orf16*	
AW1.7	1	282	570	2850	192	687	144	459	915	888	612	1146	441	1716	498	966	1152	1
		Pairwise percent nucleotide identity relative to AW1.7															
AW1.3[1)]	n.d.	100	100	100	99.5	99.6	100	100	100	100	99.7	100	100	99.9	99.8	100	99.2	3
DM18.3[2)]	1	98.6	99.6	99.9	99.5	99.4	90.3	96.7	93.4	96.2	99	99.6	99.3	99.5	99.8	99.4	99.8	1
GM16-6[3)]	2	99.3	99.6	99.9	99.5	99.6	98.6	99.3	99.6	99.7	99.5	99.5	99.8	99.6	99.6	99.3	99.5	1
ST416 pKPN-CZ[4)]	2	99.3	99.3	99	84.4	99.4	100	98.6	99.2	98.9	97.9	98.7	99.1	98.5	97.6	97.7	98.6	5
ATCC 29544[5)]	2	98.3	99.3	99.2	95.8	98.1	100	98.9	99.5	99.2	98.4	99.4	99.1	98.9	98.8	98	99.3	3

[1)]in *E. coli* AW1.3, no 5' mobile elements were detected on the contig

[2)]The length of three ORF's in *E. coli* DM18.3 differs from AW1.7 as follows: *orf5*, 492 bp; *orf6,* 957 bp; *orf8,* 909 bp. DM18.3 has an extra 1.141 kb of sequence around *orf5* and *orf6* containing different coding regions.

[3)]The length of two ORF's in *E. coli* GM16-6 differs from AW1.7 as follows: *orf5*, 492 bp; *orf6,* 1269 bp; GM16-6 has an extra 1.141 kb of sequence around *orf5* and *orf6* containing different coding regions.

[4)] The length of four ORF's in *K. pneumoniae* ST416 pKPN-CZ differs from *E. coli* AW1.7 as follows: *orf4,* 171 bp; *orf7,* 429 bp; *orf10,* 642 bp; *orf15*, 1003 bp.

[5)]The length of three ORF's in *C. sakazaki* ATCC29544 differs from *E. coli* AW1.7 as follows: *orf1,* 174 bp; *orf13,* 1710 bp; *orf15,* 957 bp.

Figure A-4. Representation of the locus of heat resistance (LHR) in *E. coli* AW1.7, AW1.3, DM18.3 and GM16-6, *K. pneumoniae* ST416 pKPN-CZ and *C. sakazaki* ATCC29544. (**A)** Representation of the LHR in highly heat resistant strains. The figure is scaled to the locus of heat resistance in *E. coli* AW1.7 (14.469 kb in size). Putative promoters and terminators sequences are indicated with hooked arrows and stem-loops, respectively. Open reading frames (ORFs) shaded in grey were identified as unique orthologs in highly heat resistant strains. The GC content of the genetic island is 61.8% while the genome average for AW1.7 is 51.1 %. **(B)** Pairwise nucleotide identity of ORFs in *E. coli* AW1.3, DM18.3 and GM16-6, *K. pneumoniae*

ST416 pKPN-CZ and *C. sakazaki* ATCC29544 to the corresponding ORFs in *E. coli* AW1.7. ORFs that differ in size from *E. coli* AW1.7 are shaded in gray and the size is indicated in footnotes. Mobile genetic elements were detected in all strains upstream and downstream of the locus of heat resistance; the number of mobile genetic elements is also indicated.

A.3.5 LHR confers high heat resistance to heat sensitive *E. coli*

To verify that high heat resistance in *E. coli* is mediated by proteins encoded by the LHR, the heat sensitive *E. coli* AW1.7ΔpHR1 and DH5α were complemented with LHR or fragments of LHR. LHR or LHR fragments were introduced in *E. coli* AW1.7ΔpHR1 and DH5α after cloning into the low-copy vector pRK767. Fragments F1, F2 and F3 encompassed about 6, 3.3, and 8 kbp, respectively. Cloning of the empty plasmid pRK767 served as control and the heat resistance of the resulting derivatives of *E. coli* AW1.7ΔpHR1 and DH5α was compared to the wild type strains (Figure A-5). Cloning the low copy number plasmid pRK767 into *E. coli* AW1.7 did not affect the strain's heat resistance (Figure A-1 and Figure A-5). Strains expressing the full length LHR were as heat resistant as *E. coli* AW1.7 while strains with plasmids containing only a portion of the LHR remained heat sensitive (Figure A-5). Complementation of *E. coli* AW1.7ΔpHR1 with the plasmid pHR1 did not alter heat resistance of the strain (Bédié et al., 2012 and data not shown), confirming that the loss of the LHR rather than the loss of the plasmid pHR1 are responsible for the heat sensitive phenotype of this strain.

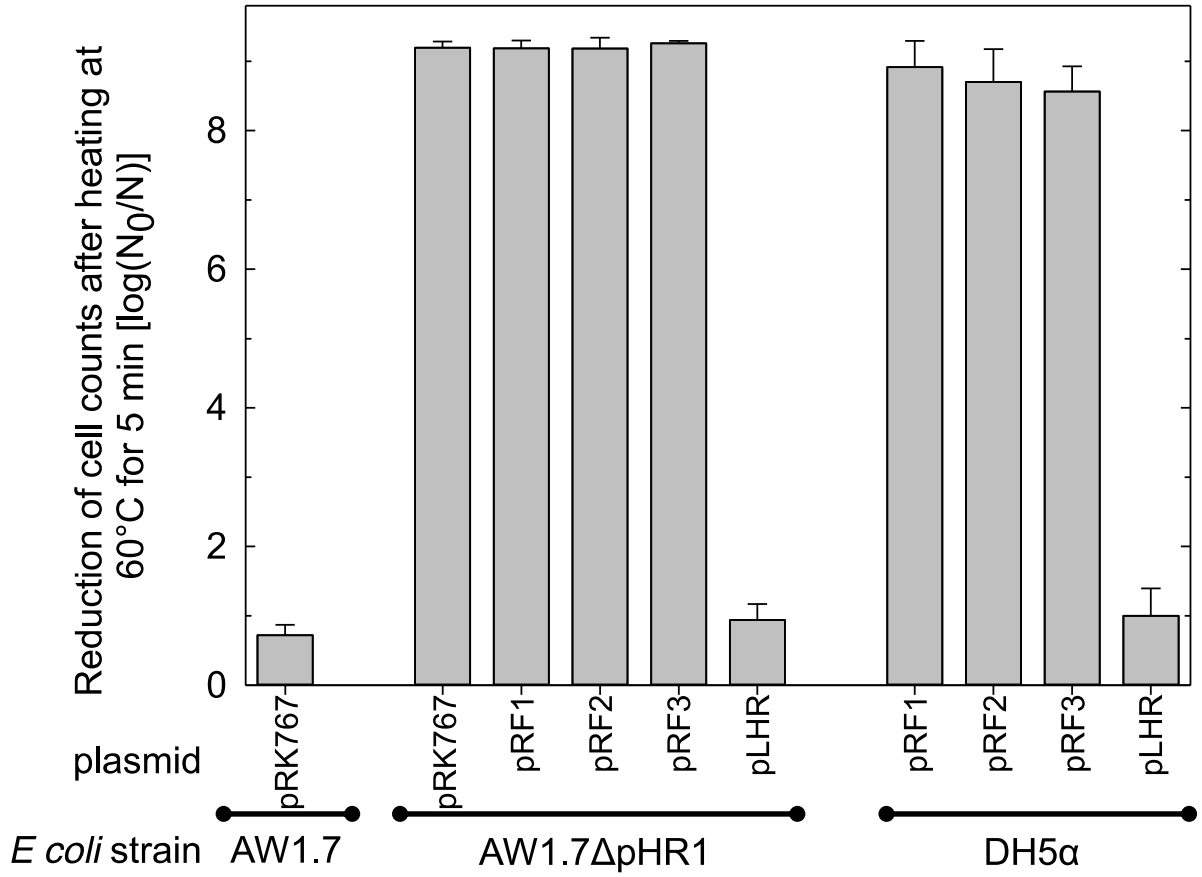


Figure A-5. Heat resistance of *E. coli* AW1.7, AW1.7ΔpHR1, and DH5α carrying the vector pRK767 or derivatives of this vector with the full length LHR or the LHR fragments F1, F2, or F3. Data are shown as means ± standard deviation of triplicate independent experiments.

A.3.6 Genes encoded by the LHR

The LHR codes for 16 putative ORFs (Figure A-4): 2 small heat-shock proteins (sHSPs); a Clp protease (Bojer et al., 2013); several hypothetical proteins with predicted transmembrane domains; a putative sodium/hydrogen exchanger; and several peptidases. Figure A-4 compares the operons in *E. coli*, *C. sakazakii*, and *K. pneumonia*. The conservation of the ORFs among *E. coli*, *C. sakazakii* and *K. pneumonia* is remarkable; most ORFs share more than 99% nucleotide identity to the corresponding genes in *E. coli* AW1.7 (Figure A-4B). *E. coli* AW1.7 and AW1.3

share 100% nucleotide identity for 10 of the 16 ORFs (Figure A-4B). In *E. coli* AW1.7, the strongest predicted promoter was located 63 bp upstream from ORF1. BPROM analysis predicted that the transcription factor OmpR interacts with this promoter. Another putative promoter is located 26 bp upstream from ORF 9 (Figure A-4A). One predicted rho-independent terminator was oriented in the same direction as the ORFs and located 177 bp downstream from ORF 16 (Figure A-4A).

In all four strains of *E. coli*, the LHR is flanked by mobile elements or putative transposases (Figure A-4B and data not shown). Accordingly, the GC content of the island is 61.8%, substantially higher than the *E. coli* average of ~50% (Figure A-4A). In *C. sakazakii* and *K. pneumonia*, the LHR is located on plasmids (Gajdosova et al., 2011; Bojer et al., 2010); however, none of the *E. coli* strains in this study possess plasmids larger than 14 kb (data not shown) and the LHR can thus be assumed to be encoded by the chromosome in the strains of *E. coli* analysed here. The high degree of sequence identity of the LHR in different species of *Enterobacteriaceae*, the presence of mobile genetic elements adjacent to the LHT, and the divergent GC content suggest that the LHR was acquired by lateral gene transfer.

A.3.7 Presence of LHR in *E. coli* and other pathogenic species

Our study and prior studies with *K. pneumonia* and *C. sakazakii* reported a correlation of the presence of the LHR and heat resistance (Figures A-1, A-4, and A-5, Gajdosova et al., 2011; Bojer et al., 2010). The LHR may thus be a marker for heat resistance in *Enterobacteriaceae* and related organisms. To determine the presence of the LHR in bacterial genomes, we performed a BLAST search using the entire LHR, excluding adjacent transposases, against the NCBI Genomes database. This analysis retrieved 41 sequences with more than 80% coverage from several species in the β- and γ-proteobacteria, including pathogenic strains of *Yersinia*

entercolitica, *Enterobacter cloacae*, *Citrobacter* sp., *Pseudomonas aeruginosa* and 16 strains of *E. coli*. The sequences were used to calculate a maximum-likelihood phylogenetic tree (Figure A-6) that shows remarkable differences from the phylogenetic tree of the bacterial species shown in the tree. The tree is divided into 2 large groups; group A is exclusively composed of sequences γ-proteobacteria (*Enterobacteriaceae* and *Pseudomonas* spp.) while group B includes sequences from β - and γ-proteobacteria (Figure A-6).

Group A includes sequences from strains of *E. coli* isolated from urinary tract infections (e.g. KTE#) and food isolates of *E. coli*. The conserved sequence identity between the most distantly related sequences from *E. coli,* DM18.3 and KTE233, is 98.9%, suggesting recent lateral transfer of the LHR. LHR sequences from *E. coli* AW1.3 and P12b, two strains that are phylogenetically closely related, cluster in separate branches of group A while LHR sequences from phylogenetically unrelated strains, e.g. *E. coli* AW1.3 and AW1.7, cluster closely together. LHR sequences from *Yersinia enterocolitica, Enterobacter cloacae, Citrobacter* spp., *K. pneumonia* and *C. sakazakii* are interspersed with LHR sequences from *E. coli* (Figure A-6). The most divergent LHR sequences in group A belong to 2 *Pseudomonas* spp. (Figure A-6).

LHR sequences in group B are represented by 13 strains of *Pseudomonas aeruginosa*, including isolates from cystic fibrosis patients. LHR sequences from other pulmonary pathogens include sequences from *Ralstonia pickettii*, *Burkoholderia multivorans* and *Stenotrophomonas maltophilia* (Figure A-6). *Dechlorosoma suillum* (now *Azospira suillum*; Byrne-Bailey et al., 2012) and *Methylobacillus flagellatus* (Chistoserdova et al., 2007) are found in freshwater and sewage and represent the most divergent LHR sequences in group B. The nucleotide identity between the most distant species from group A (*E. coli* KTE233) and group B (*Pseudomonas aeruginosa* NCAIM B.001380 K260) is 87.2 % over >80% of the entire LHR sequence. These

data provide evidence that the LHR is highly conserved and has been laterally exchanged within the β- and γ-proteobacteria.

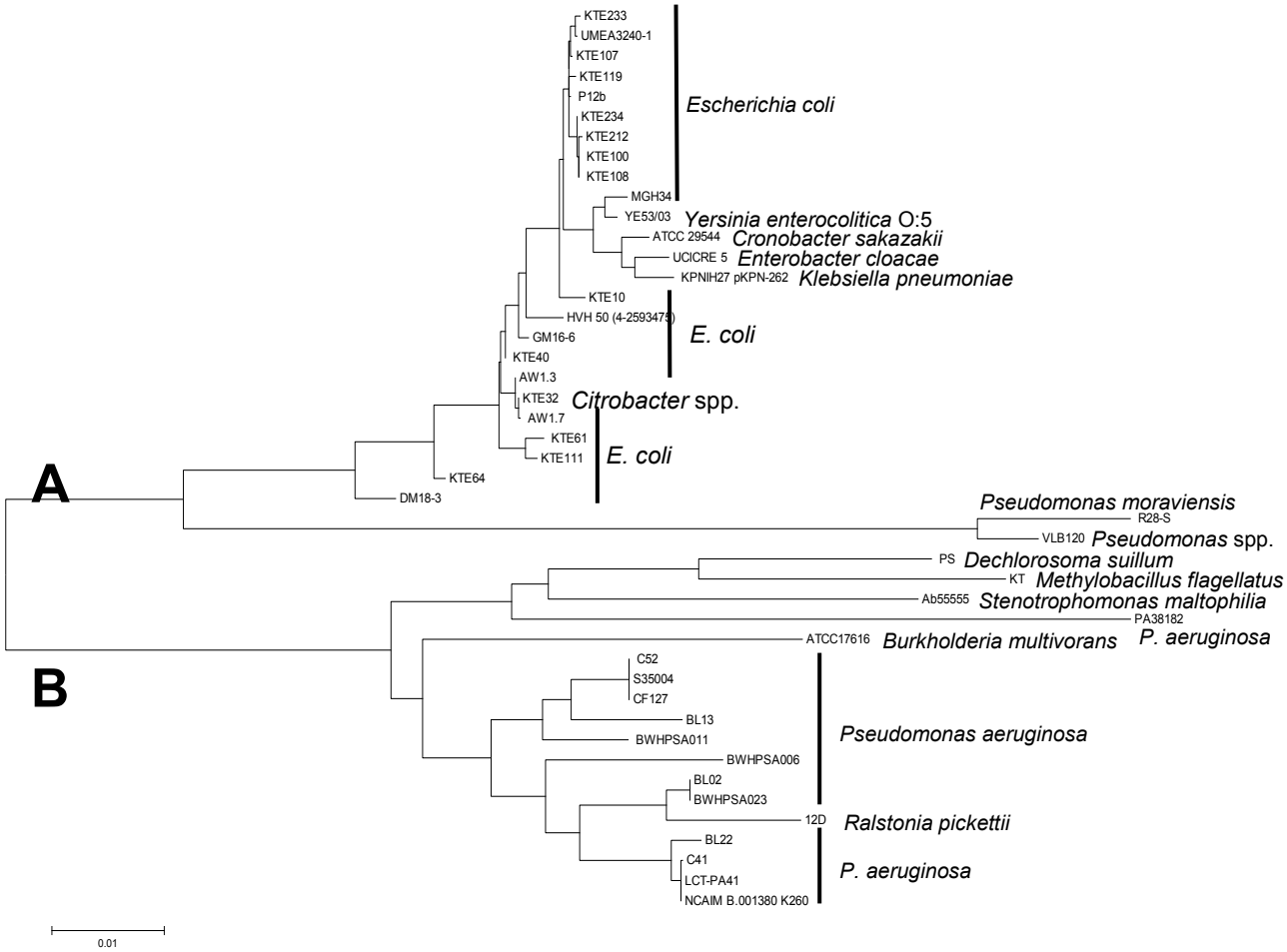


Figure A-6. Maximum-likelihood phylogenetic tree generated from an alignment of LHR sequences (>80% coverage of AW1.7) of disparate species of γ- and β-proteobacteria. The tree is separated into cluster A, represented by *Enterobacteriaceae*, and cluster B, represented primarily by strains of *P. aeruginosa*.

To determine the frequency of the LHR in *E. coli* more accurately, we searched the NCBI whole-genome shotgun assemblies (wgs) database in addition to the NCBI Genome database. This analysis retrieved additional LHR sequences predominantly from clinical isolates including UPEC and ETEC (Table A-3). Sequences covering >80% of the LHR were identified

in 66 out of 3347 strains, with an additional 15 strains found to possess 60-80% of the LHR (Table A-3). All sequences are more than 99% identical to the LHR sequence of *E. coli* AW1.7. Including genome sequences obtained in this study, the proportion of LHR-positive strains of *E. coli* is approximately 2% (Table A-3).

Table A-3. Frequency of LHR in *E. coli.* This table lists *E. coli* genomes or whole genome shotgun sequences containing the locus of heat resistance. Bioprojects were included when they contained genomes with the LHR with > 80% coverage and > 95% pairwise nucleotide identity when compared to *E. coli* AW1.7.

Origin of *E. coli* strains sequenced (ref)	**# of genome sequences**	**# genomes with LHR 80% (60%) coverage[a)]**
NCBI genome database[b)]	2263	16
Patients with urinary tract infections or bacteremia (1)	317	3 (1)
Clinical isolates of enterotoxigenic *E. coli* (ETEC) (2)	218	13 (4)
*P*atients with urinary tract infections (3)	236	15[c)]
Clinical isolates after antibiotic treatment (4)	247	21 (9)
Water isolates of O157:H12 (5)	1	1
ETEC (6)	5	1
Woman with recurrent urinary tract infections (7)	27	3 (1)
Intensive care unit patients (8)	5	2 (0)
Clinical and food isolates (this study)	28	4 (0)
	Total # of genomes 3347	**Total LHR 66 (81)**
		% positive 2.0 (2.4)

[a)] > 80% coverage and > 95% pairwise nucleotide identity when compared to *E. coli* AW1.7; values in brackets indicate BLAST hits with 60 – 80% coverage and > 95% nucleotide identity when compared to *E. coli* AW1.7.
[b)] accessed on Aug 11th, 2014
[c)] 13 of these *E. coli* strains are included in the NCBI genome database)
(1) *E.coli* UTI Bacteremia initiative, Broad Institute (broadinstitute.org) Accessed Aug 11th, 2014; (2) http://genomesonline.org/project?id=16624. Accessed Aug 11th, 2014; (3) http://www.ncbi.nlm.nih.gov/bioproject/193500 Accessed Aug 11th, 2014; (4) http://www.ncbi.nlm.nih.gov/bioproject/233951 Accessed Aug 11th, 2014; (5) http://www.ncbi.nlm.nih.gov/bioproject/PRJNA51127 Accessed Aug 11th, 2014; (6) Sahl et al., 2010; (7); Chen et al., 2013; (8) Hazen et al., 2014.

A.3.8 PCR targeting the LHR as a predictor and screening tool for highly heat resistant *E. coli*

To determine whether PCR screening for the LHR reliably identifies highly heat resistant strains of *E. coli*, 55 beef isolates of *E. coli* (Dlusskaya et al., 2011) were screened by PCR using primers targeting 3 different regions of the LHR, spanning several ORF's that are unique to highly heat resistant *E. coli.* Out of the 55 strains of *E. coli,* 13 strains were positive for all 3 LHR amplicons (data not shown) and 2 strains were positive for 2 of the 3 LHR fragments (data not shown). We selected 3 LHR positive, 3 LHR negative and the 2 strains containing a partial LHR for evaluation of heat resistance at 5 minutes at 60°C (Figure A-7). All LHR positive strains were highly heat resistant but the 2 strains containing a truncated LHR and LHR-negative strains were moderately heat resistant (Figure A-7). The results support the hypothesis that the presence of the complete LHR sequence is required for high heat resistance in *E. coli*.

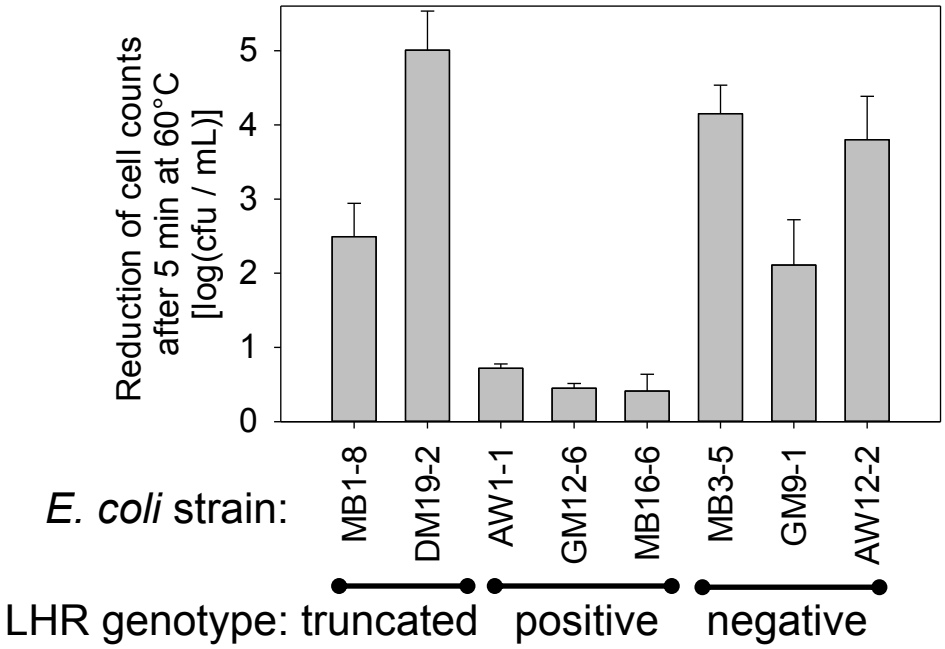


Figure A-7. Correlation of the LHR positive genotype to heat resistance in *E. coli*. Three fragments of the LHR were amplified with PCR to identify strains with a full length LHR and strains with a full length LHR. Heat resistance of 8 strains of *E. coli* representing 3 LHR-positive and negative strains, respectively, and two strains in which a truncated LHR was detected. Data are shown as means ± standard deviation of triplicate independent experiments.

A.4 Discussion

The resistance of food-borne pathogens to thermal intervention mechanisms challenges the food industry and public heath sectors, requiring a better understanding of the frequency, distribution and detection of heat resistance. This study employed comparative genomics to identify a genetic island, the LHR, which provides exceptional heat resistance in *E. coli*. Core-genome phylogenetic analysis and phylogenetic analysis of the LHR support the conclusion that the LHR is transmitted via lateral gene transfer. Transfer of the LHR occurred between diverse species in the β- and δ-proteobacteria, including enteric and pulmonary pathogens. Screening of

food isolates yielded a number of LHR positive strains, and demonstrated that the LHT is a suitable target for identifying heat resistant *E. coli*.

A.4.1 The LHR mediates heat resistance in *Enterobacteriaceae*.

Presence of the LHR in *C. sakazakii* and *K. pneumoniae* correlated to heat resistance of the strains (Gajdosova et al., 2011; Bojer et al., 2010). Of the 36 strains of *E. coli* that were analyzed both with respect to heat resistance and the presence of the LHR, all highly resistant strains carried the LHR and all strains carrying the full length LHR were highly heat resistant. Orthologs of 10 of the 16 ORFs are present in moderately resistant and heat sensitive strains, and a truncated LHR provides only moderate heat resistance. However, presence of the full length locus is unique to highly heat resistant *E. coli*. Complementation with the LHR conferred heat resistance to sensitive strains of *E. coli* only if the entire genomic island was cloned. Heat resistance of *E. coli* is thus dependent on the entire genomic island, and not on the function of a single protein.

The LHR comprises ORFs that are predicted to encode proteins with putative functions in cell envelope maintenance, turnover of misfolded proteins, and heat shock. The predicted products of 5 ORFs possess highly conserved functional domains, including sHSPs (Han et al., 2008) and several proteases. Eight ORFs contain predicted transmembrane domains, including Orfs8-10 and the proteases Orf15 and Orf16. One putative gene, *orf13,* is predicted to encode a sodium/hydrogen antiporter, which corresponds to the interplay of osmotic and heat stress in strains expressing the LHR (Pleitner et al., 2012; Orieskova et al., 2013). Orf16, a predicted membrane protease, possesses a similar domain structure to DegS, a protease involved in the activation of the σ^E stress pathway in *E. coli* (Alba and Gross, 2014). DegS types of proteases

are members of the HtrA (high temperature requirement A) family of proteins, which play a role in protein turnover in the periplasm and are induced by heat shock (Kim and Kim, 2005).

The expression of *orf3*, designated as a Clp protease ClpK, increased heat resistance in *E. coli* DH5α; however, transfer of the entire LHR was required for heat resistance in a *clpP* mutant strain (Bojer et al., 2013), suggesting an interplay of ClpP and other proteins encoded within the LHR. Heterologous expression of *orf7-orf10* from *C. sakazakii* in *E. coli* also resulted in an increase in thermotolerance (Gajdosova et al., 2011), but the heat resistance of the resulting transgenic strains was substantially lower than the level of resistance that was observed in *E. coli* AW1.7 carrying the entire LHR (Figures A-1, A-5, and A-7). Deletion of the LHR substantially reduced the resistance of *C. sakazakii* to heat (Orieskova et al., 2013).

The LHR was suggested to be transcribed as a single poly-cistronic mRNA in *K. pneumonia* and *C. sakazakii* (Gajdosova et al., 2011; Bojer et al., 2013). We identified a strong putative promoter upstream of *orf1* which is conserved in both *K. pneumoniae* and *C. sakazakii*. The promoter was predicted to interact with the OmpR, a transcription factor coordinating gene expression in response to osmotic stress (Mizuno and Mizushima, 1990). The LHR is over-expressed in response to osmotic stress (Riedel and Lehner, 2007), which corresponds to the observation that *E. coli* AW1.7 is resistant to heat only when incubated in growth media containing 1 – 4% NaCl (Pleitner et al., 2012; Ruan et al., 2011), as well as the observation that deletion of the LHR reduces the tolerance of *C. sakazakii* to osmotic stress (Orieskova et al., 2013). The LHR may thus function in response to osmotic and heat stress and its function may be partially dependent on the extracellular concentration of compatible solutes.

A.4.2 The LHR is transmitted by lateral gene transfer between β - and γ-proteobacteria.

The nucleotide identity of the LHR in the *Enterobacteriaceae* is ~99% and the LHR is consistently flanked by mobile genetic elements. Both imply recent lateral gene transfer. The differences in the phylogenetic relationship between strains *E. coli* AW1.3 and P12b support this notion. Based on core-genome sequences, *E. coli* AW1.3 and P12b are highly related and have a recent ancestor. However, their LHR sequences are much more evolutionarily distant; suggesting the strains independently acquired the LHR. Transfer of large genomic elements is well described for genomic islands encoding virulence factors, for example the LEE (Schmidt, 2010). Comparative genomics analysis of the fish pathogen *Edwardsiella tarda* indicated that the LEE of *E. coli* is also transmitted to other *Enterobacteriaceae* (Nakamura et al., 2013). Genomic islands that are transmitted by lateral gene transfer also possess environmental relevance (Juhas et al., 2009) and provide genes for sugar metabolism (Chouikha et al., 2006) or degradation of aromatic compounds (Gaillard et al., 2006). Acquiring multiple genes that require coordinated expression and protein function, e.g. LEE and LHR, can increase the overall fitness of the species.

Genomic islands do not always encode self-transfer capabilities (Shoemaker et al., 2000) and the LHR is located on the chromosome or on plasmids (Gajdosova et al., 2011; Bojer et al., 2010; this study), which may allow exchange through conjugation. Species carrying the LHR occupy similar environmental niches, such as the gastrointestinal tract (*E. coli, Citrobacter* and *Yersinia*), the urinary tract (UPEC and *Yersinia*) and sewage/fresh water (*Enterobacteriaceae*). Remarkably, transfer of the LHR is not restricted to *Enterobacteriaceae* but includes *Pseudomonas* spp. and β-proteobacteria. The GC content and predicted function of the ORFs do suggest a thermophillic origin of the LHR.

A.4.3 The LHR is present in approximately 2% of strains of *E. coli,* including food isolates and pathogens.

This study, in combination with past studies, has identified 7 LHR-positive and highly heat resistant strains (Dlusskaya et al., 2011; Ruan et al., 2011). None of these strains carry virulence factors; however, bioinformatic analyses revealed that about 2% of all the *E. coli* genome sequences or whole genome shotgun sequences contain the LHR with more than 80% coverage and more than 95% nucleotide identity. All studies on the heat resistance of LHR positive strains of *E. coli, Cronobacter,* and *Klebsiella* confirmed that the full length LHR is a reliable predictor of heat resistance. LHR positive strains of *E. coli* include UPEC and ETEC. Because both the LHR and genes coding for virulence factors are highly mobile, highly heat resistant strains of other pathovars likely also exist. A screening of about 100 strains of STEC has not identified highly heat resistant pathogens (Liu et al., 2015), but screening of 100 strains may not suffice to identify a genetic and physiological trait that is present in about 2% of strains. The identification of the genetic determinants of heat resistance provides a rapid screening tool to identify heat resistant *E. coli* in food or clinical isolates. A broader screening of strains and the assessment of their heat resistance will enable to assess the public health significance of heat resistance in *E. coli*.

This study observed a high frequency of LHR-positive and highly heat resistant strains in beef isolates (Dusskaya et al., 2011). Beef is an important vector for transmission of STEC (Scallan et al., 2012; Anomymous, 2011) and highly heat resistant *E. coli* are recovered in high numbers from inoculated beef patties that are cooked medium rare and even survive in burger patties that are cooked “well done”, corresponding to an internal temperature of 71°C (Dlusskaya et al., 2011; Liu et al., 2015). To date, the transmission of STEC was attributed to

undercooked meat (Schmidt et al., 2002); however, LHR-positive heat resistant pathogens may additionally contribute to foodborne disease. Because these organisms may survive in beef that is cooked to a core temperature of 71°C, cooking meat to a "well done" stage may not always eliminate all pathogenic *E. coli*.

A.5 References

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